

THE BIOCHEMISTRY AND PHYSIOLOGY OF BONE

Edited by

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PREFACE

In the last 25 years there has been a rapid development of new techniques and enthusiasm in their application to bone studies. A store of new knowledge has been accumulated about structure and function of bone and a growing appreciation of the skeleton as a plastic, actively metabolizing tissue. Papers dealing with these and other aspects of bone are published in an astonishingly wide range of journals throughout the world. The present treatise is the outgrowth of a belief that the time had come to collect these diverse studies into an integrated volume. Its comprehensiveness should make it of interest to many experts, in particular to histologists, to anatomists, to specialists in orthopedics and pediatrics, and to dentists; in addition I hope that many biologists, physiologists, biochemists, and pathologists will find a great deal of interest and value to them between its covers.

The study of bone has passed through a number of phases, many of the older workers were well aware of the plastic nature and metabolic activity of bone, but later a tendency to regard bones as immutable structures that one could cut and shape and treat as inanimate building material became widespread.

Frey, in his manual of histology, published nearly 100 years ago summarized what was, until relatively recently thought to be the function of bones:

"Owing to their hardness and solidity, the bones are peculiarly well adapted for the mechanical construction of the body. . . . They serve to protect internal organs, and form systems of levers." But Frey also goes on to say "The bones take part also, to a great extent in the chemical occurrences of the organism, owing to the lively interchange of matter going on in them." This is, in fact, a modern outlook on bone.

To some extent it was the mechanical investigations of bones by engineers such as Carlmann and Kochlin which tended to emphasize their structural nature and to suggest a permanence which was illusory; in fact it has been said that Kochlin designed the struts for the Eiffel tower on the same plan as the trabeculae at the head of a long bone (first described by Meyer in 1873). The bone struts of course can do what those of the Eiffel tower cannot, they can be altered in shape, size, and direction with varying stresses and strains and this is one of the characters which most distinguishes bone from an inert supporting structure.

In the last hundred years investigation of bone has proceeded actively in a wide variety of fields and in the present century the development of biochemistry has contributed a good deal to our knowledge of the nature of the organic matrix, the problems involved in calcification, and so on. The influence of vitamin deficiency on bone is well known but recent research has helped towards an understanding of the mechanism by which such changes are brought about. More recently a great deal of attention has been paid to the role of hormones in bone formation and structure. The cells of bone and problems of bone development and repair have been extensively studied. Probably the most recent field is the application of radioisotopes to the study of bone structure and function.

It would be too great a feat to expect any one person to deal comprehensively with all these facets of bone study and therefore the best solution was found to be a composite book.

In such a book a certain amount of planned overlap of chapters is both inevitable and desirable. Chapters which deal, for example, with osteoblasts, phosphatases, and calcification must be expected to have a good deal in common; in the same way the growth of bone would naturally be discussed in chapters other than that bearing such a title. In fact, one can think of this book in a sense, as a spectrum, each of the chapters representing a pure spectral color but shading indistinguishably into its neighbors.

The collecting together, on time, of 24 chapters by authors scattered over Europe, America, and the Middle East seemed an intimidating task but thanks to the cooperation and hard work of all who participated in this volume it proved to be by no means as difficult as it seemed at first.

My thanks are due to all, authors and staff of Academic Press, for their help in bringing together and getting into print so promptly the mass of material contained in this book.

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CHAPTER I

GENERAL ANATOMY AND HISTOLOGY OF BONE

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I. General Introduction

1. DEFINITION

The word *bone* is ambiguous for it is used both to denote a particular kind of hard tissue, and also to indicate one of the more or less independent elements of which the skeleton is composed. We have in fact, *bone* and *bones* (Weinmann and Sicher, 1947).

Bone, in the former sense, is a specialized type of connective tissue, characterized by the presence of cells with long branching processes (*osteocytes*) (Plate I, Fig. 1 and 6) which occupy cavities (*lacunae*)

and fine canals (*canalliculi*) in a hard, dense matrix consisting of bundles of *collagenous fibers* in an amorphous ground substance (*cement*) impregnated with *calcium phosphate* complexes.

In the formation of bone distinctive large cells of mesenchymal origin (*osteoblasts*) become surrounded by bundles of collagenous fibers which are then cemented together to form spicules or plates of uncalcified bone matrix (*osteoid*). Minute crystals of calcium phosphate complexes are deposited in the osteoid. The osteoblasts imprisoned in the matrix are converted into osteocytes.

Bone is not a static tissue. On the contrary it is constantly being reconstructed or remodelled throughout life (Amprino and Bairati, 1936), a process in which old matrix is removed at one point while new matrix is deposited at another. Where bone is being removed the matrix shows a sharply bitten surface, the "bites" or *Howship's lacunae* being occupied by large multinucleated cells termed *osteoclasts*. Although bone matrix is composed of three separate elements, viz., fibers, cement, and calcium salts, in bone removal the three constituents disappear together (Hunter and Turnbull, 1931).

2. RELATED TISSUES AND SUBSTANCES

Besides bone there are a number of other hard, calcified tissues and substances found in the animal body, some of which are not easy to distinguish from bone.

Calcified cartilage shares with bone a hard matrix, consisting of collagenous fibers in an amorphous ground substance impregnated with calcium phosphate complexa, similar and perhaps identical with that found in bone. The proportions, however, are different, for the collagen/cement ratio is high in bone and low in cartilage, while the latter is not so heavily calcified. In some parts of fracture callus (Fig. 2), nevertheless, definitive bone and cartilage are connected by a tissue in which the collagen/cement ratio, among other properties, is intermediate between that of the two tissues (Ham, 1930; Pritchard and Ruzicka, 1950). Normally, however, the tissues can readily be distinguished, not only by the differences in collagen/cement ratio but also by the shape of the cells, those of cartilage being spherical and without processes, those of bone being almond-shaped with numerous processes lying in canalliculi which are not present in cartilage. In cartilage also the calcium salts are present as a rule in coarse granular form, while in bone the crystals are so fine that the deposition appears amorphous with the light microscope.

The matrix of *dentine* is composed of similar materials to those

found in bone, but the inorganic salts are in greater concentration. There is also a canal system of dentinal tubules containing cellular processes, but the cells themselves remain outside the matrix in dentine and the tubules are more or less parallel to one another, whereas in bone the canaliculi branch and anastomose irregularly. Certain types of bone, e.g. tooth cementum, and the thin bones of fishes, may be acellular, but the presence of canaliculi differentiates them from dentine. The *crown cement* of certain rodent teeth, however, is a form of calcified cartilage.

Enamel is acellular and devoid of canaliculi and cellular processes. Its inorganic component is very similar in composition to that of bone and dentine but it is present in much higher concentration than in either. Its organic component contains no collagen, as befits its ectodermal origin. Differentiation from bone therefore presents no difficulty.

In *calcified tendons and ligaments* calcium salts similar to those of bone are deposited in a cement substance laid down between the collagenous fibers of the original tendon or ligament. Differentiation from bone may not be an easy matter, depending on establishing the presence or absence of typical osteocytes with processes lying in canaliculi traversing the calcified matrix. True bone may also occur in tendons and ligaments adjacent to merely calcified areas and gradations between the two calcified matrices may be present. For this reason Weidenreich (1928) regards calcified tendon and ligament as a form of bone.

Calcified pathological deposits resemble bone in their hardness and in the composition of their inorganic salts. Their organic matrix is a hyalinized degenerate material derived from almost any tissue, but containing a high proportion of mucopolysaccharides similar in some respects to those found in the organic matrix of bone (Howard, *et al.*, 1949; Howard, 1951). The absence of osteocytes and collagen fiber bundles, however, makes differential diagnosis a simple matter.

Some *invertebrate exoskeletons* are hardened with calcium salts, usually calcium carbonate. The calcium phosphate complexes typical of bone are only found in vertebrates. Thus in molluscs the shell is composed of calcium carbonate in either the calcite or aragonite crystalline form with only 1-2% of calcium phosphate (Bevelander, 1951). Similarly in arthropods such as the lobster the chitinous shell may be impregnated with calcium carbonate.

The inorganic component of the *eggshells of birds* is also a form of calcium carbonate.

Thus, while a number of calcified tissues resemble bone in some ways, definite diagnosis of bony tissue can usually be made on careful histological examination. The fundamental diagnostic characteristic is the presence of anastomosing canaliculi. These enable bone to support a

flourishing cell population in the midst of dense calcified matrix through which nutrient materials necessary for the life of the cells could not otherwise permeate.

3. EVOLUTION OF BONE AND CARTILAGE

Bone is characteristic of, and evidently evolved with, the vertebrates, as did cartilage. These two tissues enabled the vertebrates to develop an internal skeletal system able to support animals of much larger size than any found among the invertebrates, and capable of rapid growth without the disadvantages of the periodic molting necessary to arthropods with a rigid, acellular exoskeleton, or the complexities and redundancies of shell growth in molluscs. Moreover, being a living tissue, bone is capable of rapid adjustment to changing mechanical and biochemical demands. It has the advantage over cartilage of greater strength and rigidity, yet at the same time it has the capacity for more rapid remodelling and reconstruction as occasion demands. Bone is thus a unique tissue in its combination of strength with plasticity, and it has evidently been one of the more important factors determining the superiority of the vertebrates over the invertebrates.

The question whether bone or cartilage evolved first is difficult to decide. The most primitive living vertebrates with a skeleton, the cyclostomes, contain only a form of cartilage. The elasmobranchs, commonly regarded as having evolved very early, possess a cartilaginous skeleton (except for the dermal denticles). Most bones of higher forms, moreover, are preceded in their development by cartilaginous models. It would therefore appear that cartilage is the more primitive. However, paleontological evidence suggests that both cyclostomes and elasmobranchs are descended from bony forms, and there is no *a priori* reason why the so-called membrane bones of the skull, which are not preceded by cartilage models, should not have appeared in evolution before the cartilage bones of the axial and limb skeletons (Romer, 1947). It is possible, therefore, that cartilage is a secondary, and later, adaptation to the circumstances of embryonic life, and that where it forms much or all of the adult skeleton, bone-forming powers have been secondarily lost.

The similarities in composition of bone and cartilage, however, and the existence in fracture repair, *in vitro*, and even in normal development (secondary cartilage) of tissues intermediate between bone and cartilage, may perhaps suggest that the two evolved together, an early mutation having given rise to a cell type able to manufacture either matrix according to the dictates of environmental factors. On the other hand, there may be more than one form of cartilage-making cell, only one of

which is a variant of the bone-making cell, the others having evolved independently.

II. The Primary Constituents of Bony Tissue

1. THE CELLS

The osteocytes as a group vary widely in size, shape, and intracellular detail, as well as in density of packing and regularity of arrangement within the matrix, according to their maturity and to the kind of matrix with which they are associated. At one end of the scale there are present in newly formed trabeculae of woven bone (*q.v.*) in the embryo, and at sites of rapid growth later, closely but irregularly packed cells, rich in cytoplasm, which are almost indistinguishable in their external form and internal features from the osteoblasts from which they have so recently been derived. Their processes are relatively few and stout and freely anastomose with those of their neighbors. Their nuclei are large, round and hypochromatic. Their cytoplasm is finely granular, highly basophilic, contains numerous rod-like mitochondria, a large Golgi element, a moderate amount of glycogen, and shows alkaline phosphatase activity.

At the other end of the scale there are the mature cells of lamellar bone (*q.v.*), flattened and ovoid like almonds or melon seeds, and possessing very numerous fine processes which branch freely (Fig. 1). Such cells tend to be evenly spaced and uniformly orientated with respect to the long and radial axes of the lamellar system they occupy (Fig. 5). They are spaced much further apart than the cells of embryonic woven bone (*cf.* Figs. 7 and 8); their nuclei are flattened and hyperchromatic; their cytoplasm is scanty; and their basophilia, mitochondria, Golgi element, and glycogen content are less in evidence than is the case with the younger cells. Whether or not alkaline phosphatase activity is exhibited is debatable.

Mitoses have not been observed in mature osteocytes but there is evidence (Bast, 1921) that amitotic divisions may sometimes occur in newly-formed cells.

The bone cell in life completely fills its lacuna, appearances to the contrary being due to shrinkage during fixation. The processes of the young bone cells also probably completely fill the canaliculi in which they lie, and anastomose freely with those of other cells; but it has been suggested that in mature bone the cell processes may be retracted leaving the canaliculi empty except for tissue fluid (Ham, 1953), while anastomoses with neighboring cells are necessarily lost.

While there is no doubt that osteocytes are derived from trapped

osteoblasts in normal circumstances, there is evidence that in some situations they may be derived from the fibroblasts of fascia, tendon, or ligament without passing through an osteoblast stage (Weidenreich, 1928). Cartilage cells may also give rise to osteocytes directly according to some observers, while according to others freed cartilage cells in endochondral bone formation may become osteoblasts and give rise to osteocytes indirectly. The reticulum cells of bone marrow, and fragmented osteoclasts, are other probable sources of osteoblasts, and hence of osteocytes. The problem of the origin and specificity of the osteocyte is in fact the problem of the origin and specificity of the osteoblast, for as a general rule the immediate precursor of an osteocyte is an osteoblast, whatever may be the ultimate origin of the latter cell.

The fate of the osteocyte when bone is resorbed has not been settled. It may die, it may fuse with other cells to form an osteoclast, or it may revert to an osteoblast or reticulum cell as the matrix is removed from around it: none of these possibilities has been ruled out.

The functional role of the osteocyte is a matter for conjecture. It probably maintains the matrix in some way, possibly by facilitating exchange of materials between the tissue fluids and the matrix via the canalicular system. It may perhaps resist attempts of the tissue fluids to leach out the matrix constituents. It may preside over molecular remodelling and rejuvenation of the matrix. Its turgor may contribute to the mechanical properties of the matrix by conferring elasticity and pressure resistance. It may be a reserve cell from which osteoblasts and osteoclasts are recruited following bone resorption. It may act as a sensitive receptor to mechanical and humoral alterations in the environment and initiate appropriate changes in the matrix, though this latter role is more probably carried out by the osteoblasts and related cells which lie on the *surface* of the matrix. The osteocytes, nevertheless, might be the effective receptor cells, passing on their information to the neighboring osteoblasts for action. Whatever may be its function, however, the osteocyte is an essential constituent of bone, for when the cells die the matrix around them, although persisting for a time, eventually crumbles and is removed to be replaced by living bone. Osteocyte death is, in fact, synonymous with bone death.

2. THE FIBERS

With appropriate staining methods bone matrix is seen to be permeated by bundles of fine collagenous fibers (Fig. 3, 7, 8) some of which, at the boundaries of the matrix, continue into the surrounding uncalcified connective tissues. Chemical and electron microscopic studies

of bone collagen show it to be similar to, if not identical with the collagen of skin, tendon and the connective tissues generally, and to make up the major part of the organic matrix.

The elementary fibrous unit is the collagen fibrilla, only to be seen with the electron microscope. Closely packed aggregates of many such fibrillae constitute the primitive "fibers" which are just resolvable with the light microscope. These fibers are again aggregated into the fiber-bundles which constitute the easily recognizable fibrous unit of bone structure (Weidenreich, 1928).

In newly formed embryonic bone the fiber bundles run irregularly through the matrix, interlacing and crossing without apparent order or system, hence the name *woven bone* (Figs. 3, 8). In mature *lamellar bone* (Fig. 7) the fiber pattern is remarkably uniform and regular. In successive lamellae, however, the predominant direction of the fiber-bundles tends to change (Fig. 7). The optical and staining differences resulting from the changing fiber-bundle orientation in successive lamellae is in fact the explanation of the lamellar appearance and the reason for calling this type of bone "lamellar bone." The term is, however, somewhat misleading as the bone matrix in a lamellar system is not composed of isolatable and independent thin plates as the name and the optical appearances suggest. It is in fact a continuum. Precisely similar bone in all other respects may have its fiber-bundles orientated similarly throughout and then, being optically homogeneous, no lamellar pattern is discernable. The real distinction between the two main types of bone lies in the size and regularity of the fiber-bundles present; between bone with irregularly interlacing coarse bundles and bone with regularly arranged fine bundles. The fibers of connective tissue and bone are discussed in detail in Chapter V.

3. THE CEMENT

The presence of a cementing medium for the collagen fibers of bone matrix is generally accepted, although the evidence is inferential rather than direct. This is because it appears optically homogeneous, and its chemical nature has not been determined with certainty though it is probably a protein-polysaccharide complex (see Chapter IV). The clearest evidence comes from silver-stained sections of bone which show argentophil fibers as a discontinuous phase within an argentophobe continuous phase, the latter being regarded as the cement. Within a given lamellar system in compact bone the fibers are closely packed and the argentophobe phase is not very conspicuous, but at the boundaries of the lamellar systems there are thin but distinct apparently fiber-free zones

(Fig. 7). Similar zones are found wherever bone formation has taken place intermittently, either because deposition has been interrupted by periods of rest, or because deposition has alternated with resorption. Such fiber-free argentophobe zones are termed "cement lines," subdivided into "accretion lines" and "reversal lines" according to the past history of the region. Cement lines often appear blue against a pink background in hematoxylin and eosin sections. They afford valuable evidence of the way in which the bone has grown in much the same way as in geological stratigraphy lines of unconformity in rock formations indicate phases of denudation interrupting sedimentation.

The early formation of bone also affords good evidence for the existence of a cementing substance between the fibers. The pre-osseous matrix shows collagen fibers very distinctly with ordinary histological stains such as Weigert's hematoxylin and Van Gieson, but as the earliest bone trabeculae are formed these fibers lose their distinctness and the matrix appears hyaline, even before the calcium salts have been precipitated. Silver impregnation methods nevertheless show the fibers still to be present, and morphologically unchanged (Fig. 3). The obvious conclusion is that a cementing substance of similar refractive index to collagen has been deposited around the fibers.

The chemical nature of the cement in bone matrix has not been fully determined but there are good reasons for believing that it contains mucopolysaccharides (See Chapter IV). First of all, in cartilage, where a similar histological masking of collagen fibers takes place in development, large quantities of the acid mucopolysaccharide, chondroitin sulphate, can be demonstrated chemically and histochemically. This and other mucopolysaccharides are also found universally in the loose connective tissues, though in much smaller amounts, as a ground substance for the collagen fibers (Gersh and Catchpole, 1949). In fact there seems to be a universal association between collagen and mucopolysaccharide in the connective tissues, and it would be surprising if bone were an exception. Chemical analyses of bone demonstrate a small proportion of hexosamines and hexuronic acid, which are almost certainly degradation products of mucopolysaccharides (Rogers, 1949, 1951). The best evidence, however, comes from histochemical studies of developing bone, for the newly formed trabeculae, before calcification, are metachromatic to such dyes as toluidine blue and, moreover, give a strong positive reaction with the periodic-acid-Schiff (P.A.S.) tests of McManus and Hotchkiss. The calcified matrix gives a strong P.A.S. reaction, and also shows metachromasia, but only after decalcification (Pritchard, 1952). This combination of staining reactions almost certainly indicates the presence of acid mucopolysaccharides in the matrix. It would appear,

in fact, that the organic matrices of bone, cartilage, and loose connective tissues are qualitatively similar in chemical composition, the differences in properties depending very largely on the proportions of the primary constituents, collagen and mucopolysaccharide, present.

4. ADVENTITIOUS CONSTITUENTS

Bone normally develops in an area of loose or dense fibrous tissue, and while most of the collagen fibers of the bone matrix appear to be formed at the time of ossification, the fibers of the preceding tissue tend to be incorporated in the matrix as well. In this way fascial sheets, aponeuroses, tendons, and ligaments may become incorporated in, and anchored to, the bone matrix. Such adventitious fibers are generally known as *Sharpey's perforating fibers*. In some places these adventitious fibers may comprise a large proportion of all the fibers present in the matrix, e.g. in the linea aspera of the femur, in tooth sockets, and adjacent to cranial sutures. Elastic fibers also may in some places become incorporated in bone. Adventitious fibers are only to be found as a constituent of coarse-fibered woven bone: they are never seen in fine-fibered lamellar bone. If present in adult compact bone they lie in tracts of persisting coarse-fibered bone *between* the lamellar systems.

There is possibly another organic constituent of bone present around the osteocytes, for the matrix immediately surrounding them stains more heavily with hematoxylin and other dyes than elsewhere and, according to some observers, is more resistant to destructive agents; for on maceration with strong acids corpuscular bodies can be isolated in the shape of bone cells and their processes, as if a cast of the lacuna and its canaliculi had been taken. It is possible that a keratin-like cuticle lines the space in which the cell and its processes lie but it is more likely that the phenomena mentioned are due to the existence of a relatively fiber-poor, cement-rich region immediately round the cell similar to the so-called "capsule" around the cartilage cell. Alternatively, mucopolysaccharides may be more concentrated or more highly polymerized in this region (Ham, 1953).

5. THE INORGANIC MATRIX

The hardening material present in bone has long been known to consist principally of calcium phosphate. However, the existence, on chemical analysis, of other elements and radicals such as magnesium, sodium, fluorine, carbonate, and citrate, and the readiness with which many foreign elements can be incorporated into the bone matrix, e.g. alkaline earths and heavy metals, has made it difficult to determine

whether such substances are simply mixed with the calcium phosphate, or chemically combined with it to form a complex molecule in which anion and cation exchanges with the tissue fluids are free to occur. The X-ray diffraction pattern of bone mineral indicates a crystalline structure, and recently electron microscopic studies have demonstrated the existence of minute tabular crystals of regular size and shape (Robinson, 1951). It would appear that in spite of variations in chemical composition, the bone mineral shows a remarkably constant microcrystalline structure. This strongly indicates that the essential inorganic constituent is reasonably constant in chemical composition, adventitious substances present either being capable of replacing the primary constituents in the crystal lattice without altering the crystal form, or else being adsorbed to crystal surfaces without affecting the lattice. The small size of the crystals affords relatively large surfaces for such adsorption to take place. Fortunately there exist a series of natural minerals with very similar composition, properties, and crystalline structure to those of bone. These are the "apatites" in which calcium phosphate is the primary constituent, and adventitious constituents are present, as in bone. It has not been easy to determine which particular apatite most closely resembles bone, and there are still sharp differences of opinion. However, the consensus of opinion is now that the bone mineral is a hydroxyapatite, a complex of tricalcium phosphate and calcium hydroxide (See Chapter X). The large carbonate portion is thought to be principally in the form of calcium carbonate, and, with other adventitious constituents, to be adsorbed on the surfaces of the hydroxyapatite crystals. This view accounts for the chemical composition, X-ray diffraction pattern, size and shape of crystals, and for many other chemical and physical phenomena exhibited by the bone mineral. Some workers, however, maintain other views, such as that the primary constituent is a carbonato-apatite in which calcium carbonate, and not calcium hydroxide, is combined with calcium phosphate in the lattice; or that the crystals are essentially a hydrated form of tricalcium phosphate; but general opinion has swung away from these hypotheses.

In very young newly formed bone the matrix stains differently from (e.g. having a greater affinity for alizarin), shows a much faster turnover rate with radioisotopes, and is generally more chemically reactive than, older matrix. This has given rise to the view that the bone mineral when first laid down is either a secondary calcium phosphate, or else a simple tricalcium phosphate, not an apatite. Electron microscopy and X-ray diffraction studies, however, indicate no differences in crystalline structure between old and new bone, and so it seems likely that the differences mentioned are due to physical rather than chemical factors,

e.g. proximity to blood vessels, less dense packing of crystals; or perhaps to differences in the organic matrix which supports the crystals.

The relationship of the bone crystals to the fibers and cement of the organic component of the matrix has long been debated. In an early experiment of Von Ebner's bone was calcined to remove all organic material and then on histological examination it was seen that, in place of the fibers, there were numerous very fine canals in the residual inorganic matrix. This seemed to indicate that only the cement was calcified. In recent years the electron microscope has, however, revealed some interesting facts. The elementary fibrillae into which the collagen fibers can be resolved are seen to be encrusted with minute crystals with their long axis bearing a constant relationship to the long axis of the fibril, and with a strong tendency for them to be spaced at intervals corresponding to the natural periodicity of the fibrillar banding. The cement cannot be seen with certainty under these conditions but the inference is that the crystals lie within it rather than within the fibrils, although they certainly lie within the "fibers" as seen under the light microscope, for these are composed of many fibrillae around each of which there appears to be a layer of crystal-containing cement. The argument is, in the final analysis, a sterile one, for approaching the molecular level of organization no structure can be *within* another. The chief conclusion to be drawn is that bone matrix exhibits an extraordinarily intimate association of the three primary components, *viz.*, fibers, cement, and crystals; and that the structural unit of each of these components is an aggregate of relatively few similar molecules. See Chapter VI for a detailed discussion of this problem.

III. Types of Bony Tissue

In the final analysis all bony tissue consists of an intimate mixture of collagen fibers, cement substance, and calcium salt crystals, permeated by a system of lacunae and canaliculi for the osteocytes and their processes. These primary constituents, however, may be combined in different ways. Thus different samples of bone vary in the size and orientation of their fiber bundles; in the proportion of cement to fibers; in the number of cells in a given volume; and in the spacing and orientation of the cells: while the cells themselves vary in size, shape and numbers of processes. These variables give rise to distinctive histological types of bony tissue, which have been classified in various ways by different authors. A satisfactory classification, however, of all the types of bone which exist, from that found in the scales and teeth of cartilaginous fishes to that in the compact bone of adult mammals, is not easy

to arrive at Weidenreich's (1928) classification is probably the best put forward so far. He distinguishes a type of bone with coarse fiber bundles (coarsely bundled bone, fibrous bone) existing in two main varieties (1) in which the fiber-bundles are *parallel* and (2) in which they are irregularly *woven* or plaited; and a type of bone with fine fiber-bundles (finely bundled bone, shell-like bone), again in two varieties, (3) in which the fiber-bundles are parallel, and (4) in which they are stratified, the fiber-bundles running in different directions in neighboring strata (lamellar bone). (5) There is also a type of bone in which coarse and fine bundles are intermixed.

a. *Parallel-Fibered, Coarsely Bundled Bone*

This type of bone is found in some fish scales, in the skeletons of fish and amphibia, particularly near tendon insertions, and in ossified bird tendon. Its fiber characteristics are those of the corium or tendon which has been ossified, few new fibers being added.

b. *Woven, Coarsely Bundled Bone*

This is widely distributed in the scales and teeth of cartilaginous fishes, in the flat bones of amphibia and reptiles, in the skeleton of the mammalian embryo, at sites of attachment of strong fasciae, tendons, and ligaments to adult mammalian bones, near cranial sutures, in the alveolar bone of the jaws, in the bony labyrinth, in fracture callus and in pathologically-formed bone of all kinds.

c. *Parallel-Fibered, Finely Bundled Bone*

This is found in the long bones of birds, in newly-formed endochondral bone in mammals, and in the simple Haversian systems of the mammalian fetus.

d. *Stratified or Lamellar Finely Bundled Bone*

This is characteristic of the adult skeleton of mammals, including man. The skeletons of large fossil reptiles and birds, dinosaurs and pterodactyls, were similarly constituted.

e. *Finely and Coarsely Bundled Bone*

These are intermingled in many situations, either as a temporary phase in the replacement of coarsely bundled by finely bundled bone, or as a permanent feature where tendons and ligaments get strong anchorage and send great numbers of perforating Sharpey fibers into the underlying bone as in the tooth sockets, or the linea aspera of the femur.

Of the various types of bone mentioned only the woven, coarse-bundled bone (or simply "*woven*" bone, Fig. 8), the lamellar finely-

bundled (or "lamellar" bone, Fig. 7) and the *mixed* type are of importance in the mammalian skeleton. *Woven bone* comprises the skeleton of the early embryo, but it is gradually replaced by lamellar bone, starting well before birth and continuing until almost the whole of the adult skeleton is composed of lamellar bone. Some woven bone, however, persists in tooth sockets, near cranial sutures, in the osseous labyrinth, and near tendon and ligament attachments, usually mixed with lamellar bone. In the mammal woven bone is therefore, for the most part, a temporary expedient. It is characteristically formed at the rapidly growing margins of a developing bone just beneath the fibrous periosteum. Lamellar bone, on the other hand, is formed slowly and deliberately on pre-existing bony or cartilaginous surfaces and is essentially a *replacing*, not a primary, tissue. Woven bone, as it were, makes the running and extends an osseous territory into the surrounding connective tissues: lamellar bone follows in its wake, replacing it, and consolidating the position occupied.

Whenever bone formation is violently reactivated in the adult, in consequence of fracture or other pathological circumstance, woven bone is the first type to be formed. Eventually, however, as the process slows down, lamellar bone is formed, and finally the woven bone is almost entirely replaced by lamellar bone as in normal development.

Woven bone can arise in three distinct ways:

- (i) as an ossification of a pre-existing stout fibrous structure such as a tendon or ligament, in which case the tendinous or ligamentous fibers are carried into the bone almost unchanged and form the major component of the matrix (bundle-bone, Sharpey bone);
- (ii) as an ossification in mesenchyme, in which virtually all the fibers of the bone matrix are *ad hoc*, newly formed ones; and
- (iii) as an ossification "in membrane," in which most of the fibers of the bone are newly formed but a proportion are carried in from the preceding fibrous membrane.

Histologically woven and lamellar bone are easily distinguishable, especially in silvered preparations (cf. Figs. 3 and 8 with 5 and 7). The former shows coarse fiber-bundles of varying size up to 30μ in diameter, running in a random, interlacing fashion through a cement-rich background. The cells are large, irregular in shape and size, densely packed and with comparatively few processes. The matrix tends to be basophil because of the abundance of cement. In lamellar bone on the other hand the fiber-bundles are fine, only $2-4\mu$ in diameter, and are regularly arranged in parallel or concentric curving sheets, successive sheets differing in their predominant fiber direction, so that an area of lamellar bone has a stratified appearance. The cells are regular in size and shape

but generally flatter and smaller than those of woven bone. Moreover, they are fairly regularly but relatively widely spaced and their orientation conforms with that of the lamellar system as a whole. The processes of the cells and the canaliculi in which they lie are extremely numerous and have a predominant radial or transverse orientation with respect to the lamellar system as a whole (Fig. 6). Woven bone thus gives the impression of disorder and hurried formation; lamellar bone of order and deliberate formation.

It is probable that lamellar bone is constructed on sounder mechanical principles than woven bone, giving greater strength for a given volume of materials; and it is probable that the replacement of woven bone by lamellar bone in evolution, reflected in a similar replacement in mammalian ontogeny, has been an important factor in the adaptation of the mammal to a highly active life on land where strength combined with lightness of skeleton is a necessary condition of survival essential for the rapid movements on which survival must often depend.

IV. Organization of Bony Tissue

1. GENERAL

So far we have been dealing with bone as it appears under high magnification with the microscope, and we have classified bony tissues according to the way in which the elementary constituents are combined. At lower magnification, and macroscopically, bony tissue exhibits other levels of organization which arise from the fact that the elementary bony tissue is not built up into large amorphous bony masses, but instead, as it accumulates, a pattern and a grain is imposed upon it independent of the much finer pattern and grain of cells, fibers and crystals which has already been described. This coarser pattern is occasioned by a number of factors, including (i) the relationship of bone matrix to vascular spaces, (ii) the relationship of bone matrix to the honeycomb of calcified cartilage at the epiphyseal line, (iii) intermittency of matrix deposition and removal, and (iv) conformity of bony architecture with the prevailing external forces of pressure and tension acting upon it.

Factors (i) and (ii) tend to give a honeycomb or "cancellous" structure to bone, firstly because bone matrix and blood vessels tend to alternate (Fig. 3) and secondly because the bone matrix is built up around the cartilage scaffolding. The third factor tends to give bone matrix a laminated, "plywood" type of structure, reminiscent of geological stratification (Figs. 5 and 7). The fourth factor tends to organize the cancellous bone into "trajectorial systems," that is to say, specially strengthened bony tracts are developed within the general cancellous network along which pressure and tension forces are conducted and

distributed. Probably orientations of similar import are present in compact bone also (Murray, 1936).

Because bone matrix is everywhere interrupted by vascular-connective tissue spaces and canals, it has an essentially porous structure. On the basis of pore-size alone, without regard to the type of bone matrix involved, three main varieties of bone may be distinguished, viz. *compact*, *coarse cancellous*, and *fine cancellous*. In compact bone the pores are not visible to the naked eye and even under the higher powers of the microscope they are relatively inconspicuous interruptions in an almost solid matrix (Fig. 4). They may be perhaps 10–20 μ in diameter and separated by intervals of 200–300 μ or more. In coarse cancellous bone on the other hand the spongy architecture is easily visible to the naked eye as the pores may be from a millimeter up to a centimeter in diameter. There is evidently more, or at least as much, vascular connective tissue as bone matrix in a given volume of this kind of bone. In fine cancellous bone the architecture is only readily appreciable under the lower powers of the microscope, for the pores are only 30 and 200 μ in diameter. The bony partitions, however, are very thin so that a given volume of this type of bone also contains more vascular connective tissue than bone matrix (Fig. 3).

Although most bone can be readily classified into one of these three types, transformations of fine cancellous bone into coarse cancellous bone, and then into compact bone and back to coarse cancellous bone again are regular features of fetal and adult life, so that areas of bone can usually be found where intermediate conditions are present. The classification is, therefore, not an absolute one.

In the mammal compact and coarse cancellous bone are characteristic of the adult skeleton, and fine cancellous of the fetus. Fine cancellous bone may reappear in the adult in fracture callus and in pathological bony conditions generally.

In the mammal, again, compact and coarse cancellous bone are composed for the most part of finely bundled lamellar bone, whereas fine cancellous bone may be composed of (a) woven bone alone, (b) woven bone overlaid with lamellar bone, or (c) lamellar bone surrounding spicules of calcified cartilage.

In lower vertebrates both compact and cancellous bone alike are composed of coarsely bundled bone, either in its woven or parallel-fibered form. No true lamellation exists, but a pseudo-lamellation or stratification may appear because of the deposition of successive layers of coarsely bundled bone separated by cement lines. In true lamellar bone the stratification does not depend on cement lines, but on differences in orientation of the fiber-bundles in adjacent lamellae. In parts

of the bird skeleton compact bone may be composed of finely bundled bone, yet not have a stratified appearance because the fiber-bundles are all running in the same direction.

2. THE LAMELLAR SYSTEMS OF MAMMALIAN COMPACT BONE

Sections of mammalian compact bone show, at moderate magnification, three main lamellar patterns or systems—the concentric, the circumferential, and the interstitial. The concentric lamellar (Haversian) systems are the most conspicuous as a rule because of their rounded form with clear external boundaries (cement lines), central fine vascular canals (Haversian canals) and lamellation like the section of an onion (Fig. 5). These Haversian systems are of course in reality solid cylinders (except for the fine central canal) extending for considerable distances in the long axis of the bone. When the three-dimensional concept is desired, the term "*osteon*" is preferable to "Haversian system" as the latter term is too strongly associated with the two-dimensional appearances in cross section.

Between the Haversian systems the angular intervals are packed with so-called "interstitial lamellae," which from their architecture are obviously the remnants of past, largely eroded, Haversian systems.

As the free surfaces of a mass of compact bone are approached the Haversian systems disappear and are replaced by circumferential lamellar systems with much smaller curvature, conforming to that of the surface of the bone as a whole. It must be stressed that each of these three types of lamellar system is composed of identical finely bundled bone matrix and that stratification of lamellae, osteocytes, canaliculi, etc. is precisely the same in all. Only the completeness and curvature of the lamellae differ.

Besides the cement lines marking the extreme boundary of each Haversian system, other cement lines, straight, curved, indented, or branching, course in all directions through compact bone. The indented lines mark the limits of an earlier period of erosion before deposition started again (reversal lines), and the straight and curved lines, usually parallel to each other, mark resting stages in a process of advancing, but intermittent, bone deposition (resting lines).

Individual Haversian systems vary considerably in size and hence in the number of constituent lamellae. They may be almost perfectly circular in section, or may be round or flat ellipses or ovoids. In successive lamellae, the fiber-bundle direction may change through any angle from 0 to 90°, the stratification being most easily apparent when the angle is large. In ordinary stained sections, even though the individual fiber-bundles cannot be seen, the different directions of the fiber-bundles

in successive lamellae lead to sufficient differences in staining intensity for the lamellar architecture to be faintly discerned (Fig. 4). When the fibers are stained with silver the lamellation is quite evident (Fig. 7). With the polarizing microscope the lamellation is even more evident because the degree of rotation of the beam of polarized light varies from 0 to 90° as the fiber direction changes from being parallel to being at right angles to the direction of the incident light. The successive lamellae therefore appear as alternate light and dark bands when viewed through the analyzing prism.

The fiber pattern of an Haversian system is even more complicated than has so far been indicated. When a given lamella is followed longitudinally, the predominating fiber direction changes at successive levels because the fiber-bundles of such a lamella are not all parallel, but are regularly plaited or woven, the woof and web bundles crossing at about 45°. Moreover, fiber-bundles regularly leave one lamella and pass into the next. The fiber-bundle system within a given osteon is thus more of a continuum than the appearances in transverse section would suggest, and the superficial view that the osteon is composed of separate concentric cylinders is strictly erroneous. The lamella is therefore not a true structural unit: it is rather an optical artefact, and the osteon has its best analogue, not in an onion but in a machine-wound spool of cotton thread. (See also Chapter V for comments on the Haversian systems.) The osteon itself appears to have more of the properties of a natural structural unit, as it is apparently isolated from its neighbors by a cement "line" or wall. However, the osteones, if followed far enough along the axis of a bone, are seen to branch and anastomose with neighboring osteones as the vessels contained within the central canals branch and anastomose. The truth would appear to be that bone is *not* made up of structural units, in the sense of building blocks, at any level of organization above the sub-microscopic. The universal habit of speaking and writing as if it were, though perhaps a necessary descriptive convenience, is dangerous when it leads to a belief in the real existence of such units, and when elaborate mechanical analyses are founded on such beliefs (Murray, 1936). Bone, in fact, is a continuum, and its apparent unitary features are due to its conforming with external factors such as blood-vessel patterns, and not to the exhibition of any inherent property of growing, crystal fashion, by the accumulation of similar structural units.

Within a given osteon the osteocytes in their lacunae may occupy any site, without reference to the lamellar pattern (Figs. 5, 6, 7). They are, however, usually constantly orientated or polarized with respect to the osteon as a whole (Fig. 5), their longest axis being in the direction

of the long axis of the osteon, the intermediate axis being concentric with the lamellar system, and the short axis being radial. The cell processes in their canaliculi branch off from every aspect of the cell and lacuna, but soon turn so that they run radially for the most part, those of adjacent cells anastomosing freely (Fig. 6). Those nearest the central canal anastomose with the processes of lining cells of that canal, but those nearest the periphery of the osteon do not usually cross the cement line into neighboring osteones, though a few may do so. The canaliculi and fiber-bundles interlace in systematic fashion, the former passing at right angles through the meshes of the woven sheet of fiber-bundles which, as previously described, constitute the individual lamella as seen from its surface.

The central canals vary in size, the smallest having room only for a capillary blood vessel and a few resting osteoblasts closely applied to the bone matrix, while larger ones may contain an arteriole, one or more venules and a capillary plexus in addition to littoral osteoblasts and mesenchymal connective tissue. Larger canals still are best called Haversian spaces, and may contain bone marrow, fatty or hemopoietic, and the walls may be lined with active osteoblasts in some places, osteoclasts in others. Finally, Haversian spaces grade into frank marrow spaces and cavities. The Haversian spaces, as opposed to the canals, are associated either with Haversian systems in process of resorption, or reformation, or both simultaneously. Other vascular canals (Volkmann's canals) run through compact bone at right angles to the Haversian canals, which generally run parallel to the long axis of the bone as a whole. Volkmann's canals open either on to the periosteal or on to the medullary surfaces of the bone, and do not appear to influence the pattern of the bone matrix, for the lamellae are not arranged concentrically around them. Volkmann's canals carry blood vessels to and from the predominately capillary system inhabiting the Haversian canals. Nerve fibers have rarely been described in them or in the Haversian canals but probably exist, being difficult to demonstrate for technical reasons. The presence of lymphatics is very doubtful.

3. THE STRUCTURE OF COARSE CANCELLOUS BONE

The cancellous component of the adult mammalian skeleton is similar in fine structure to that of compact bone, except that complete osteones are present only in the thicker trabeculae, and for the most part the matrix consists of an irregular, haphazard "breccia" of osteon fragments equivalent to the interstitial lamellae of compact bone. Cement lines are abundant and very irregularly indented, indicating a complex past history of resorption and redeposition. Haversian canals are few,

the matrix evidently being nourished, via the system of canaliculi, from superficial vessels lying in the marrow spaces. Here and there fragments of calcified cartilage may be found embedded between the lamellar systems, evidently surviving remnants of the epiphyseal growth cartilage which have escaped the complex remodelling processes. Similar fragments of cartilage are normally found in the compact bone near the epiphyses and have a similar significance. The trabeculae of coarse cancellous bone are covered with resting osteoblasts closely applied to the surface of the matrix, with here and there areas of more active osteoblasts, and in other places, osteoclasts, indicating sites where deposition and resorption respectively are still taking place.

4. THE STRUCTURE OF FINE CANCELLOUS BONE

This type of bone is characteristic of the fetal skeleton, and of secondary centers of ossification in the epiphyses of growing bone, but is also found in early fracture callus and other pathologically induced new bone. It is of two main varieties, depending on whether the bone is being formed directly in membrane, or is replacing cartilage.

a. *Fine Cancellous Intramembranous Bone*

When first formed, fine cancellous intramembranous bone has a superficial resemblance to wire netting in histological sections, thin anastomosing trabeculae of woven bone alternating in a very regular fashion with spaces filled with loose embryonic connective tissue containing a central blood vessel (Fig. 3). A regular palisade of active osteoblasts covers the surfaces of the trabeculae. From the periphery of a mass of such bone pointed bony trabeculae project freely into the surrounding connective tissue, each covered with a single layer of osteoblasts except at the free tip where these cells form a rosette. Almost everywhere, but especially at the tips of the projecting superficial trabeculae, coarse fiber-bundles pass into the bone matrix from the surrounding connective tissue. Many of these bundles are obviously destined for inclusion in new bony trabeculae and hence are termed "*osteogenetic fibres*." With ordinary connective tissue stains the fiber-bundles lose their distinctness as they pass into the bone matrix owing, as we have said before, to a deposit of cement around them of similar refractive index. When stained for calcium salts the junction of the osteogenetic fibers with the bone matrix is often found to be uncalcified: in other words, a layer of "*osteoid*" commonly intervenes between osteogenetic fibers and fully calcified bone matrix. Such osteoid borders are not obligatory, however, as McLean and Bloom (1940) have shown.

The regular, even-meshed network of woven bone just described is

of short duration, for almost as soon as it appears remodelling processes begin. Many of the more centrally placed trabeculae are wholly resorbed, the osteoblasts being replaced by osteoclasts, and the vascular-connective tissue meshes, running together, forming large irregular marrow spaces. Other trabeculae, however, become thickened by surface additions, first of more woven bone, and later of fine-fibered bone, and simple lamellar systems appear, superimposed on a scaffolding of residual woven bone. In this way the very fine, regular network of woven bone is replaced by a coarser, more irregular network of mixed woven and lamellar bone. Eventually almost the whole of the original bone will disappear and lamellar bone will take its place, the lamellar systems becoming progressively more organized until the adult pattern of Haversian systems appears with its complementary interstitial and circumferential lamellar systems. The Haversian systems are formed by the addition of successive lamellae inwards from the walls of a largish marrow space carved from the original network of fine cancellous woven bone, until only a small central canal is left containing the pre-existing blood vessel. During this process a regular palisade of active osteoblasts is seen on the surface of the innermost lamella, but when the last lamella has been deposited these cells are transformed into a relatively inconspicuous layer of flattened cells lining the central canal.

b. *Fine Cancellous Endochondral Bone*

The growth cartilage at the epiphyseal line of a growing bone, just prior to its replacement by endochondral bone, has a very orderly, regular structure, consisting of a honeycomb of calcified cartilage matrix, the interstices of which are occupied by hypertrophic, degenerating cartilage cells. The cells die and disappear and are replaced by primitive marrow tissue which removes the transverse matrix partitions but leaves most of the vertical partitions intact. These later act as a scaffolding on which bone matrix is deposited and so the newly formed endochondral bone has an architecture prescribed for it by the cartilage which it replaces. In longitudinal sections such bone appears to consist of regularly spaced, longitudinal, and apparently isolated, bony trabeculae, separated by vascular marrow spaces. Each trabecula has a central core of calcified cartilage, and a peripheral layer of fine-fibered bone matrix. On its surface there is the usual palisade of active osteoblasts. In transverse sections, however, a cancellous architecture is apparent, and the bone superficially resembles the network of woven bone previously described. But on detailed examination major differences are seen, for the endochondral bony trabeculae are composed of fine-fibered bone, while the intramembranous trabeculae are primarily of woven bone.

Moreover the endochondral trabeculae have calcified cartilage cores which are not present in the intramembranous trabeculae.

Once formed, however, both types of fine cancellous bone undergo similar remodelling, some trabeculae being strengthened by surface accretion, others being removed entirely or in part by osteoclastic action, with the result that the older endochondral bone has a much coarser mesh, and much thicker trabeculae, while much of the calcified cartilage is lost. Only a minor part of the endochondral bone is destined to form compact bone with Haversian systems. Most of it is remodelled into the coarse cancellous bone of the adult skeleton, or else is removed entirely to make way for the marrow cavity. The endochondral bone of the secondary centers of ossification is formed in the same way as, has a similar architecture, and suffers similar remodelling to, endochondral bone formed from the growth cartilage. Apart from the occasional remnants of calcified cartilage in its make up, the remodelled endochondral bone is indistinguishable from remodelled intramembranous bone, both consisting of fine-fibered lamellar systems.

5. REMODELLING PROCESSES

The transient nature, in the mammal at any rate, of the fine cancellous bone formed directly in "membrane" or indirectly by replacing cartilage, and the processes of superficial accretion leading to the strengthening of some trabeculae, and of resorption leading to the removal of others, has already been described. Likewise the tendency for woven bone to be replaced by lamellar bone has been stressed, and we have considered the aggregation of successive lamellae into lamellar systems of which the Haversian system or osteon, is the highest expression. Even the osteon is not a static feature, however, for any section of compact bone, at any age, will show areas where older osteones are being resorbed and others where new ones are being formed. Osteon removal takes place by irregular enlargement of Haversian canals into Haversian spaces with the replacement of osteoblasts by osteoclasts, while new osteon formation is brought about by narrowing of Haversian spaces due to the centripetal deposition of successive concentric lamellae, and the reappearance of osteoblasts. As each interval between the termination of a phase of removal and the beginning of a phase of formation is marked by a cement "reversal" line, the pattern of such lines in a given area of lamellar bone affords graphic evidence of the complex rearrangement of lamellar systems which has taken place in the past. The interstitial lamellae are also obvious remnants of once complete Haversian systems and witness to the universality of the remodelling process.

V. "Membrane" and "Cartilage" Bone

Two types of bone formation are commonly described (1) *ossification in membrane*, or intramembranous ossification, and (2) *ossification in cartilage*, or endochondral ossification; bone formed by the former process being termed "membrane bone," and by the latter "cartilage bone." An extension of this concept leads to the description of whole bones of the skeleton as "membrane bones" or "cartilage bones." These terms need clarification and explanation, for as they stand they may be misleading.

First of all, the processes of bone deposition "in membrane" and "in cartilage" are fundamentally similar except that woven coarse-fibered bone is the usual type initially formed "in membrane" and fine-fibered bone "in cartilage." Both types, moreover, as a result of remodelling, give rise to lamellar bone with Haversian canals, etc. and become histologically indistinguishable except for the isolated remnants of calcified cartilage that may persist in bone formed "in cartilage." The terms "in membrane" and "in cartilage" therefore simply indicate the tissue being *replaced* by bone, and do not imply any fundamental differences in the structure of the bone itself.

It follows that "membrane bone" and "cartilage bone" are not distinct histological types, but differ only in their early developmental history. Further, the designation of a whole bone as "a membrane bone" or "a cartilage bone" is not based on adult histology, and does not mean that the entire bone has been formed by intramembranous or endochondral ossification exclusively. Almost all so-called cartilage bones are largely comprised of bone formed in membrane, while many so-called membrane bones have large components developed in cartilage. All that is in fact meant by the term "a membrane bone" is that, in its first appearance in the embryo or fetus, it is found replacing a pre-existing "membrane" of condensed mesenchyme. A "cartilage bone," on the other hand, is preceded by a cartilaginous model of the future bone which has differentiated from the mesenchyme before any bony tissue has been formed. The cartilage which is often found in the course of the development of a membrane bone is termed "secondary cartilage" because it does not appear until ossification has started. Thus we have the paradoxes that the clavicle and mandible are classified as membrane bones although much of the bone present has been formed initially in cartilage, while the long bones generally are classified as cartilage bones although the shafts, which make up the major portion of these bones, are almost entirely formed in membrane.

The term "ossification in membrane" perhaps needs still further clarification. It really refers to three distinct processes, according to the nature of the "membrane" that is ossified. In the case of the flat bones

of the skull vault and the shafts of the long bones generally, the term membrane is applicable, for ossification occurs in pre-existing, obviously fibrous structures, the membranous skull (desmocranum) and the periosteum respectively; and pre-existing fibers, together with a great number of newly formed ones, are incorporated in the newly formed bone matrix. In the case of the bones of the face, e.g. the maxilla and mandible, bone first appears in an area of mesenchyme in which definitive collagen fibers are not present until immediately before ossification begins, and such as do appear are at once incorporated in the bone matrix. In the third type bone develops among the dense fibers of a pre-existing fascia, aponeurosis, tendon, or ligament, and the newly formed bone is largely composed of such fibers, although some new fibers are almost always added at the last moment. These three types of woven bone are easily distinguishable before secondary remodelling takes place.

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PLATE I

(Magnifications are approximate)

FIG. 1. Osteocyte showing cell body and numerous fine protoplasmic processes. Schmorl's method. $\times 1275$.

FIG. 2. Periosteal callus from a fractured rat femur showing gradation from bone to cartilage. Weigert's haematoxylin and Van Gieson. $\times 95$.

FIG. 3. Embryonic woven bone. Sheep mandible. Wilder's silver method. $\times 95$.

FIG. 4. Lamellar bone showing Haversian systems. Transverse section of rabbit ulna. Haematoxylin and eosin. $\times 95$.

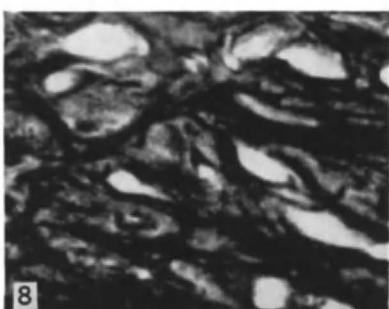
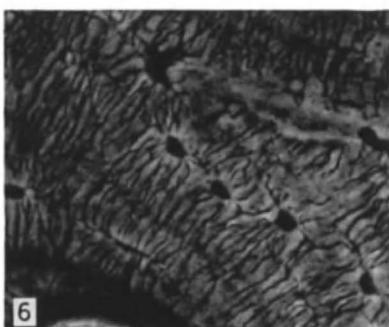
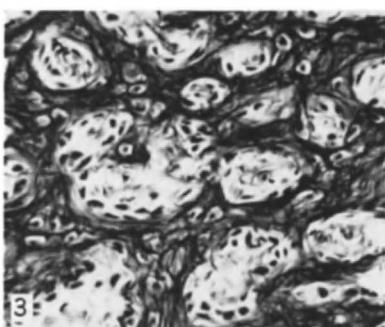
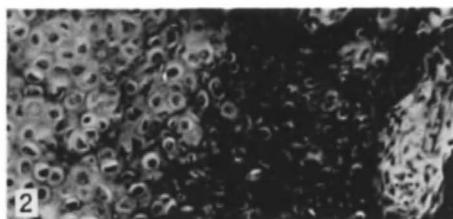
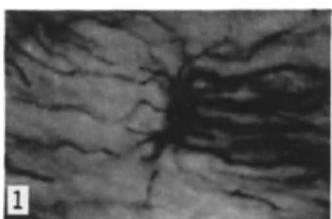
FIG. 5. Haversian system. Transverse section of rabbit ulna. Phase-contrast. $\times 190$.

FIG. 6. Parts of two Haversian systems. Transverse section of rabbit ulna. Schmorl's method. $\times 415$.

FIG. 7. Lamellar bone. Adult human parietal. Wilder. $\times 725$.

FIG. 8. Woven bone. Human fetal parietal. Wilder. $\times 850$.

PLATE I



CHAPTER II

BONE AS A MECHANICAL ENGINEERING PROBLEM

G. H. BELL

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I. Introduction

The subject matter of this chapter develops from some questions which seem to the writer to deal with problems of first-rate importance to the animal in whose body the bones are contained. At the risk of violating the convention which bans teleology from scientific writing it may be said that the bones of the skeleton are "adapted" to support the body, to aid locomotion, and to protect important parts. It is not the intention, however, to indulge in philosophical speculations as to how bones exhibit through their form and structure a high degree of mechanical fitness for their functions. The scientific attitude to such problems is expressed admirably by D'Arcy Thompson (1948) On Growth and Form, Chapter XVI, and we can accept his approval for searching for the "physical causation by which the material structure was so shapen to its ends."

This then is the justification for investigating bones as structures by "engineering" methods. In this chapter the shape, strength and elasticity will be thought of as the important features; the microscopic or molecular details will be regarded as of only secondary importance (*pace* other contributors to this volume) as these are merely the means by which the structural properties are achieved. This utilitarian attitude is exemplified at the every day level by the patient (and doubtless also by his surgeon) who enquires when his fractured femur will be able to bear his weight. He is most unlikely to be deeply concerned, even if he chance to be a histologist, with the details of osteoblastic activity.

Especially at the extremes of life the strength of the bones is of interest because at these ages the retention of minerals or the intake of vitamins may be deficient. This, however, is a static aspect of this biological problem. Since bone is a dynamic, living tissue which shows marked structural alterations in response to changes of stress and to vascular, endocrine, and nutritional influences it is proper to enquire how these alterations affect its physical characteristics. It is as well to say at the outset that in many cases the answers to such problems are disappointingly inconclusive while others are still controversial.

Many of these aspects of the physiology of bone are capable of investigation by direct observation, or by simple physiological or engineering methods. It is not surprising, therefore, that important scientific contributions began to appear about 200 years ago. The older work has been described in the concluding chapters of a most delightful monograph by Sir Arthur Keith (1919). Koch (1917) gives many older references and a valuable review is provided by Murray (1936). A synopsis of some of the material presented here has already been published (Bell, 1945, 1952).

II. Shape and Size

The femur has, in a wide range of animals, such a characteristic shape that anyone familiar only with the human form of the bone has little difficulty in recognizing non-human femora. Indeed even in the embryo the characteristic head, trochanter, and condyles are recognizable at an early cartilaginous stage. No doubt a similar, though perhaps not so striking, statement could be made about most bones of the skeleton. Characteristic shapes of skulls in races and in families are well known. The general shape of a bone must, therefore, be handed on by some hereditary mechanism. The nature of the mechanism is rendered, if anything, more incomprehensible by the beautiful tissue culture experiments of Fell (see Chapter XIV of this book) in which the growing chick femur retained its shape although isolated from all other tissues and deprived of the circulating blood which might bring hormonal substances. This self-differentiation continued in the absence of nervous influences and of muscular pull. It can thus be asserted that the general shape of this bone is determined partly by hereditary factors and partly by intrinsic factors. John Hunter described this power of self-differentiation somewhat picturesquely as a form of consciousness. Without invoking this real but mysterious power it is difficult to explain the remarkable recovery in form shown after fractures in children. It is difficult to control the fragments of a femur fractured at birth and indeed

good results may be eventually obtained in the absence of any attempt at control. The fragments are usually firmly united in 2 or 3 weeks and, even if they unite overlapping at right angles, remodelling is often so extensive and so efficient that it may not be possible from examination of X-ray photographs taken a year later to decide which femur had been broken (White, 1944). Older children also show considerable ability to smooth away such deformities but the adult can do this to a small extent only. Since in the adult muscular, nervous and hormonal influences are in full flower it is difficult to escape the conclusion that in the child the power of self-differentiation is resident in the bone itself very much as in the tissue culture experiments just quoted. But perhaps too much credit should not be given to this power since the final result of bone grafting to repair a gap may be the achievement, especially in young persons, of a bony structure complete with medullary cavity and with something much better than a distant resemblance to the original bone. This transformation occurs even if a graft of tibia is used to replace a gap in another bone such as the radius and even if living bone or dead bone or bone chips are used as the grafting material. No experiments or clinical cases could be found to show whether this transformation can occur in the absence of function or muscular attachment. Examples of successful grafting of this kind are given by MacEwen (1881, 1912), Bond (1913-14), Evans (1913-14), White (1944), Mowlem (1944), Wilson (1951) and many others. (See Chapter XVI).

Many illustrations can be given to show that the shape and size of a bone are modified by external factors, especially muscular action. For example Stieve (1927) removed two or three toes with their metatarsals from newborn rabbits. Eight to twelve months later he found the remaining foot bones thickened but not altered in length. He quotes similar cases in man where the remaining bones became shorter and thicker. Recently Ross (1950) described a laborer who had lost all the fingers of his right hand except the little finger. When he was seen, 30 years after the accident, the remaining phalanges and corresponding metacarpal had greatly increased and the man had a powerful and useful finger. Watt and Williams (1951) provide another illustration of the effect of activity. They found that rats fed on a rough diet requiring considerable mastication had heavier and thicker mandibles, especially at the sites of muscular attachment, than had rats on a soft pappy diet. The loss of muscular activity may produce atrophy of bone as in the experiments of Washburn (1947) and Wolffson (1950) and in the work to be described later on nervous influences on bone growth. Washburn (1947) removed one temporal muscle from newborn rats and examined the mandible 3 to 5 months later. The coronoid process, to which this muscle

is attached, is well developed at birth but disappears entirely when the muscle pull is lost. Similar experiments by Wolffson (1950) showed that after removal of scapular muscles the shape and size of the scapula was considerably diminished as compared with the control unoperated side. It is not to be inferred, however, that loss of muscular pull always results in bony atrophy. Appleton (1924-5) removed the gluteal muscles from the great trochanter of young rabbits without producing any alteration in the ossification or shape of the epiphysis. Furthermore Keck (1914) has described a one-month-old child showing congenital bifurcation of the leg with the tibia in one compartment and the remainder of the leg in the other. The tibia was nearly of normal shape although it had no muscular attachments; the lower half of the fibula, presumably because of its muscular attachment, was increased in size.

Although muscular action may be the main cause of hypertrophy it may not be the sole cause as Wermel (1935) demonstrated. He removed the radius or the tibia from various young animals and described the changes seen 2 to 4 months after the operation. The remaining bone, ulna or fibula, on the operated side was considerably thickened but also shorter than that on the control side. The thickening may be attributed to increased stresses on the remaining bone but this is not the whole explanation since the ulna thickened even if the nerves to the limb were cut to reduce muscular activity.

The accessibility of the tibia makes it especially suitable for observation or experiment. Although its triangular section is not the best for resisting stresses it is still a strong bone; it may, according to Messerer (1880) and Bernhard (1924), require 1000 kg hung on the tibia to break it. Bernhard described four cases of talipes equinus, three with resection of knee joint and all with atrophy of leg muscles, in which the tibia carried the weight of the body; in all the tibiae were round in section. If the anterior leg muscles are removed from a young dog the characteristic triangular cross-section is lost (Fick, 1857). All these findings are consistent with the idea that the shape of the cross section is a compromise between the intrinsic forces (due to weight bearing) which are best served by a circular section and the extrinsic forces (the pressure of the muscles on the lateral aspect) which produce the triangular outline with a prominent anterior crest. In the child, before walking begins, the tibial section is rounded. The following case described by Brittain (1942) may be another illustration of the balance of forces. "An injured workman had suffered more than 12 operations of bone grafting, both tibiae being used. Each bone had hypertrophied enormously." The results of muscular pressure in flattening the shape of the tibia can be compared with the effect of long-continued pressure

such as is produced by blood vessels or expanding cysts, or slowly growing benign tumors which, as is well known, produce similar alterations by a process of remodelling so that the smooth depression is lined by compact bone. The so-called triangular section of the tibia is extremely variable as examination of saw cuts across a few tibiae will soon reveal (Macqueen, 1951) and this variability has frustrated attempts in this laboratory to investigate the elastic properties of this bone in man during life along the lines suggested by animal experiments (Weir, Bell, and Chambers, 1949).

As this chapter is concerned with what may be termed the gross properties of bones, it is proper to refer to the work of Mack, Brown, and Trapp (1949), and Mack (1950). A microphotometer has been applied to roentgenograms of living human bones taken alongside step wedges so that a graph of the amount of mineral matter at any cross section of, for example, a hand or foot or knee can be obtained. It appears that the ends of long bones (such as the femur) and also the calcaneum change rapidly in density when the calcium intake is altered whereas the phalanges alter only slowly. As much of the work described in this chapter suggests that, apart from rickets, the quality of the bone material remains relatively constant, it seems quite justifiable to use the amount of mineral matter (obtained from the density of the X-ray photograph) to indicate the amount of bony material present. As so few methods of investigating bone in man are available, it will be of interest to learn the results which accumulate by the use of this laborious and expensive method.

III. Growth of the Skull

Observations on the growth of the skull are often difficult to reconcile among themselves or even with those just made on the growth of the tibia. In Washburn's (1947) experiments already quoted, the loss of the temporal muscle affected the form of the bone at the point of insertion but did not alter the shape of the skull at its origin. This may be due to the reduction of stress produced by the large area of origin but it might mean that the formation of the skull is determined by other more important factors. The cranium at all stages of growth is a good fit to the growing brain and does not appear to restrict its growth. The fact that the two grow in step with one another is responsible for the difficulty in deciding which sets the pace. *In utero* the intracranial pressure must be offset to some extent by the external pressure of the liquor amnii. The loss of this equilibrium at birth does not apparently affect the concomitant expansion. The condition of hydranencephaly

(Hamby, Kraus, and Beswick, 1950; MacDougall, 1951) is worth quoting as it could be interpreted as meaning that the box determines the size of the contents. Children suffering from this disorder appear to be normal, at least for the first few weeks of life, even to the experienced pediatrician in spite of the severe cerebral agenesis. The cranium may contain only the cerebellum, the basal nuclei and a small portion of the occipital lobes. The frontal and parietal lobes are then represented by one vast cyst but there is at first usually no evidence of increased tension. In other words, in spite of the absence of a large part of the brain, the brain case is normal. In experiments designed to investigate the growth of the brain Barron (1950) made incidentally some interesting observations on the growth of the skull which are relevant here. In three different sheep fetuses about 73 days after insemination he destroyed large parts of the cerebral hemispheres by operating through the uterine wall. The fetuses were examined about 60 days later near the end of the gestation period and inspection of the skulls, unfortunately not checked by actual measurement, showed no difference in shape and capacity from those of normal controls. The rise of intracranial pressure in hydrocephalus in infants produces enlargement of the cranium and in long standing cases accessory bones may appear in the dura. When the sutures are closed a rise of internal pressure can produce very little expansion but various parts of the internal surface of the skull may be eroded (Russell, 1949). Premature synostosis of the cranial sutures (for example, scaphocephaly) interferes with the growth and function of the brain unless the closed suture lines are excised. Weinnoldt (1922) gives examples of this condition.

In old age there may be atrophy of the frontal lobes and new bone may be laid down between the dura mater and the inner table of the skull, the area of new bone corresponding to the area of atrophy (Harris, 1933); the external shape of the skull is not altered. If one assumes, and it seems reasonably safe to do so, that the brain and bone changes occur simultaneously and also that the intracranial pressure is not likely to decline in these cases, it is difficult to account for this senile hyperostosis. In mild degrees of this atrophy the hyperostosis, by following up the retreating cerebrum, could deepen the depressions for gyri and blood vessels (Weinmann and Sicher, 1947). Examination of such a skull might easily lead to the conclusion that the gentle pressure of the contents had led to resorption of bone with deepening of the depressions. It would be difficult to disprove either explanation. This filling up of a space may be compared to the situation after a dental extraction where the socket soon fills up with immature bone in the absence of pressure. The final shape of the jaw, however, may be

determined by the presence of other teeth or the use the jaw is put to. Such examples are a warning against looking for a complete explanation of bone growth in terms of applied forces. It is difficult to imagine mechanical reasons for the formation of ectopic bone in the eye (Ballantyne, 1940), or in the lung (Davson, 1945).

IV. Cancellous Bone

The head and neck of the femur have exercised a strange fascination over the minds of investigators since the time of Meyer and Culmann. The latter, an engineer, examined a frontal section of the upper part of a femur exhibited by Meyer (1867) who was an anatomist and found in the pattern of the cancellous tissue a resemblance to the pressure and tension lines "that he had taught his pupils to draw in a crane" (Murray, 1936). Keith (1918) has pointed out that F. O. Ward in his "Human Osteology" of 1838 had expressed the same idea less elaborately when comparing certain architectural features of the femoral neck to a triangular bracket supporting a street lamp. This idea has found acceptance by the tidy-minded and mathematically inclined including Wolff (1892) who enunciated the Law of the Transformation of Bone, but it is vigorously rejected by a group of forceful writers who find many flaws in this trajectorial theory. Many in both groups tend to treat the bones as if they were merely static weight bearers isolated from the rest of the body. D'Arcy Thompson (1948, pp. 989 and 1018) asks us, on the contrary to keep in mind the whole animal. He points out that the bony skeleton provides the struts (compression members) of the mechanical structure while the muscles and ligaments act as ties (tension members). After describing various types of bridges, both those designed by the engineer and those formed by the vertebral column with its ligaments and muscles he says ". . . as biologists, we may go as far as to say that even the bones themselves are, only in a limited and even in a deceptive sense, separate and individual things. The things that link bone with bone, cartilage, ligaments, membranes, are fashioned out of the same primordial tissue, and come into being *pari passu* with the bones themselves."

The internal cancellous structure of many bones, especially the bones of the leg and foot, adds greatly to their strength and makes this contribution at the minimum cost of material. The trajectorial theory asserts that the lines of the bony trabeculae correspond to the lines of maximal pressure and tension as shown by mathematical analysis. Further, the thickness of the trabeculae is greatest and their spacing smallest where the stress is greatest (Koch, 1917). The pattern of the

trabeculae in the cancellous tissue of the head of the femur and in other situations such as the calcaneum has such a regularity and constancy that one supporter of the trajectorial theory, perhaps with more wit than justification, described the pattern as a crystallization of the lines of force.

The trajectorial theory requires for its substantiation two things (1) proof that tension or pressure can produce trabeculae and (2) demonstration that the trabeculae cross at right angles as the lines of maximum pressure and maximum tension must do. Neither of these conditions is easy to satisfy nor is the assertion that the trabeculae are, in fact, trajectories easy to disprove. There is no need to describe or illustrate these trabeculae here as they are to be seen in many anatomical text books.

The long paper of Koch (1917) contains several much quoted diagrams of trajectories in models and an analysis of the stresses in a single pair of normal femora. He is a convinced upholder of the theory that the trabeculae are produced by tension and pressure as demanded by the trajectorial theory. Since several critics had dismissed the theory by showing that the trabeculae do not always cross at right angles he was careful to point out how easy it is to be deceived by the cursory inspection of two intersecting curved lines. Two curves approaching one another with their concavities facing may appear to meet at an acute angle even when they are, in fact, at right angles at the point of intersection; apparently obtuse angles may be formed by the converse process. Koch believes that the stresses due to weight bearing on the femur in man are much greater than those due to the action of the muscles. He calculates that, even in running, there is a factor of safety, i.e. ratio of breaking stress to actual stress due to the weight of the body, of 6 in the weakest section. He also calculates from their cross section that the thigh muscles could develop only one seventh of the maximum strength of the femur, but no attempt was made to calculate the bending stress in the femur produced, for example, by the adductor magnus muscle. It still has to be decided whether body weight or muscles produce the greater bending stress. The work of Koch has recently been extended by Marique (1945).

Jansen (1920) is a vigorous critic of the theory and asserts that the muscular forces may exceed the forces due to the body weight. The bone elements on the upper convex surface of the femoral neck are stretched by the body weight but compressed to a greater extent (he says) by the glutei, obturator, pyriformis and gemelli muscles attached to the pelvis. He gives illustrations of the trabeculae in coxa vara in which the angle between the head and neck of the femur is nearer to a right angle

than in the normal bone. In weight bearing in coxa vara there must be a greater than normal stretch on the bony elements on the convex upper part of the femoral neck and more than normal compression on the compressed concave side. Since there is, in fact, less bone on the tension side and more on the compressed side the evidence is against the notion that tension produces bony trabeculae. In addition to the frontal section, which had been seen by Meyer, Jansen shows several sections of the femur in other planes in which the lines of the pattern certainly do not cross at right angles. Cursory inspection of the lower end of the femur might give the impression that the trabeculae are parallel as the trajectorial theory demands but closer examination shows that they are quite irregular in places and are braced by oblique trabeculae which could be either in compression or in tension according to the posture of the subject.

Jansen also analyzes the cancellous pattern of the ankylosed knee joint and the vertebrae of scoliotic spines to show that pressure and not tension is the effective stimulus which causes deposition of bone. If tension and compression are equally potent osteogenic agents he asks why do tendons not ossify or why does the dura mater, which is under the same tension stresses as the skull, not ossify? On the other hand, it might quite properly be asked why does compression not cause ossification of intervertebral discs. Koch (1926-7) claims that in certain peculiar instances, as in the tendons of the kangaroo's tail, tension can produce bone but it is difficult to exclude pressure effects as the tail is often used as a third foot.

The simple conclusion is, of course, that stress is not the only factor operating. Cancellous tissue occurs in bony outgrowths which have no observable function, fine cancellous tissue is found in the bones of the paralyzed limb of poliomyelitis. Even after amputation of a large part of the femur the head and neck of the stump may show fine spongy trabeculae; two cases are well illustrated and discussed by Townsley (1944). The spongiosa is much thinned but the systems of trabeculated plates are still recognizable. In neither of these cases did the stump bear weight but the hip muscles although atrophied were still able to act on the stump. Weinman and Sicher (1947) also give an illustration of the head of a femoral stump.

The trajectories as drawn in a diagram are only a mathematical expression as are the "lines of force" around a magnet. The resemblance between the diagram and the spongiosa depends on the number of lines drawn but this number, as Triepel (1922) points out, is the purely arbitrary choice of the illustrator and the beautiful lattice pattern would be replaced by solid black if an infinite number of lines were drawn.

It is doubtful if trajectories can be drawn with certainty except for homogeneous solid bodies. Bone is not solid and is histologically not homogeneous. The ratio of twisting to bending stress, 0.27 (Bell, Cuthbertson, and Orr, 1941), also shows that bones are not homogeneous (or isotropic). One opponent (Dixon, 1910) of the trajectory theory believes that the cancellous tissue in the upper part of the shaft of the femur is arranged in spirals, the disposition of the lamellae being such as to resist shearing.

Using polarized light Milch (1940) studied the stress (photoelastic) patterns in suitably loaded catalin (phenyl formaldehyde resin) models of the femur. It is difficult to know how far this material represents the actual conditions obtaining in a non-homogeneous non-isotropic material like bone.

This discussion about the relation between stress and bone formation should not be dismissed as mere academic speculation because the stress acting at a fracture site may be almost the only factor under the surgeon's control. Charnley (1953), mainly as a result of his experience of compression arthrodesis of the knee, believes that compression induces bone formation. In this operation the joint surfaces of the femur and tibia are sawn off, two nails are pushed transversely through the ends of the bones and are approximated by turnbuckles. With S. L. Baker (Charnley, 1953) he has demonstrated union of the two bones by bony trabeculae in biopsy specimens taken 4 weeks after the operation. He believes that cancellous tissue with its rich blood supply is a more favorable site for demonstration of the value of compression than is compact cortical bone. Watson-Jones (1952) criticizes Charnley's conclusions by saying that his work only proves the value of immobilization and that the bones unite in spite of the pressure. It could also be claimed that the good contact obtained by pressure prevents shearing of trabeculae growing across the fracture and so leads to early union. Even if these theoretical objections are accepted Charnley's conclusions on the value of compression cannot be easily set aside. Friedenberg and French (1952) investigated in 27 dogs the healing of fractures of the ulna which were compressed by a calibrated spring at the end of an intramedullary nail. Over a certain range of pressure union was more rapid than at higher or lower pressures but it was not possible to decide whether the rapid union was due to good fixation or to stimulation of osteogenesis. At the fractured ends of a bone the lacunae become empty for about a centimeter and the ends of the compact bone, which were in contact in these experiments, may be regarded as dead. These experiments are, therefore, not properly comparable to Charnley's clinical ones where the pressure was borne by living cancellous bone. Eggers,

Shindler, and Pomerat (1949), by three saw cuts in the cranium of a rat, made a tongue of bone which was pulled to one side by rubber bands or cotton threads attached to hooks set in the skull. As callus was produced most abundantly in the saw cut which was narrowed, the authors suggested that osteogenesis was stimulated by a "contact compression factor." Even Charnley is, however, unable to accept this evidence, as the forces involved compared with those in his own experiments must have been quite small and healing would in any case occur more quickly in the narrowed saw cut. An apparent anomaly occurs in the jaw where a tooth can be moved along by gentle pressure on it, as in orthodontic treatment; bone is absorbed where there is pressure and laid down where there is traction. It is, however, important to recall that a tooth is attached to and slung in its bony socket by a fibrous layer, the periodontal membrane, and the force applied to the tooth is not directly applied to bone.

The evidence although by no means conclusive is on the whole in favor of the idea that compression stimulates osteogenesis. It may be that the situation is radically changed by the intervention of a fibrous layer such as the periodontal membrane in the instance mentioned above or the periosteum in the case of an aneurysm pressing on bone. In these cases bone absorption tends to occur.

V. Bone Strength

The work described up to this point has been concerned with the general effects of forces but has only rarely dealt with the problem quantitatively. The earliest work available for consultation was that of Messerer (1880) in which the strength of the various parts of the skeleton, including the skull and long bones, taken fresh from cadavers is listed. The bending and twisting moments required to break the bones were measured and, what is more surprising, the breaking stresses were calculated in some instances. The testing machines used are illustrated and are the same in principle as used by the present author 60 years later (Bell, *et al.*, 1941). Messerer's results are still valid. Evans and Lebow (1951, 1952) have recently investigated the tensile strength of specimens from various human bones and compared the strength of compact bone from different parts of the same bone. The tensile strength varied a little in the range 11,000 to 12,000 lb/in.² according to the site of the specimen but a significant increase (32%) in tensile strength was observed in specimens dried at room temperature.

In planning the work done by the author and his colleagues, the first essential was to decide on the method of measuring the strength of the

bones and in this we were guided by the accumulated experience of engineers on more familiar structural materials. The more usual and more reliable tests consist in finding the load necessary to break a test sample. The engineer is, of course, usually able to choose his specimen and to cut it and shape it to suit his testing machines. This is not advisable with bone because the shaping of a specimen would certainly open up Haversian or vascular channels which would be sites of weakness. It is necessary, in other words, to use whole bones carefully cleaned to avoid surface damage. It seemed reasonable on biological grounds to use tests which produced straining actions likely to occur in the living animal. Four kinds of straining action are possible—axial compression, axial tension, bending and twisting. There are great technical difficulties in obtaining axial compression; a slight eccentricity of loading gives fallaciously low results and it is difficult to prevent the failure (collapse) of the ends of the bone where the force is applied. In any case true axial loading seldom occurs in the body; for example, the line joining the head and condyles of the femur lies mostly outside the shaft. The weight of the body produces bending of the femur and the action of the muscles attached to it must also produce bending, for example the adductor

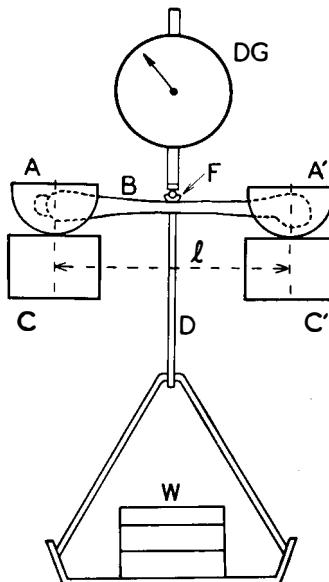


FIG. 1. Apparatus for measuring elasticity and bending strength of bones. *B*, bone. *A*, *A'*, semi-cylindrical ends cast on to bone ends. *C*, *C'*, supports. *D*, hook covered by layer of fiber *F*. *W*, load. *DG*, dial gauge. *l*, span. Reproduced by kind permission of the Editor, from *J. Bone and Joint Surgery* **31B**, 445 (1949).

magnus attached to the shaft must have a bending action when the head and the condyles are fixed. These considerations demand a bending test as being an approximation to the physiological actions. It is most unlikely that axial tension occurs in life; even in skeletal traction the stress must be borne by ligaments and muscles. Twisting must, of course, occur frequently in life and twisting tests are easy to carry out, if difficult to evaluate. Although a case can be made out for impact tests, since bones are frequently broken by impact, they are difficult to evaluate. Even where a member such as a piston rod is subjected to impact engineers place greater reliance on tests using static loading than on impact tests.

Since it is necessary at this stage to differentiate clearly between the strength of a bone and the strength of the bony material (breaking stress) brief descriptions of the method of carrying out bending and twisting tests will be given. The bending test is carried out as in Fig. 1. The actual dimensions of the apparatus depend, of course, on the size of the bones. Semi-cylindrical pieces A and A' of hard resinous material are cast on the ends of the bone B to prevent axial rotation and to give an accurately measurable span l . The bone is laid horizontally on two solid steel supports C and C' . A strong wire D is hooked over the bone at F (where the wire is protected with red fiber) and carries a pan for weights W . The bending moment M at the point F is $\frac{WL}{4}$. As the weights

are increased the bone sags and the amount of the sag "y" is read off on a dial gauge DG . The weight is increased gradually until the bone breaks. The bending moment at breaking is a measure of the strength of the bone as a whole; it is obviously dependent on, among other things, the size of the bone. The stress or force per unit area, which is greatest on the outer bony elements at F the mid-point of the shaft, can be calculated from a knowledge of the external and internal dimensions of the cross section of the shaft. Stress = $\frac{WLD}{8I}$ where D is the external depth

of the mid-section and I the moment of inertia of the cross section. If W is the load required to break the bone the formula gives the breaking stress on bending S_B , which is a measure of the quality of the bone material and, since it is expressed as force per unit area, it is independent of the size of the bone. By fixing one end of the bone and applying a load through a lever to the other the twisting moment at breaking can be found; this is, of course, dependent on the size of the bone. The breaking stress on twisting S_T can be calculated by means of a well known formula if the dimensions are known; S_T is also independent of the size of the bone. It was found that the scatter of results was very

much greater in the twisting tests and in later experiments only S_B has been measured. Since the strain or elongation of the bony elements at the mid-point of the bone can be calculated from y it is possible to calculate Young's modulus of elasticity (or stress/strain), which is best thought of as a measure of the stiffness of the material. Two errors enter into these calculations, the first biological and the second mathematical. It is not possible to dissect out bones adequately and carry out the necessary measurements without allowing them to dry a little. Tests showed that this error is not serious (but see Evans and Lebow, 1951) and in any case it applies to all the experiments. The second error arises from the assumption made in the calculations that the bones have a regular elliptical cross section. This is, of course, not quite true. The variations in the cross section along the shaft also introduce a slight error for which an allowance can be made. The information gained about the quality of normal bone will be described first and then the variations obtained under experimental conditions.

The breaking stress in bending in rat femora was usually about 27,000 lb/in.² or 19 kg/mm². The value obtained in a few puppy bones and in some sheep bones (Bell and Weir, 1949) was of the same order. These values are higher than those given by Rauber (1876) which lie between 9.25 and 12.41 kg/mm², but this may be due to a difference in method, as he used little pieces taken out of human bone. The values given by Messerer (1880) for humerus, femur, and tibia (male and female) are scattered over the range 10.4 to 19.8 kg/mm². The breaking stress on twisting in our experiments was 9,500 lb/in.² (6.7 kg/mm²). Messerer (1880) obtained the value of 5.75 kg/mm² in the femur of a 29-year-old man. Hard wood, cast iron, and mild steel, give values of approximately 10,000, 40,000, and 70,000 for bending and 1000, 24,000, and 40,000 for twisting. The ratio of breaking stress in twisting to that in bending was found in our experiments to be about 0.3, which is lower than that found in isotropic material like steel (about 0.5) and much higher than that found in wood (about 0.1). There is evidently some weakness in bone at the interconnections between longitudinal fibers; it is certainly not isotropic. Young's modulus is about 1.6×10^6 lb/in.² (1100 kg/mm²) in rat bones. Rauber (1876) has given results of 2000 kg/mm² for human bones. These values are about one tenth of that for cast iron and about one twentieth that for steel.

These figures show clearly what a remarkable material bone is since it has, although three times lighter, the strength of cast iron and is very much more flexible. No doubt this flexibility is biologically useful since it helps bone to absorb sudden impacts. But bone is remarkable in another respect since it remains elastic up to about three quarters of

the breaking stress. In other words the load-deflection graph (W against y), or the stress-strain graph, shows no deviation from a straight line until about three quarters of the breaking load or breaking stress is reached and even after that the falling over of the curve is relatively slight. (This has since been confirmed by Evans and Lebow, 1952). The strain at the upper limit of elasticity is about 1.4% while that at breaking is about 1.8%. Our original work (Bell, *et al.*, 1941) was begun just before the war when nutritional propaganda was directed to increasing milk consumption on the pretext of avoiding mineral imbalance. The question arose as to what would happen to bone strength if the calcium intake was drastically reduced. It did not seem feasible to obtain sufficient bones at post mortem from children with reliable histories and experience showed that such work would almost certainly have been futile. Our first experiments were carried out on rats about 6 weeks old which received an excellent diet varied in respect only of the salt mixture so that a range of diets containing 0.075 to 1.39 g calcium per 100 g diet was obtained. After 8 weeks on this diet the animals were killed and the femora were removed for the bending and twisting tests. Maximal values of bending and twisting moments and thickness of the cortical bone at the mid-point of the femoral shaft were reached on a diet containing 0.36% calcium; increases beyond this produced no improvement. On a diet containing 0.2% calcium the intake of calcium was down by 20% and the strength of the bones fell by 20%. At the lowest level of calcium the intake of calcium was about one third of the maximum and the bones were about half as strong as those produced on an adequate diet. In spite of the range of dietary calcium offered to the animals the external dimensions of the femora were unchanged and the breaking stress in bending and twisting were unaltered as was the calcium percentage in the bones. The sole alteration, and this occurred only when dietary calcium was less than 0.36%, was in the thickness of the femoral cortex. It is to be noted that a luxus intake of calcium did not produce what might be called "super" bones. If the shaft of the femur could be compared to a factory chimney it could be said that on a good diet over a wide range of calcium intake the quality of the bricks is always the same (the best) and the external dimensions and shape are the same. The only possible variation is in the number of bricks forming the thickness of the walls up to a certain limit since the central space cannot be encroached on to more than a certain fixed amount. It is easy to see now that random human specimens would have been unlikely to yield useful information as breaking stress measurements do not reflect at all great changes in dietary intake of calcium provided, as shown below, that the vitamin D supply is adequate.

The picture is, however, quite different when rachitic bones are examined (Bell, Chambers, and Dawson, 1947; Weir, *et al.*, 1949). In these experiments, each lasting 4 weeks, rats about 50 g in weight were divided into three groups *R*, *N*, and *S*. *R* received the well known rachitogenic diet, Steenbock No. 2965; *N* received this diet with the addition of vitamin D; *S*, which acted as controls, received a good stock diet. The *R* group showed gross evidence of rickets; the epiphyseal lines of *N* were quite narrow but growth was rather poor. The bones produced on the poor diets were, of course, smaller and their strengths poor as shown by the bending moment at breaking. The average ash content of the femora in *R* was 36%, in *N* 43%, and in *S* 60%, and the corresponding breaking stresses S_B were 12,000 lb/in.² (8.4 kg/mm²), 18,500 lb/in.² (13 kg/mm²), and 27,000 lb/in.² (19 kg/mm²); Young's modulus *E* in the three groups was 0.6×10^6 lb/in.² (420 kg/mm²), 1.0×10^6 lb/in.² (700 kg/mm²), and 1.6×10^6 lb/in.² (1120 kg/mm²). The three diets produced significant differences in percentage of ash, S_B , and *E*. Although inspection of the graphs suggests a high correlation between percentage of ash and S_B and between percentage of ash and *E*, rigorous statistical treatment does not allow such a claim (Perry, 1949). The stress-strain diagram of the group *N* bones showed for a given stress a much greater strain but the elastic limit was reached at the same strain as before, namely about 1.4%. The deviation from linearity at higher loads is markedly greater in the rachitic bones than in the controls. The undoubtedly high correlation between S_B and *E* suggests that the quality of bone material could equally well be measured by *E*. As *E* can be obtained without destroying the bone it is at least theoretically possible to design a method of measuring bone quality that could be applied in man. Unfortunately the difficulties as mentioned earlier are very great since there seems to be no easy method for determining the shape and size of the cross section of a living bone.

The poor quality of the bone in rickets could theoretically be due to (1) an alteration in the mineral part of the bone, (2) an alteration in the protein matrix, (3) or simply to the greater ratio of collagen to mineral matter. No defect in the inorganic material could be detected in the rachitic specimens. The ash had the same Ca/P ratio (2:1) and the same percentage of Ca and P as the control bones. The X-ray diffraction patterns of all the bones were the same with the same degree of orientation in a direction parallel to the bone length. (This, however, is at variance with the results of Reed and Reed (1942, 1945). At the time of these experiments the quality of the protein could be judged only by X-ray diffraction, which gave the same picture in all cases, but recently in this laboratory Perry (1954) has examined by chromatographic methods a large number of rat bones produced on rachitogenic

and normal diets. The protein of the bone was hydrolyzed and desalting and run with various solvents. There was no difference in the amino-acid content either qualitatively or, as far as could be judged, quantitatively. The amino-acid pattern was similar to that of collagen from other sources. Furthermore, she found no difference in the specific gravity of fragments of bones from the three groups. This means that a given amount of mineral matter or matrix must have occupied about the same volume in the rachitic and normal bones. In the 1947 experiments the ratio of breaking stress on twisting to breaking stress on bending was the same in groups R, N, and S. The only difference that has been demonstrated between rachitic and normal bones is in the ratio of inorganic to organic material; the actual materials seem to be the same in both cases.

Now that we know some of the static physical properties of rachitic bone, it is worth while speculating on the cause of rachitic bowing, a prominent feature of human and canine rickets. Macewen (1880) thought that the curves were not likely to be due to muscular action since the muscles are weak and flabby in rickets. Bowing was seldom seen in the upper limbs unless the child crept about on all fours and so used them for weight bearing. The changes in shape were seen mainly in the lower extremities although both extremities were equally affected by the disease process as judged by the swelling of the epiphyses. None of our rats, some of which had very severe rickets, showed bowing of the limbs even though, as Perry (1954) showed, the rachitic bones had to support a relatively greater body weight as shown by an increased ratio of total body weight to weight of femur. Harris (1933) gives X-ray photographs of rachitic rabbits and guinea pigs with but little bending of the bony shafts in the limbs. Weakness by itself is not sufficient to cause bending. Some of our animals, (Bell *et al.*, 1941) on a very low calcium diet had such fragile bones that extreme gentleness in dissection was needed to keep them intact for the mechanical tests but these bones were of normal shape. Again the poorest rachitic bones had less than half the breaking stress of normal bones and yet showed no bowing; puppy bones of the same breaking stress showed bowing (unpublished experiments). This leads to the conclusion (Bell *et al.*, 1947) "that the rachitic deformities occurring in man and the dog cannot be explained on simple static structural properties, such as those which would be considered by an engineer dealing with a steel tube; the deformities are more likely to be due to an abnormal reaction of the living animal to the bending forces, perhaps best described as a disorder of bone modelling. It is, however, not easy to see why one species should show rachitic deformities and another none at all."

The "stresscoat" technique has been used recently to investigate the

strain when bones are loaded. Küntscher (1935) declared that bones were too complicated in shape to allow calculation of the forces acting at any one point when the bone is loaded. The strain could, however, be shown experimentally by covering the bone with colophonium resin and then applying a force; the stretched lacquer cracks, when a certain strain is reached, in lines at right angles to the greatest extension. Küntscher showed clearly the increased stress around weak spots such as the mental foramen when the jaw was compressed. More recently Gaynor Evans and his colleagues studying similar problems have used an improved brittle lacquer called "stresscoat" which is used industrially to detect weak spots in machine parts (Evans, 1948; Evans and Lissner, 1948; Evans, Lissner, and Pedersen, 1948; Pedersen, Evans, and Lissner, 1949; Evans, Pedersen, and Lissner, 1951). This stresscoat cracked when extended about 0.001 inch per inch (0.001 cm per cm). The cracks were filled with a dye or India ink to facilitate photography during the application of the load to the bone (usually the femur). The stress may be calculated if the modulus of elasticity of the bone is known. In this way the site of maximum strain, and, therefore, maximum tensile stress, can be shown for any chosen form of loading, either static or dynamic. As the common fracture sites of the femur are similar to those obtained by loading the femur, these authors conclude that most fractures in life occur under tension stress. The methods and results of methods of measuring strain in bones have been recently reviewed by F. G. Evans (1953).

VI. Hormonal Effects

The endocrine glands are well known to have an influence on bone growth and on bone shape. It is not possible to imitate experimentally all the conditions occurring clinically but the effects of administration of some hormones on the physical properties of rat bone have been investigated (Bell and Cuthbertson, 1943). Injection of anterior pituitary extracts containing growth promoting activity into young rats accelerated growth and produced heavier and stronger bones but the increase in strength was due to the increase in the dimensions, the bone quality as judged by breaking stress was perhaps a little lower than in controls. Injection of large doses of estradiol produced slightly heavier bones with increase in dimensions but no alteration in bone quality. Parathyroid hormone treated animals also produced bigger and stronger bones without any alteration in bone quality. Animals given thyroid extract in their food nearly up to the maximum tolerable amount lost weight and had weaker bones than controls but the quality of the bone material was unaffected.

The striking feature of these hormonal experiments is the complete

lack of success in altering the quality of the bone material or in producing bony deformities. It may be that here again we are confronted with species difference in susceptibility to hormones but again it seems necessary to invoke some disorder of the modelling process to account for the deformities seen clinically. Eventually, it may be found true that, in the absence of rickets, bone is formed according to a single recipe from which the body cannot depart. Even in the case of rickets there is no need to assume that any abnormal material is laid down.

VII. Nervous Influences on Bone

While it is well known that bony changes accompany nerve lesions, there is considerable doubt as to the precise relationship between the two. If bone possessed no nerves then any alterations occurring after, say, section of a nerve could be described as secondary and merely a consequence of disuse, lack of muscular pull, or even of circulatory changes. But both clinical and histological observations show that bone does possess nerves. Osteotomy under local anesthesia is painful unless a local anesthetic agent is put into the bone as the cut deepens; tension of the periosteum gives rise to pain as does inflammation of bone. Using silver methods, de Castro (1925, 1930) has shown that nerve fibers travel along with the blood vessels in growing bone (but not in adult bone) and end in contact with the osteoblasts; these nerves were regarded as autonomic in origin. Hurrell (1937) has, however, been able to demonstrate a rich nerve supply in adult bone passing either with the nutrient artery to the marrow vessels or from the periosteum to end near the bone cells. There is no evidence for the passage of nerve fibers from the cells of the anterior horn of the grey matter of the spinal cord to bone (Corbin and Hinsay, 1939). Since there is apparently an anatomical basis for it the possibility of nervous influence on bone growth cannot be easily excluded.

The effects of nerve lesions on bone have been studied both experimentally and clinically. In many experiments, however, the work is somewhat unsatisfactory as few animals were used and the observations were qualitative rather than quantitative. There is always great difficulty in differentiating between nervous and muscular influences since nerve section leads to inactivity of muscles and it is usually impossible to detach muscles from bone without interference with the nerve and blood supply of the bone. Schiff (1854) and Naase (1880) reported atrophy of bone after section of sciatic and femoral nerves in dogs and attributed it to the loss of some trophic factor and muscular impairment. Grey and Carr (1915) showed that section of the posterior roots of the lumbosacral plexus in a dog brought about no bony change whereas

section of anterior roots caused atrophy in the bones of the hind limb. Earlier and more rapid atrophy was, however, produced by immobilization in plaster of Paris. These experiments were confirmed on rabbits. No acceleration of the atrophy produced by immobilization was seen after simultaneous ligation of the femoral artery or section of the sciatic nerve. Elloesser (1917) in his work on experimental production of Charcot joints also showed that section of the posterior roots caused no bone atrophy. Excision of the brachial plexus in puppy dogs has been carried out by Pottorf (1916), Howell (1917) and Allison and Brooks (1921); similar experiments on rats are reported by Armstrong (1946). In all, growth in length was much less affected than growth in girth. Tower (1937) cut the lumbosacral posterior roots and transected the cord above and below this region; the bones of the hind limbs were of normal length but were thinner and the protuberances for muscular attachments were poorly developed. There was gross wasting of muscles. McMaster and Roome (1934) found that section of the sciatic nerve did not alter the healing time of fractures of the fibula. Grey and Carr (1915), Pottorf (1916), and Allison and Brooks (1921) could find no difference between the bone changes produced by immobilization and those produced by nerve section; the final result depended on the amount of disuse.

The injuries of both world wars have provided many examples of bone atrophy following nerve injuries in man (Fleischauer, 1915; Reidel, 1916; Lehmann, 1917; Meliwa, 1917; Brailsford, 1941; Howard, 1945; Abramson, 1948). The tendency to fracture in atrophied bones is noted and incidentally soft tissue ossifications in the affected area. The atrophy of limb bones in anterior poliomyelitis is too well known to need any specific reference.

The lesions described in the previous paragraphs might be conveniently referred to as lower motor neuron lesions. Lesions of the higher parts of the brain also affect bone growth but less obviously. Injury of one cerebral hemisphere causes a decreased growth rate in the opposite half of the body but it would not be wise to describe this as an upper motor neurone lesion since postcentral lesions are often found as in the patients investigated by Penfield and Robertson (1943). These cases showed a "comparative moderate smallness" of the body on the opposite side to the lesion in the postcentral gyrus, often in the presence of a normal precentral gyrus. The original damage occurred before the age of two years and gave rise to epileptic attacks. The cortex was displayed under local anesthesia so that the position of the lesions was accurately defined.

Bone growth is known to be modified by circulatory alterations, the best known example of this being the increased bone growth at or near

inflammatory processes. Conversely, reduction of blood supply can lead to bone atrophy or delay in healing of fractures, both human and experimental. It is thus important to enquire if a nervous lesion which produces alteration in bone does so not directly but indirectly through some alteration of blood flow. There seems little doubt that the sympathetic system is often involved in anterior poliomyelitis; indeed it is difficult to see how the cells of the lateral horn could escape (Smith, Rosenblatt, and Limauro, 1949). Signs of such involvement are angiospasm with cyanosis, Horner's syndrome, upset of anal and bladder sphincters, ischemic muscle pain. The poliomyelitis virus has been demonstrated in the abdominal sympathetic (Sabin and Ward, 1941). Various authors have reported relief of some of these conditions after sympathectomy (Robertson, 1934; Collens, Foster, and Wert, 1947). It might be that the good effects were due to relief of vasospasm.

If the vasospastic element in poliomyelitis is accepted, then it is proper to enquire into the effect of sympathectomy on bone growth. All experiments on animals show that unilateral sympathectomy by itself has no observable effect (Bacq, 1930; Cannon *et al.*, 1929; Simon, 1930); McCullagh, McFadden and Milroy, 1930; Harris, 1930; Bisgard, 1933; Harris and McDonald, 1936. Harris (1930) and Harris and McDonald, (1936) have described four children suffering from Hirschsprung's disease whose left lumbar sympathetic chain was removed; the left leg subsequently grew longer than the right. Harris and McDonald (1936) report on 46 patients who had a short limb as a result of acute poliomyelitis; they found that after sympathectomy the discrepancy decreased if the original paralysis was not too extensive and if the operation were performed before the age of 6 years. More certain methods of controlling bone length have since been discovered and this work does not seem to have been followed up. The results of experiments on the effect of sympathectomy on healing of fractures are equivocal: Fontaine (1926), Colp and Mage (1931), and Palma (1925) claimed that it accelerated healing but McMaster and Roome (1934), Keay and Moore, (1933), and Pearse and Morton (1931) found either no influence on healing or even a retardation.

Stinchfield, Reidy, and Barr (1949) studied 166 adults who had suffered from poliomyelitis before the age of 11 years; the final discrepancy between limb lengths depended not on the age of onset but on the discrepancy between the respective muscle strengths of the two limbs. Gullickson, Olson, and Kottlee (1950) who studied 88 patients with paralysis of one lower limb as a result of anterior poliomyelitis found low correlations between muscle strength and bone atrophy. Gillespie (1954) seems to have been the first to investigate this relationship experimentally and quantitatively in animals. He found that there was a

highly significant degree of correlation (+0.79) between the weight of the long bones of the paralyzed limb expressed as a percentage of the weight of the bones of the opposite normal limb and the total weight of the skinned paralyzed limb expressed as a percentage of the weight of the opposite limb. (About nine tenths of the weight of the limb is due to muscle). Such a high correlation makes it clear that disuse is certainly the main factor in atrophy; if there is a neurotrophic influence, it must be very small. The state of affairs after posterior root section is somewhat anomalous since the animal drags the affected limb along behind it and undoubtedly uses it less than the sound one; there is, however, no muscle atrophy and no loss of bone substance. Perhaps disuse in this connection should be construed as loss of pull by innervated muscle fibers. It should be noted that denervation of a limb does not prevent growth completely; muscle pull can only be a factor modifying the rate of growth. In some of these experiments sympathectomy was carried out at the same time as anterior root section. This procedure did not alter the bony changes produced by anterior root section alone, except that there was a slight (1.2%) but statistically significant increase in length. It may be concluded that the bony atrophy is not primarily due to diminution of blood supply. Gillespie's experiments lasted 2 months only—it may be that longer experiments would yield somewhat different results.

The X-ray picture of the bones of a disused limb shows loss of density which is often described as decalcification or osteoporosis. Gillespie's work shows clearly that these terms should not be applied. The quality of the bones of the paralyzed limbs as judged by breaking stress, elasticity, percentage of ash, and specific gravity, was very nearly the same as that of the control bones. The changes in the bones of the paralyzed limbs were due almost entirely to a reduction in the quantity of bone material, partly by a reduction in the external dimensions and partly by a reduction in the thickness of the cortical shell. These remarks apply also to the bones in experimental hyperthyroidism and to those produced on a low calcium diet.

It has long been known that the sex hormones have important influences on bone growth but it is only recently that their effects on bone atrophy have been investigated. Armstrong, Knowlton, and Gouze (1945) using rats and Halvorsen (1949) working on rats showed that testosterone and estradiol reduced bone atrophy in paralyzed limbs as judged by their weight and ash content. Gillespie (1954) has repeated these experiments and has found that these hormones significantly reduced the difference between the bones of the normal and paralyzed limbs of rats in respect of total weight, ash weight, and bending moment at the breaking point without, however, affecting the quality of the bone as

indicated by breaking stress. In view of the known effects of prolonged administration of sex hormones it is doubtful if they could be used clinically to prevent bone atrophy.

VIII. Strength of Healing Fractures

Lindsay and Howes (1931) and McKeown *et al.* (1932a) fractured the fibulae of rats with scissors and then at various intervals after the operation dissected out the fibulae and measured their strength. The bone was rested on two supports a fixed distance apart and the load was applied in two equal parts at two points adjacent to the point of fracture. Breaking strength in their experiments means load in grams required to break bone. The fibulae had no strength before the 6th post-operative day but the strength mounted until the 15th day and then diminished coincident with the formation of a medullary cavity. From the 24th day, as the cortex thickened and the callus reorganized, the strength rose to a new high level. The fracture was firmly healed by the 45th day. The rise in strength from the 6th to the 15th day coincided with deposition of salts in the callus as shown by X-ray photographs but after this the strength bore only a slight relationship to density in the X-ray photograph. These authors found that the unfractured left fibula could not be used as a control for the fractured right fibula since the left fluctuated in strength simultaneously with the right; they suggested that there may be a general skeletal reaction to the injury but they had no clue as to the mechanism. Since there is some evidence that a high fat diet increases the intake of calcium, McKeown *et al.* (1932b) repeated their previous experiments with the high fat diet and found that the primary callus was formed as before by the 15th day but was not so strong as on the standard diet; healing was assumed to be complete on the 42nd day (as compared with the 45th on the standard diet) but the strength reached was less. It is difficult to account for this as the vitamins A and D and the Ca and P were the same in both diets. On a high carbohydrate diet (McKeown *et al.*, 1932c) the healing strength did not reach the level of the bones produced on the standard or on the high fat diet by the 15th day; thereafter the strength increased very slowly without the preliminary loss seen in earlier experiments. Observations on a low calcium diet (McKeown, Harvey, and Lumsden, 1932) show a considerable slowing in the process of repair.

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CHAPTER III

THE GROUND SUBSTANCE OF CONNECTIVE TISSUE AND CARTILAGE

BENGT SYLVE

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I. Introduction

Recent trends in biology and medicine clearly show the need of additional information on the structure, physiology, and pathology of mesenchymal tissues. Although a considerable amount of observation has already been collected, many fundamental problems are still incompletely understood. In particular, this applies to the so-called ground substance of connective tissue, which so far offers little facility for detailed investigation. This presentation will necessarily be restricted to an outline of *the fundamental structural aspects and methods of study* under normal conditions. For details the readers are referred to available reviews by Merkel (1908–1909), Biedermann (1913), Hueck (1919–1920), Maximow (1927), Standenath (1928), Studnicka (1929), Was-

serman (1929), Bauer (1934), Le Gros Clark (1945), Gersh (1949–1950), Favilli (1951), and others, and further to the interesting conference reports published by the New York Academy of Sciences, the Josiah Macy Jr. Foundation, and other bodies during the last few years.

Many experiments and tentative suggestions relating to the possible physiological significance of ground substance materials and other fields of research dealing, for instance, with connective tissue as a whole with reference to metabolism, permeability, spreading, etc., are omitted in this chapter.

II. Historic Survey

Since the time when Johannes Müller coined the name “Bindegewebe” about 1820 to 1830 and Th. Schwann in 1839 first used the term “Zwischensubstanz” histologists have recognized in the light microscope an interfibrillar amorphous material in mesenchymal tissues. During the 19th century, observations on many embryonic and adult connective tissues led to the view that the intercellular spaces contained fibrous materials of different kinds and diameters, and further a gelatinous, mucoid, or fluid phase without orderly structure. This matrix material was seldom visualized in the regular adult connective tissues, but appeared in larger amounts in embryonic tissues (Holmgren, 1940, and others), the umbilical cord, and in various specialized mesenchymal regions, such as the vitreous body. At this time a certain relationship was presumed between the formation of the fibrous protein collagen and the ground substance; the fibers developed in the ground substance, which gradually decreased in amount and finally became sparse or disappeared. Remnants were thought to be left in the adult state as an interfibrillar “cement” substance or somehow incorporated into the collagen fibers.

The morphogenesis and texture of ground substances thus became intimately associated with the various hypotheses on fibrogenesis as either (1) an *intracellular process* (Flemming, 1897, 1903–1906; Mall, 1901–1902; Studnicka, 1907; Meves, 1907; Ferguson, 1912; and others), (2) an *epicellular process* (Laguesse, 1903–1904, 1914–1915, 1919, 1920, 1921; Golowinski, 1907; Meves, 1910), or (3) an *extracellular process* (Henle, 1841; Mall, 1890–1891; Merkel, 1893–1895, 1908–1909; Renaut, 1903–1904, Nageotte, 1916; and others), in the last-mentioned case perhaps in part induced by material derived from fibroblasts. More recently the last-mentioned theory got support from tissue culture data (Maximow, 1929; McKinney, 1929; Momigliano-Levi, 1932; Olivo, 1933; Doljanski and Roulet, 1933; and others), from studies *in vivo* using the

transparent chamber technique (Stearns, 1940), and lately from electron microscope studies on *in vitro* fibrogenesis (Porter, 1951).

More fundamental insight into the structure of collagen was obtained during the 1920's through work in colloid and macromolecular chemistry, and later on by the X-ray diffraction method (reviewed by Bear, 1952), and by electron microscopy. In 1926, Heringa and Lohr expressed "la formation des fibres collagènes comme un phénomène de gélatinisation d'un sol fibrillaire." In 1927, Nageotte described the first experiments of dissolving rat-tail tendon collagen and ichthyocol in dilute acid, thus obtaining a clear, viscous solution from which collagen could be reconstituted by addition of salt. Similar experiments were performed by Fauré-Fremiet (1933) and Fauré-Fremiet and Garrault (1937), and later on by other workers in Russia (Tustanovsky, 1947; Orékhovitch, *et al.*, 1948; Belozerski *et al.*, 1952) and in the United States (Highberger, Gross and Schmitt, 1950). Other interesting reconstitution experiments (Highberger, Gross and Schmitt, 1951; Gross, Schmitt, and Highberger, 1952; Jackson, 1953; and others), illustrate the possible relation between fiber formation and acid polysaccharides. During developmental conditions ground substances on the whole are assumed to contain the necessary protein building blocks and enzymes for fiber organization. The various sources of such materials are not known in detail (see Section VII).

It was early recognized that embryonic and certain other ground substances contained "mucin" or rather a mucoid material, protein, and water. The mucoid was first extracted by Rollet (1858) and later on analyzed by others. The non-organized protein constituent(s) presented only a faint basophilic reaction with aniline dyes, and was difficult to visualize. In many cases, the ground substances were identified by their metachromatic reaction only, which led to fallacies in terminology and gave the mucoid component too much emphasis. Recent advances in carbohydrate chemistry have to some extent clarified the nature of the extracted polysaccharides; little is known about the bulk of protein constituents.

Our microscopic classification of mesenchymal tissues was based on the varying amounts and characteristics of extracellular components, e.g. the distinction between loose, adipose, and dense connective tissue. In the outstanding works of Hansen (1899, 1905), the intercellular components of hyaline cartilage were defined as collagen and chondromucoid. From a physiological point of view, the presence or absence of blood vessels forms another important basis of classification in *vascular* and *avascular* connective tissues, implying marked structural and meta-

TABLE I
NATURE AND APPROXIMATE AMOUNTS OF ACID POLYSACCHARIDES OBTAINED BY
EXTRACTION FROM VARIOUS MESENCHYMAL TISSUES

(Due allowance should be made for the simultaneous occurrence of other carbohydrates not yet identified)

Tissues	Nature of Identified Polysaccharides	Approximate Extraction Yields		References
		Calculated per fresh tissue weight	In per cent of solid matter	
A. EMBRYONAL <i>Umbilical cord</i>	—	—	—	Data not obtainable
Human	Hyaluronic acid and	0.6-0.8%	4-5	Jeanloz and Forchielli, 1950
	Chondroitin sulfate (?)	About 0.1%	1	Meyer and Chaffee, 1941
	Hyaluronic acid	—	3.3	Follett, 1948
B. ADULT STAGES				
<i>Skin</i>				
	Human	Hyaluronic acid and	20 mg/100 g	less than 0.1
Pig	Chondroitin sulfate (?)	15 mg/100 g		Pearce and Watson, 1949
	Same as above	Total amount about 130 mg/100 g		Watson and Pearce, 1950
Rat	Same as above	Total amount about 15-30 mg/100 g		Meyer and Chaffee, 1941
<i>Tendon</i>				Snellman, Malmgren, and others (unpublished)
Guinea pig	Chondroitin sulfate	—	0.6-0.7	Wrete, 1950
<i>Aorta</i>				
Guinea pig	Chondroitin sulfate and others (?)	—	—	Levene, 1903; Meyer; and others

<i>Heart valves</i>				
Guinea pig	Chondroitin sulfate and others (?)	—	—	Meyer, 1953b
<i>Sclera</i>				
Guinea pig	None	—	—	Meyer and Chaffee, 1940
<i>Cornea</i>				
Cattle	1. Hyaluronic acid ester sulfate; or 2. Chondroitin sulfate, and others; or 3. Chondroitin sulfate, chondroitin and keratosulfate	Less than 0.5% 4.2 1.8	1.8 4.2 1.8	Meyer and Chaffee, 1940 Woodin, 1952 Meyer <i>et al.</i> , 1953
<i>Synovial fluid</i>				
Human	Hyaluronic acid	0.2–0.4%	—	Sundblad, 1953
Cattle	Hyaluronic acid	0.2%	—	Meyer, Smyth and Dawson, 1939
<i>Vitreous body</i>				
Cattle	Hyaluronic acid	0.04% 0.01%	— —	Meyer, Smyth and Gallardo, 1938 Alburn and Williams, 1950
<i>Nucleus pulposus</i>				
Cattle	Chondroitin sulfate and others (?)	2.7%	About 20	Malmgren and Sylvén, 1952
<i>Hyaline cartilage</i>				
Cattle	Chondroitin sulfate and others (?)	Various figures re- ported; ranging between 4-13%	12-40	Hirsch, 1944, and others Einbinder and Schubert, 1950
<i>Bone</i>				
Spongy	Chondroitin sulfate and others (?)	—	—	Meyer, 1938
Cortical				Rogers, 1949, 1951

bolic differences. The more or less avascular connective tissues, such as cartilage, cornea, and vitreous body, contain unusually large amounts of mucoid material (Table I) and exhibit specialized functions.

In recent years considerable progress in various fields has emphasized the complexity and importance of ground substances during the development and repair of normal and pathological mesenchymal tissues.

III. The Term "Ground Substance"

The popular term "ground substance" is not very satisfactory but the difficulty in reaching a more exact terminology has so far prompted its use. Authors use the term in different senses and nobody seems quite sure of its delineation. From a morphological point of view it may be appropriate to define ground substances as the *amorphous*—non-structured to the lower limit of resolution of the electron microscope—*interfibrillar material located in the extracellular compartment and presenting some functional and morphogenetic relations to fibrocytes and connective tissue fibrils* (cf. Dorfman, 1953; Gross, 1953). Such material is seen during embryonic development, during repair, and in specialized connective tissue organs in the adult, but in most adult loose connective tissue it is sparse or perhaps even lacking. In systemic connective tissue disorders of various etiology, a variety of alterations afflicting fibrocytes, fibrils, tissue metabolism, water transport, etc. will involve similar qualitative and quantitative changes in the composition of the extracellular material.

As to the chemical composition of the ground substances the presence of one or more of the acid polysaccharides is considered characteristic, even if it is not ubiquitous. Since ground substances also are physiologic transport pathways in dynamic equilibrium with the cells and blood plasma, substances which do not constitute virtual components may occur.

The meaning of the term "amorphous" may be taken in different ways. The histologists found during the development argyrophilic granules or fine fibrils embedded in an amorphous matrix, in which nowadays much thinner submicroscopic fibrils are seen. Some authors might include these finest fibrils in the ground substance proper.

For the present time we are apparently at a loss to give an exact definition and will therefore resort to mere accounts of data. It seems justifiable to use the broad term ground substance in a topical sense for descriptive purposes only if we remember that it denotes a non-specified material housed in a three-dimensional compartment—very similar to what Claude Bernard called "milieu interne." The term "intercellular matrix" might then be used synonymously.

IV. Distribution and Physical Characteristics

Ground substances together with water occupy considerable interstitial tissue space during the embryonic and other developmental stages, but then decrease in volume in most vascularized mesenchymal tissues parallel with the increasing amounts of fiber structures. There are, however, some exceptions. First, in the avascular group large volumes of ground substance are still found during adult stages. Second, some special structures, such as the hair (Holmgren, 1939; Sylvén, 1949) and teeth papilli (Letterer, 1932; Holmgren, 1940), and some hormonal target organs contain much ground substance under certain conditions.

Due to the varying composition, characteristics, and amounts of extracellular components, the enumerated tissues present widely different physical characteristics, plasticity, elasticity, transparency, etc. The mass distribution figures of the extracellular compartments of different connective tissues and cartilage (Clemons, Freeman, and Angevine, 1954) are not known in detail, but may perhaps in some cases be roughly estimated from other figures on contents of water and solid matter, respectively. In the avascular group, the vitreous body and the normal nucleus pulposus present characteristics similar to three-dimensional lattice gels of gelatin with comparable water content (Sylvén *et al.*, 1951). Wharton's jelly is known to have similar characteristics. Normal hyaline cartilage has a more rigid structure with a larger solid content and a rubber-like elasticity to compression (Hirsch, 1954), which makes it suitable as a shock absorbing material in joints. Solid films of protein-free hyaluronates have characteristics very similar to cellophane, and form excellent material for sheaths above a certain minimum level of water content.

The remarkable transparency of the cornea is not yet fully elucidated, but seems associated with a carefully defined water and polysaccharide content (cf. Hertel, 1933; Heringa, 1949; and others), which factors in part condition the regular arrangement in space of the collagen fibers. This arrangement most likely results in the continuous transitions in refractive indices and the transparency to light (Casperson and Engström, 1946).

It is generally inferred that ground substances *per se* have a gel structure with marked plasticity, but direct evidence is mostly lacking. Some gel characteristics may be noted depending upon the composition and the salt and water content, but if and when they are true gels or sols in a physicochemical sense belongs to the realm of speculation. It is further stated that the materials are viscous, optically homogenous (isotropic), and devoid of structure (amorphous) at the maximum resolution of the light microscope. Native preparations of hyaluronate

and chondroitin sulfate (Blix and Snellman, 1945) as well as native synovial fluid (Partridge, 1948) show birefringence of flow indicating the presence of asymmetric macromolecules. Highly oriented protein-free, but not salt free, hyaluronate fibers show a marked positive birefringence to light (Sylvén and Ambrose, 1955), which warrants further investigation.

V. Microscopy of Ground Substances

1. IN THE FRESH STATE

In spreads or teased preparations of fresh adult mammalian loose connective tissues and fasciae inspected under ordinary light microscopes no ground substance is in general visible, and therefore its existence has been denied many times. The old hypothesis about a "Kittsubstanz" uniting the thinner collagen fibers together into larger bundles which in turn would form thin membranes as expressed by Flemming (1876), and others, lacked convincing proof (Nageotte, 1916, and others). This view, however, later on got support from Laguesse (1903-21) and more recently from Day (1947a), Day and Eaves (1953), and others. The latter inferred from data obtained by acid- and/or salt-treated thin spreads of fresh connective tissue inspected under dark-ground illumination, that the ground substance was made up of one amorphous part and, in addition, small refractile granules and a delicate cobweb-like fibrillar network. He got the impression of membranous structures, which could be broken up by trypsin treatment but were unaffected following incubation with testicular hyaluronidase. This picture was only obtained when the tissues were treated at pH 3.6-3.9, and Day suggested that an interfibrillar protein component had been precipitated near its isoelectric point. Trypsin treatment apparently led to loss of fibrillar cohesion and gross elasticity of the tissue, suggesting that the large collagen bundles owe their cohesion in part to a "cementing substance" of protein nature in which the fibrils are embedded. Although this interpretation may be questioned, Day's interesting experiments presumably indicate the presence of some protein material between the large collagen fiber bundles in normal loose connective tissue. The cohesion between the finest collagen fibrils, on the other hand, is explainable by electrovalent and other lateral bonds, and there seems to be no need or evidence from other sources to assume additional links by extraneous proteins.

In several other kinds of fresh connective tissues, such as embryonic, umbilical, vitreous body, the renal papilli, etc., rich in ground substance and poor in collagen fibers, and further in hyaline cartilage, the inter-

fibrillar matrix is easily visualized *in situ* as a gelatinous material presenting some opacity and optical homogeneity. In fresh frozen sections supravitally stained in aqueous media for a short time and subsequently inspected under the microscope, a uniform adsorption of dye is observed in the ground substance. *In vitro* cultures of fibroblasts growing in plasma with added embryo juice (Porter, 1951; and others), as well as available transparent chamber studies (Clark and Clark, 1933; Stearns, 1940), have been more suitable for studies of fiber formation than of ground substances as such.

2. STAINING OF MORDANTED TISSUES

Following fixation, the visible ground substances generally appear as amorphous, granular and/or finely fibrillar material. The various artifacts brought about by the usual chemical fixation procedures seem deleterious for fine structure analysis of water-soluble material, which renders a detailed review of old morphological observations unjustified. Most of the involved artifacts may, however, largely be circumvented by the use of freezing and vacuum dehydration of fresh tissue samples, which method is reported to give improved morphological results in the study of ground substances (Gersh and Catchpole, 1949; Sylvén, 1951; Sylvén *et al.*, 1951, and others), and further facilitates histochemical analysis.

Since no specific constituent(s) seems to occur in connective tissue ground substances, selective staining reactions cannot be expected. For the demonstration of ground substances *per se* it is probably sufficient to apply any basophilic stain bringing out the cell borders and then define the ground substance by its topical distinction and faint basophilia. A review of current staining methods and their alledged selectivity for various constituents belongs to the field of histochemistry. Present interest in these methods, however, calls for some comments as to the evaluation of staining results.

With reference to *metachromasia* with the basic aniline dyes such as toluidine blue and Azure A, a metachromatic dye-substrate interaction seems first to depend on the availability and distribution of electronegative charges on the surface of the substrate molecules (Sylvén, 1954b). A negative staining result may mean various things: (*a*) that such substrates are really absent; (*b*) that such a constituent is present but linked in a way so the surface charge density has decreased below a certain minimum level; or (*c*) that the topical substrate "concentration" in the tissue is below the threshold value for a positive reaction. Model experiments indicate that a number of non-charged sugars and polysaccharides of low-charge density do not produce

metachromasia. The reaction is not specific for polysaccharides; it may be said to indicate the presence of negative colloidal polyelectrolytes in general. The sensitivity with respect to concentration ranges and states of molecular aggregation of various substrates is not known. Slightly diverging data on the alleged metachromasia of purified hyaluronic acid at various concentration ranges (above 1%, 1.8% and 0.5%, respectively) are reported by Meyer (1950a), Sylvén and Malmgren (1952), and Persson (1953). The metachromatic reaction seems in this case not very sensitive and not directly suitable for the distinction between various types of charged polysaccharides. Some data on the occurrence of metachromasia in various ground substances are reviewed by McManus (1954).

The current *periodic acid-aldehyde Schiff reaction* (McManus, 1946; Lillie, 1947; Hotchkiss, 1948) is well known from a chemical point of view (Jackson, 1944), but when used as a histochemical tool it presents unsettled questions relating to specificity and evaluation of results (McManus, 1950; and others). Positive results have previously been ascribed to the presence of hyaluronic acid in spite of the fact that this polysaccharide consumes very little periodic acid (Jeanloz, 1950; and others), and is moreover not stained in model experiments (Davies, 1952; Glegg, Clermont, and Leblond, 1952). Apparently, other carbohydrates should be assumed to be responsible for a positive staining of ground substances. The positive reaction of reticulin and basement membranes was ascribed to the presence of other sugars (Glegg, Eidinger, and Leblond, 1953). These may presumably occur in small amounts also in connective tissue cells, collagen (?) and some PAS-positive ground substances, but have so far not been completely identified (cf. Consden, 1953). A detailed review on the distribution of PAS-positive material(s) in the adult rat is published by Leblond (1950), and further data are found in papers by Glegg *et al.* (1952), and others.

The methods discussed above are further combined with enzymatic digestion experiments using various crude hyaluronidases and other enzymes, in order to reach a closer identification of the stained substrates. This approach, however sound and useful it may seem under carefully specified conditions, still leaves much to be desired. If metachromasia is abolished after digestion with some partially purified hyaluronidase, this may mean *either* that a polysaccharide in question (hyaluronic acid and/or chondroitin sulfate) has been degraded, *and/or* that the electronegative charges of the substrate have been blocked by the added enzyme protein.

Present histochemical tests need experimental corroboration to a

considerable extent, preferably by other methods, and are not alone sufficient for the identification of ground substance components. For further details of staining methods and results the readers are referred to papers by Bensley (1934), Sylvén (1938, 1941, 1945), Krauss (1944), Hale (1946), Dempsey and Wislocki (1946), Wislocki, Bunting, and Dempsey (1947), Grishman (1948), Gersh and Catchpole (1949), Bunting (1950), Dempsey (1950), Lillie (1951), Immers (1954), and others, recent reviews by Persson (1953) and McManus (1954), and histochemical manuals by Gomori (1952), Lison (1953), and Pearse (1953).

3. ELECTRON MICROSCOPY

A number of electron microscopic observations have been made on mesenchymal material prepared in different ways by means of teasing, physical and chemical fragmentation, washings, etc. and finally drying on the grids. Since these procedures involve some artifact handling, the results have to be further corroborated on ultra-thin sections. Due allowances have still to be made for possible artifacts by drying.

Varying amounts of an amorphous material embedding thin fibrils of different diameters (about 200–400A) have been seen in fragmented sections of umbilical cords (Wolpers, 1944), in smears of fresh adult and newborn infant skin (Gross and Schmitt, 1948), in fresh frozen sections from calf and human nuclei pulposi (Sylvén *et al.*, 1951), in sections and squash preparations from cartilage (Wolpers, 1944; Schwarz and Vester, 1953; Martin, 1953), and in washed suspensions of fragmented vitreous bodies (Matoltsy, Gross, and Grignolo, 1951). The amorphous ground substance is also seen in electron micrographs of teased embryonic chicken connective tissue and in fragmented tendons (Pratt and Wyckoff, 1950; Wyckoff, 1952). Spreads of mouse fascia have been investigated by Day and Eaves (1953) both in fresh condition and after swelling at pH 10.3. The ground substance was interpreted as delicate membranes built of a fine fabric of fibrils embedded in a structureless material. In pictures of suspensions of fragmented cornea and sclera, sheets of ground substance were observed surrounding collagen fibrils (van den Hooff, 1952). Pictures of purified hyaluronate dried from aqueous solutions showed various thin fiber structures and stellate sheets (Gross, 1948).

The vitreous body presents similar facilities for study as the nucleus pulposus due to a low content of solid matter. Electron micrographs (Fig. 1 and 2) of fresh frozen sections (thickness 0.2–0.5 micron) from the bovine vitreous show a dense network of long slender fibrils with an average diameter of 100–200A, and indications of a granular fine-

structure suggesting an axial periodicity of about 330–660A (cf. Matoltsy, 1952). Numerous almost uniformly distributed *microcrystalline granular deposits* are embedded in this network, thus representing the "amorphous" ground substance *per se*. The diameter of the granules

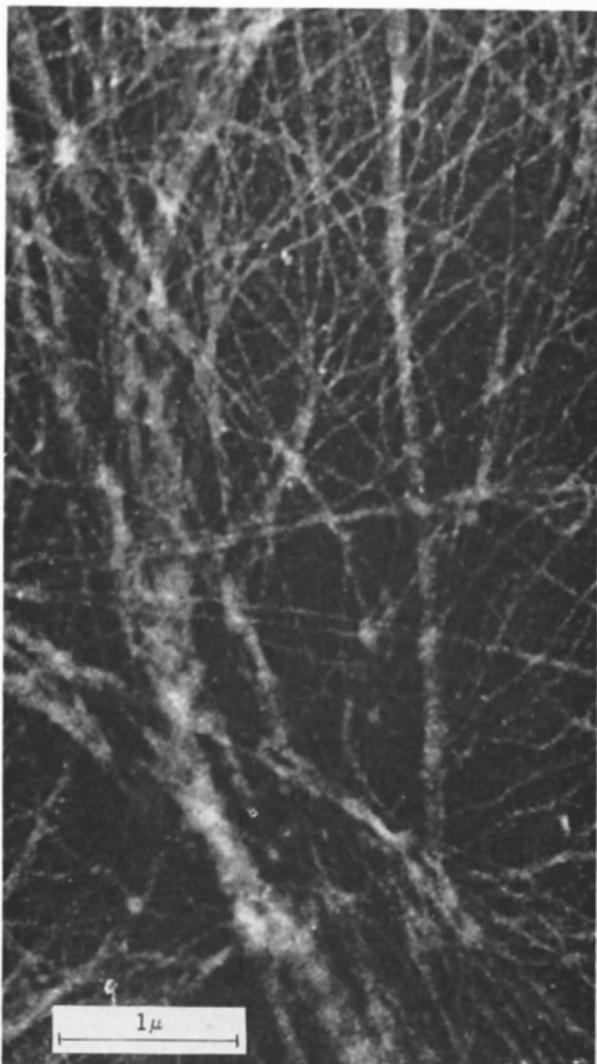


Fig. 1

FIGS. 1 AND 2. Electron micrograph of fresh frozen sections, otherwise untreated, of bovine vitreous body. Thickness 0.2–0.5 micron. Magnification 30,000 times. The numerous granular deposits represent the interfibrillar ground substance. (Fernández-Morán and Sylvén; unpublished work.)

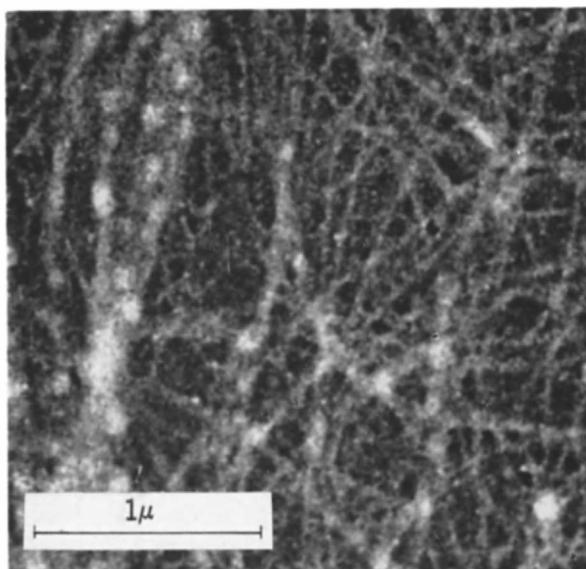


Fig. 2

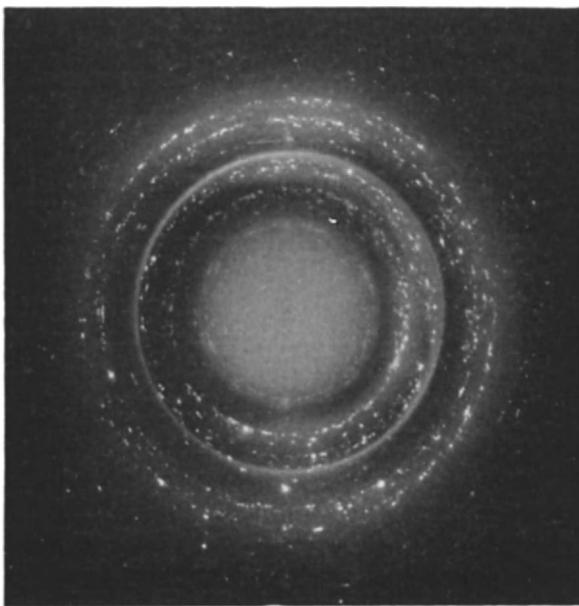


Fig. 3

FIG. 3. Electron diffraction pattern obtained from the granular microcrystalline ground substance material in an area of approximately 500 square microns of a dried fresh frozen section of calf vitreous body. Taken with the specimen in the object holder position of an RCA EMU microscope fitted with a 200 micron condenser aperture and an intermediate lens. (Fernández-Morán and Sylvén; unpublished work.)

varies between 300–1000A. In general, the granules were found adhering to the filaments, aligned longitudinally. In places, the granular deposits were obtained as aggregated "sheaths" around the fibrils, most likely an artifact produced by drying. By means of the "selected area" electron diffraction method (Fernández-Morán, 1951) it was possible to record diffraction patterns from these deposits (Fig. 3). Since the technique of electron diffraction used in these experiments only recorded short interplanar distances, the observed effects were probably due to the presence of salts (cf. Scott and Packer, 1939; Draper and Hodge, 1949).

In this place it is tempting to speculate why no mature collagen fibrils are seen in the normal vitreous body. Is some necessary factor(s) for the organization of collagen absent, or is some inhibiting material present? The answer may be that the fibroblasts are absent.

These preliminary electron microscopic results suggest that most ground substances as seen in the light microscope may be resolved into a network of narrow fibrils with diameters between about 100 to 400A and with an axial periodicity of about 640A or less. Some non-structured fibrils seen in the pictures from the vitreous body may consist of polysaccharide material. The "amorphous" or, in the writers opinion more correctly, *finely granular ground substance* is apparently distributed in the spaces between the fibrils. This will lead to the concept that the fibrous protein components form a more prominent part of ground substances than was previously understood. The coarser argyrophilic fibrils demonstrable in the light microscope may be considered to correspond to fiber bundles coated by such ground substance as discussed by Tomlin (1953), and others. The supposed organization of ground substance will be outlined below (Section IX).

VI. Chemistry of Ground Substances

Present insight into the composition of connective tissue ground substance is meager. Only in the case of avascular tissues, namely the vitreous body, nucleus pulposus, cornea, and cartilage, where we deal with a limited number of structural elements and cell types, available data may with some security be referable to ground substances as such. Ground substances from connective tissue have not yet been isolated and analyzed by chemical means. A few basic constituents probably occur in most ground substances but other components may be present. The amounts of the postulated constituents evidently vary in different regions, during different developmental stages and physiological conditions. For descriptive purposes the synovial fluid may be accepted as a fairly well-known model (Ropes *et al.*, 1947; Sundblad, 1953). In this

connection the assumed components are briefly summarized and a few comments added.

1. THE POLYSACCHARIDE PART

In most mesenchymal tissue ground substances one or more acid polysaccharides are assumed to occur, possibly in a high-molecular state; either hyaluronic acid and/or chondroitin sulfate (see Table I). Carbohydrate components of other nature and origin should further be expected in tissue extracts (Connsden, 1953), such as glucose for nutritional purposes, blood group polysaccharides, and the serum mucoprotein(s) (Winzler, *et al.*, 1948; Weimer, Mehl, and Winzler, 1950; and Schmid, 1953). The quantitative yields of polysaccharide material (Table I) are generally low following extraction of adult vascularized tissues, but larger in adult avascular and embryonic tissues.

2. THE PROTEIN PART

In general terms, it is accepted that ground substances contain non-structured protein material. This is inferred from data on synovial fluid, the vitreous body, and cartilage, and from the fact that following aqueous extraction of mesenchymal tissues the polysaccharides are mostly obtained as protein complexes, often together with large amounts of other (non-conjugated) water-soluble proteins of unknown origin. Mucoprotein complexes are partially extractable from defatted and dehydrated tissues using hot water (Partridge, 1948), and in larger yields using dilute NaOH (Jorpes, 1929) or concentrated salt solution, e.g., CaCl₂ (Meyer and Smyth, 1937). Partridge suggested that the water-soluble protein of cartilage derived from the "disordered" collagen of the "cementing" substance. In extracts from tendon and heart valves, Meyer (1952) obtained a protein containing tyrosine and tryptophane suggesting a non-collagenous origin. The normal synovial fluid contains 8 to 10 times more protein than hyaluronic acid (Sundblad, 1953). The native synovial hyaluronic acid-protein complex obtained by ultrafiltration appears to have a polysaccharide: protein ratio of about 3:1 (Ogston and Stanier, 1950, 1953). Day (1947a) inferred that the ground substance of adult normal connective tissue contains mostly protein digestible by trypsin (24 hours at 20°C). Dempsey and Haines (1949) reached a similar opinion. It seems apparent that the nature of the suggested protein components and their possible mode of linkage with polysaccharides (cf. Meyer, 1952) are poorly known but of considerable interest, particularly with a view to their possible role in fibrogenesis.

3. ELECTROLYTES

Average figures on the gross contents of inorganic ions in various mesenchymal tissues, including cartilage and bone, are available (Everett, 1946). Little is, however, known of the topical ionic concentrations in the soft ground substances. There are good reasons to expect that ground substances rich in polyelectrolyte material, possibly exerting cationic binding effects, will show different ionic distributions as compared with the extracellular phases of other connective tissues poor in polyelectrolytes. Available isotope distribution studies are not referable to ground substances *per se*, and are therefore omitted from discussion. The electrolyte concentration of tendon and adipose tissue has been reported to approximate that of a serum ultrafiltrate (Manery, Danielson, and Hastings, 1938). In the avascular nucleus pulposus, Na predominates over Cl, and only small amounts of K, Ca, and Mg are present (Malmgren and Sylvén; unpublished data). Data on cartilage show a similar excess of Na over the Cl content (Logan, 1935; Iob and Swanson, 1937-1938; Manery and Hastings, 1939; and others). The large amount in cartilage of chondroitin sulfate, offering (partly) ionized sulfate and carboxyl groups, may account for the cationic binding capacity (Neuman, Boyd, and Feldman, 1952; and others). Hyaline cartilage is further characterized by a low content of calcium (Baginski, 1929; and others); its high sulfate content decreases considerably prior to calcification (Logan, 1935; and others).

Variations in the normal electrolyte content of ground substances, in particular those rich in polyelectrolytes, may influence the physicochemical characteristics of the polysaccharide and protein complexes as to degrees of hydration, charge distribution, viscosity and particle sizes. The electrolyte effects on colloidal matter are well known and recently discussed (Butler and Conway, 1953). The postulated adrenal regulation over connective tissue ground substances may in part be effected via such mechanisms.

4. WATER

Part of the extracellular tissue water is considered "bound" to proteins and polysaccharides of the ground substance, forming a hydration phase (cf. McMaster and Parsons, 1950). Evidence to this effect may easily be obtained in the avascular types of mesenchymal tissues rich in polysaccharide material with a large water-binding capacity. Clear distinctions between such hydration water and more labile "interstitial" (immobilized) water are, however, not yet possible (cf. Robinson and McCance, 1952). The problems pertaining to the state and transport of water in various mesenchymal tissues have been studied by different

techniques (cf. McMaster, 1941; McMaster and Parsons, 1939, 1950; and others), but the experimental conditions do not justify a correlation of these results directly with ground substances *per se*. Other factors, such as interfacial diffusion under the influence of surface flow (Crisp, 1946), the presence of interfibrillar spaces ("pores") in gelatinous ground substances (cf. Friedman, 1930), etc., have to be further elucidated. Direct measurements of diffusion rates of various organic and inorganic compounds by means of an interferometric technique have been performed on fresh sections of bovine nuclei pulposi (Paulson *et al.*, 1951). Similar determinations, however, can not be made on hyaline cartilage due to its optical inhomogeneity. Additional information on tissue and ground substance permeability may be obtained by using tritium-labelled water (Renkin, 1954).

5. OTHER CONSTITUENTS

Apart from the components outlined above, a variety of metabolic and other materials are expected passing either way between the cells and the vascular system.

Great caution is justified in the interpretation of chemical and physiological data assumed to pertain to ground substances in the strict sense. Present pitfalls and limitations have been recognized and discussed during recent years by several investigators (cf. Meyer, 1953b, 1954; Sylvén, 1954a, b; and others). We have to recall that this field of research presents unsolved problems as to sampling of the inhomogeneous starting materials. Available extraction methods are often rough and will lead to artifact results in so far as the isolation of the *native* components is concerned. The separation of various polysaccharide materials of *similar physical and chemical characteristics* is difficult, and artifact complexes with proteins are obtained. It seems thus apparent that chemical extraction data obtained on whole tissues cannot alone provide a sufficient basis for interpretations as to the topical localization of various components. For this purpose *other auxiliary methods* such as staining reactions (Section V), histochemistry, digestion experiments using enzymes, spectrophotometry, etc., must be looked for. These points are illustrated by the fact that we are still not capable of finding by direct means the loci of the small amounts of hyaluronic acid extracted from adult skin and other loose connective tissues. Is it evenly distributed in the ground substance, deposited in basement membranes, or does it originate from any of the intermingling cell types? These and other problems relating to the polysaccharides of fibroblasts and tissue mast cells during normal conditions, repair, and pathological disorders are waiting more definite answers (Sylvén, 1954a). Present information is

also too limited for a discussion of the topical origin of non-collagenous protein components found in tissue extracts.

VII. Genesis of Ground Substance Materials

Ground substances of connective tissue and cartilage were previously often considered as generic entities, and concepts of their formation were based on series of microscopic observations, mostly performed on embryonic materials (cf. Baitsell, 1924/25; and reviews by Standenath, 1928; Wassermann, 1929; and others). It will, however, now seem more adequate to discuss the origin as well as the dynamics of each one of the various components separately as they are isolated and studied. The difficulty will perhaps arise to decide whether a certain substance really belongs as a virtual component of ground substances or is just passing through the extracellular compartment.

Presently, little can be said about the sources of various components which are just superficially known. Water and electrolytes may safely be assumed to derive from the blood. Proteins, peptides, and amino acids, including so-called collagen precursors, are probably in part derived from the protein pool (Gitlin and Janeway, 1953; and others) and in part from the fibrocytes and chondrocytes, respectively (Porter, 1951; and others). The origin of the postulated polysaccharide material has been the subject of extensive speculation for years. Currently, the following sources are considered: (1) During developmental stages, the *fibrocytes* may secrete a metachromatic and/or PAS-positive material (Laguesse, 1920; Bensley, 1934; Teilum, 1946; Gersh and Catchpole, 1949; Holmgren, 1949; Mancini and Lustig, 1950; Jackson, 1954; and others). (2) The tissue *mast cells* may under certain conditions liberate metachromatic material (in one chemical form or another), contributing to the metachromasia of ground substances in particular during repair (Staemmler, 1921; Sylvén, 1938, 1941; Aykroid and Zuckerman, (1938-1939). Asboe-Hansen (1951) believes that the mast cells, under the influence of hormonal action, secrete the hyaluronic acid of ground substance. (3) It may be derived from the *blood* (Renaut, 1903). In so far as connective tissues proper are concerned, no convincing evidence exists in favor of any one of these hypotheses. In the case of cartilage and related mesenchymal tissues circumstantial data support the view that chondroitin sulfate may be elaborated by the chondrocytes (Hansen, 1905; Le Gros Clark, 1939; Clark and Clark, 1942; Follis, 1952; Dziewiatkowski, 1952; Layton, 1951; and others). In the granulation tissue of healing wounds, the local tissue mast cells seem to release their cytoplasmic material simultaneously with the appearance of the strongly

metachromatic component of the ground substance (Sylvén, 1941). Final chemical evidence as to the composition of this same material is, however, still lacking (Sylvén, 1954a). The origin of mineral ground substance materials in bone is discussed elsewhere in this book.

VIII. Some Physiological Aspects

Although different functions have been tentatively ascribed to ground substances of connective tissue and cartilage and to its postulated components, respectively, little can be said without additional knowledge of the components during the different physiological conditions under question. It seems still too early to correlate functional aspects to postulated degrees of "polymerization" or aggregation, water content, solubility properties, etc. as has been claimed by various investigators (Gersh, 1951; Persson, 1953; and others). However, present information seems to give some hints as to the functional significance of ground substances in specified cases.

There is enough evidence to suggest that the polysaccharide components of ground substance probably influence *fibrillogenesis*, both during the embryonic differentiation and later on during repair. This is illustrated by experiments on the *in vitro* reconstitution of collagen (cf. reviews by Gross, 1950, 1953). It should be noted that the serum mucoprotein(s) of Winzler and Schmid exerts a marked organizing influence (Highberger *et al.*, 1951). It may further be assumed that the ground substance during stages of fibrillogenesis also contains all necessary protein building blocks (collagen precursors).

Secondly, the relative preponderance of ground substance materials in the avascular connective tissues seems to have an *essential physiological significance in so far as the metabolic transport systems are concerned in such tissues*. The real physicochemical mechanisms may not yet be understood, but it seems probable that the living cells in association with the nature of the ground substances are of fundamental importance for the nutrition and maintenance of these tissues (Sylvén, 1951; Paulson *et al.*, 1951). Chondroitin sulfate has been claimed to "stabilize" the structure of tendons (Jackson, 1953; Jackson and Ball, 1953).

The small amounts of ground substance polysaccharides present in adult loose connective tissues of the skin and internal organs (Table I) makes it difficult to ascribe very important functions to this scanty material, unless it were localized in special structures. Other functions have recently been reviewed by Dallemande (1950), Gersh (1951), Ragan (1952), Robb-Smith (1952-1953), Persson (1953), and others.

1. HORMONAL INFLUENCE

The structural organization (differentiation) of connective tissues and cartilage is influenced by hormones as well as by vitamins. For special functional purposes, some connective tissue regions are hormonal targets, e.g. the sexual skin regions of monkeys (Ogston, Philpot, and Zuckerman, 1939), the cock's comb (Hardesty, 1931; Ludwig and Boas, 1950), and the pubic symphysis (Catchpole, 1950; Perl and Catchpole, 1952; Catchpole, Engel and Joseph, 1954). Marked increase in dermal ground substance polysaccharide is noted as sequence to thyroid hypo-function (Halliburton, 1893; Asboe-Hansen, 1950b, 1951). The endocrine relations of hyaline cartilage have been extensively studied by various authors (Silberberg and Silberberg, 1938, 1940a, b, 1941). Present ideas pertaining to the possible role of adrenal hormones for the regulation of ground substance components and tissue permeability have been recently reviewed by Dorfman (1953) and Seifter *et al.* (1953). Other data suggest that the ground substance mucopolysaccharides are influenced by gonadotrophins, sex, and other hormones (reviewed by Iversen, 1954). The incorporation or fixation of labelled sulfate ($^{35}\text{SO}_4$) into polysaccharides of embryonic and wound tissues is impaired by cortisone but enhanced by addition of DOCA (Layton, 1951; and others; review by Boström, 1954). The endocrine influence on the formation of granulation tissue constituents was described by Ragan (1950), Taubenhaus and Amromin (1950), and Taubenhaus (1953).

IX. Possible Organization of Ground Substance

The following tentative ideas of a possible organization of ground substance at a supramolecular level *only refer to the AVASCULAR types of mesenchymal tissues*, rich in ground substance. The structural organization of bone matrix is discussed elsewhere in this book.

In such avascular tissues, the components previously mentioned (Section VI) seem to be united in an orderly way so that the fibrous proteins are hydrophilic (Sylvén, 1951; Day, 1952) and form together with the other parts characteristic three-dimensional cobweb-like gel systems. The electron micrographs suggest that part of the "amorphous" material is attached to the fibrillar surfaces as a uniform, fine-granular coating (Figs. 1 and 2; pictures by Sylvén *et al.* (1951) from nucleus pulposus; by Martin (1953) from cartilage, and others). Most of the polysaccharide-protein complex is removable by simple washing, but some rests are usually more firmly attached to the surface of the fibers. As mentioned above, the interfibrillar matrix may present a micro-crystalline structure in electron diffraction experiments (Fig. 3). Pertinent diffusion

studies on fresh frozen sections of nuclei pulposi further suggest the occurrence of diffusion pathways, called "pores," in between the fibrous networks (Paulson *et al.*, 1951).

A hypothetical arrangement of these components fulfilling the specifications just described probably requires a regular pattern (Sylvén, 1951). The polysaccharide molecules would presumably be somehow attached to the available surface charges of the collagen fibrils, forming highly hydrated shells. If a "free" (interfibrillar) protein component exists, it may be attached to the polysaccharide and thus directed into the interfibrillar "pores" (cf. Day 1952). The water-binding capacity of the system would then depend on the molecular state and available charges mainly of the polysaccharide moiety. The state of aggregation of the main components is expected to vary with the modes of linkages, ionic environment, and other factors.

Gersh (1949-1950), Gersh and Catchpole (1949), and others have discussed the degrees of "polymerisation," hydration, and viscosity of loose connective tissue ground substances mainly with respect to its carbohydrate components. These speculations may seem reasonable but will need experimental evidence.

The physical characteristics of such a hypothetical gel model will be influenced by variations in the quantitative ratios of the component parts per unit tissue volume. The changes observed during aging in hyaline cartilage (Hirsch, 1944) and the nucleus pulposus (Hirsch *et al.*, 1952) involve a continuous increase in the amount of fibrous proteins, while the polysaccharide amounts remain the same or decrease. This leads to an increase in extracellular mass, a lowered polysaccharide: protein ratio, and in turn a diminished water-binding capacity.

The imagined model needs substantial experimental support and is subject to modification as new evidence comes along. Present attempts to elaborate a comparable model system by experimental means do not seem promising. We would request the presence of a certain number of living cells per tissue volume, which by their activities might facilitate transport through the intercellular matrix. These speculations cannot be extended to the vascularized connective tissues poor in ground substance. Other data on the crystalline structure of the inorganic matrix of bone seem to tend to a similar hypothesis. Furthermore, the suggested pattern may afford a basis for the interpretation of various physiological phenomena (swelling and others) not discussed in detail.

X. Concluding Remarks

The present need for additional insight into the structure and composition of ground substances has been repeatedly emphasized. Although

considerable advances have been made in recent years, most of our concepts are mingled with speculations, and many fundamental questions remain unanswered. The ground substances of avascular mesenchymal tissues are fairly well known in comparison with those present during repair and in embryonic material. The paucity of ground substances in soft adult connective tissues has so far prevented its adequate isolation and analysis. Those who are acquainted with the chemistry of this field will be able to present a long list of points to be further investigated (cf. Meyer, 1953b, c). In this connection just a few comments will be made.

Since methods for isolation of ground substances from other connective tissue elements are not yet available, we have to look for physical and histochemical methods for their study *in situ*. The histochemical attack may seem fruitful but requires the elaboration of more refined methods. Further extraction and identification of polysaccharides from various connective tissue sources of heterogeneous composition appears of limited significance as long as the exact *localization(s)* and *function(s)* of such polysaccharides are unknown. It is just a very reasonable postulation from chemical and physiological data that hyaluronic acid and chondroitin sulfate are parts of ground substances of adult connective tissues. Moreover, these acid polysaccharides (Table I) are built up of a few kinds of very similar carbohydrate units. As long as we do not know what particular physiological function(s) are to be ascribed to the various polysaccharides, it makes little difference to our concepts which one is present in one tissue or another. Further investigations may reveal the occurrence of other polysaccharides (serum mucoprotein? heparin??) and other substances playing a fundamental role in the formation of fibrous proteins. It is a personal feeling that we have to learn more about these and related problems by studying how Nature itself during embryonic development and repair elaborates the various components of ground substances.

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C H A P T E R I V

THE ORGANIC MATRIX OF BONE

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I. Introduction

1. DEFINITION OF "MATRIX"

When the inorganic portion of a bone is dissolved with acid there remains a model of the bone in a coherent elastic material which is

sometimes referred to as the organic matrix (Le Gros Clark, 1945). The term matrix, which is widely used in descriptive anatomy, is derived from a Latin word meaning womb or uterus. This derivation implies the two main ideas governing the present use of the term.

- (a) A substance or medium enclosing other bodies.
- (b) A medium in which something is developed.

The organic portion of bone is known to consist of an intricate fibrillar network which encloses the minute inorganic crystals in its interstices. Further, this organic network, which remains substantially unchanged after treatment of bone with acid, in some ways resembles the tissue from which bone is formed by calcification, a process which involves the deposition or development of mineral crystallites within the pre-existing matrix.

This chapter describes the organic constituents of the intercellular substance of bone, but only passing reference is made to the visualizable structure of the collagen fibrils, which is dealt with specifically in Chapter V.

The above definition of matrix, being based by implication on the existence of mineral crystallites, is justifiable only when applied to bone or its precursors. The interfibrillar "ground substance" or cement of connective tissue in general is also sometimes referred to as matrix (Jacobson, 1953), and care is necessary to avoid confusion of the different meanings of a term which has been applied to various tissue components whose finer structures were almost unknown at the time.

2. THE CHEMICAL BALANCE SHEET FOR BONE

a. *The chemical constituents of bone*

(i) *Inorganic material.* The inorganic material is the constituent present in largest amount in bone, the principal ions being calcium and phosphate. Hydroxyl and bicarbonate ions occur in smaller but significant amounts, while magnesium, sodium, potassium, and fluoride ions are minor constituents. See Chapters VI & X for details of the chemical constitution and form of bone salt.

(ii) *Collagen.* A protein of the collagen family (see Section II) is the principal organic constituent of bone tissue. Collagen is the characterizing component of all vertebrate connective tissue and is laid down in the form of long slender fibrils by the activity of fibroblast cells (Fitton Jackson, 1953). (See Chapters I & V for details.)

(iii) *Water.* Bone, like all tissues, contains a substantial amount of water. This forms the medium for the diffusion of inorganic ions, sugars, and other small organic molecules necessary for cell nutrition and also allows the escape of waste products. The proportion of water is less in

bone than in softer tissues, it does, however, account for at least 20% of the weight.

(iv) *Citrate*. Shear and Kramer (1928) suggested that the citrate ion might play some part in the calcification process. The actual demonstration of citrate in bone is, however, a comparatively recent discovery (Dickens, 1940). This organic anion is usually considered to be associated with the inorganic crystal lattice (Neuman and Neuman, 1953). Its probable role in calcification has been discussed by Dixon and Perkins (1952). (See also Chapter XI in the present volume.)

(v) *Polysaccharide*. Bone contains a small quantity of a material rich in polysaccharide, the component sugars of which have been investigated recently (Hisamura, 1938b; Rogers, 1951; Masamune, Yosizawa and Maki, 1951). Eastoe and Eastoe (1954) have shown that it also contains a protein component. The composition of this protein-polysaccharide complex will be discussed in Section III.

(vi) "Resistant" protein. The existence in bone of small quantities of another protein which offers more resistance to solution in boiling water than collagen does, has been reported by several workers (Bogue, 1922; Rogers, Weidmann, and Parkinson, 1952). A more detailed discussion of the resistant protein is given in Section III.

(vii) *Other substances*. Little information is available concerning the existence in bone tissue of substances other than those already mentioned. Harris (1932) has speculated on the occurrence of glycogen in association with phosphatase in bone, but was unable to demonstrate it experimentally. Small quantities of lecithin of the order of 0.2% have been reported as being present in bones by Nerking (1908). It would appear doubtful whether true bone tissue contains lecithin, however, since Eastoe and Eastoe (1954) have reported the complete absence of fat in compact bone. Cells and small blood vessels must also make some contribution to the composition of bone.

The relationship between the histological elements of bone and the substances they are known or are likely to contain is illustrated on page 84. No idea of the relative amounts of each substance so contributed is given, however. The sizes of the various components are shown in Fig. 1.

b. *Relative proportions of the various constituents*

The proportions of the individual substances present in bone vary over a fairly wide range. Thus different relative proportions may be found according to the particular bone considered and the species. There is also a variation in composition in different parts of the same bone and with the age of the animal.

Inorganic matter, collagen, and water are the principal constituents

<i>Tissue element</i>	<i>Substance</i>
Intercellular substance	Inorganic crystallites Fibrils Cementing substance Portion surrounding cells
Osteocytes	Hydroxyapatite (plus CO_3^{2-} , Mg^{++} Na^+ , K^+ , F^- , and citrate) Collagen Mucopolysaccharide ? Resistant protein ?
Blood vessels	Soluble inorganic salts Cell proteins Carbohydrates Fats
Residual blood in vessels	Collagen Elastin
All above	Salts, plasma and cell proteins Water

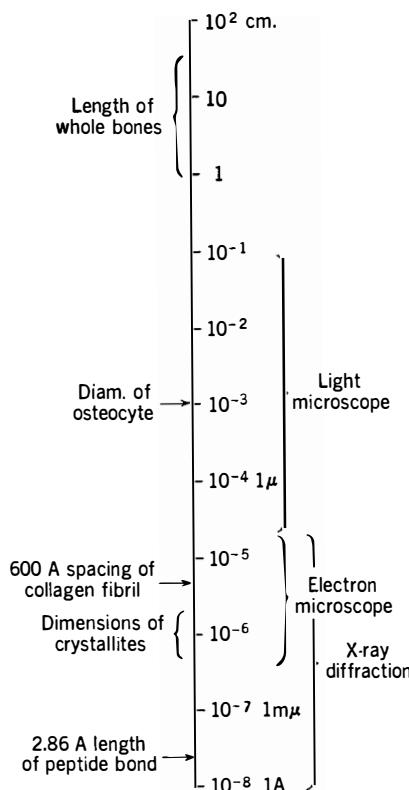


FIG. 1. Sizes of the components of bone tissue.

which together make up at least 96% of mammalian bone tissue. Some of the earlier analyses of bone were carried out on poorly defined material which was often closer to whole bones than clean bone tissue, as shown by the high proportion of fat sometimes reported (Gerngross and Goebel, 1944). Since the water content of bone rapidly diminishes after death and during the preparation of the tissue for analysis, *the percentage of each constituent is best expressed in terms of the oven-dried bone.* This avoids misleading effects due to an arbitrary variation in the moisture content.

A good overall impression of the variation in composition of bone tissue may be obtained from the recent work of Rogers *et al.* (1952). These workers determined the total nitrogen content of carefully cleaned samples of bone tissue and also distinguished the soluble substances appearing in the acid used for demineralization, the collagen, and the residue from autoclaving. They expressed their results in terms of the nitrogen appearing in each fraction. Eastoe and Eastoe (1954) considered that the total nitrogen content of these constituents are as given in Table I and these values have been used in calculating the actual percentages of each nitrogenous constituent (shown in Table II) from the nitrogen values of Rogers *et al.* Since the results are expressed in terms of oven-dried bone tissue, the "difference from 100" given in the last column is a reasonably accurate estimate of the percentage of inorganic matter.

TABLE I
TOTAL NITROGEN CONTENT OF VARIOUS ORGANIC FRACTIONS OF BONE
(Eastoe and Eastoe, 1954)

	Per cent N in dry, ash-free material
Demineralized bone	18.1
Water soluble fraction (assumed gelatin or degraded gelatin)	18.4
Bone collagen (free from mucopolysaccharide and resistant protein)	18.6
Resistant protein	14.6

There is a wide variation in the total organic matter content of the different bones of the rabbit. Compact bone invariably contains less organic matter than cancellous bone in the same member. The lowest value for organic matter is for the highly calcified femur shaft. Comparison of the values for total organic matter in rabbit, ox, and human femur shafts reveals remarkably large species differences, the values increasing in the ratio 3:4:5 respectively. There is a relatively small

TABLE II
CONTENT OF NITROGENOUS CONSTITUENTS IN RABBIT, OX AND HUMAN BONE
(Calculated from Rogers, Weidmann and Parkinson, 1952)

Type of Bone	Percentage of nitrogenous constituent on weight of dry, defatted bone				Difference from 100
	Total nitrogenous organic matter	Soluble in acid	Collagen	Insoluble on autoclaving	
Rabbit, femur, shaft	15.8	0.52	14.2	1.18	84.2
	25.0	1.10	23.6	0.19	75.0
	18.5	0.54	16.0	0.47	81.5
	23.7	0.63	22.6	0.19	76.3
	21.4	0.43	19.5	1.34	78.6
	28.1	0.46	26.5	0.24	71.9
Ox, femur*	25.8	0.60	24.1	0.34	74.2
	A1	20.4	0.48	18.0	79.6
	A2	20.0	0.36	17.8	80.0
	A3	21.1	0.40	19.6	78.9
	B1	19.8	0.48	18.4	80.2
	B2	19.4	0.39	18.2	80.6
	B3	20.6	0.53	18.6	79.4
	C1	19.8	0.53	18.2	80.2
	C2	21.6	0.49	19.7	78.4
	C3	23.3	0.47	21.3	76.7
	D1	21.3	0.54	19.3	78.7
	D2	22.2	0.48	20.8	77.8
	D3	23.1	0.58	21.7	76.9
Human, femur, age 2.5 years	Epiphysis	25.5	0.46	23.8	74.5
	7	26.7	0.55	25.0	73.3
	10	27.4	0.51	25.6	72.6
	15	27.2	0.56	23.8	72.8
	27	26.4	0.37	23.8	73.6
	41	26.2	0.47	24.3	73.8
	65	25.7	0.52	23.3	74.3
		25.8	0.55	22.3	74.2

* The positions of samples of ox femur in the bone are shown in Fig. 2.

variation at different points within a single ox femur shaft, lowest values for organic matter being reached towards the center and away from the bone surfaces. The proportion of organic matter in human femora definitely falls with increasing age, this variation is, however, small compared with the large changes in mechanical properties over the same period.

The citrate ion is a comparatively minor constituent of mammalian bone, for which recorded values range from 0.2 to 2% (Table III). Higher concentrations have been recorded in the bones of fishes.

TABLE III
THE CITRIC ACID CONTENT OF BONE FROM VARIOUS SPECIES

Species and bone	Citric acid (g/100 g bone)	Reference
Ox, bone from foreleg	0.272%	
red marrow of this bone	0.041	
Kitten, bone (including marrow) from ribs and legs	0.373	
cartilage, from ribs and leg joints	0.033	
"Steamed bone meal," ox and sheep	1.42	
Puppy, normal	1.31	Dickens (1941)
after prolonged administration of parathyroid hormone	1.66	
Kitten, normal	0.644	
rachitic	0.345	
Man, femur	1.62	
collar bone	1.87	
rib	1.75	
vertebra	0.89	
Gull, pelvis	2.08	
shoulder blade	2.63	
thigh	1.20	
breast bone	2.67	
humerus	0.60	
parietal bone	1.75	
coracoid bone	1.57	
vertebra of neck	1.78	
Herring, spine	5.25	
Mackerel, spine	1.60	
Cod, spine	2.01	
Frog, spine	0.29	

The mucopolysaccharide portion of bone is present only in small amounts. Hawk and Gies (1901) found approximately 0.4% of osseomucoid in the moist demineralized bone prepared from both ox femora and ribs, while Eastoe and Eastoe (1954) obtained a value of 0.24% for ox femur, expressed on air dried bone tissue. These figures may be low, since it is difficult to extract the protein-polysaccharide complex completely. Seifert and Gies (1904) have extracted complexes of the same general type from the bones of several species of mammals, birds, and fish.

It is not possible to obtain a definite figure for the resistant protein content of bone, since this material passes gradually into solution on continued heating with water. Since collagen dissolves in water at a finite rate, there is no point at which all of the collagen and none of the

resistant protein may be said to have gone into solution. Thus the apparent resistant protein content decreases with increase in the time of heating and the temperature of the extraction water.

c. Completeness of the Chemical Analysis for Bone

A complete chemical analysis would provide a useful check on our knowledge of the composition of bone. It would either exclude or point out the possibility of the occurrence of hitherto unknown but important minor constituents. It is difficult to carry out such an analysis, because several of the known constituents of bone are of indefinite composition and are correspondingly awkward to characterize and estimate. Eastoe and Eastoe (1954) have attempted to obtain a complete analysis for compact bone (Table IV).

TABLE IV
COMPOSITION OF AIR-DRIED COMPACT BONE TISSUE
[OX FEMUR DIAPHYSIS]
(Eastoe and Eastoe, 1954)

	Per cent by weight
Inorganic matter, insoluble in hot water (Probably including up to 1% of citrate)	69.66
Inorganic matter, soluble in water	1.25
Collagen	18.64
Mucopolysaccharide-protein complex	0.24
Resistant protein material	1.02
Fat	0.00
Carbohydrate, other than mucopolysaccharide	0.00
Water (lost below 105°)	8.18
	<hr/> 98.99

Bone from the center part of the ox femur shaft, between lines B and C (Fig. 2), from which all extraneous tissues had been removed, was reduced to a powder by a method involving little exposure to heat. The powder was spread out in a thin layer to allow its moisture content to reach equilibrium with the air, it was then placed in sealed bottles and used in this state as a reference material (Chibnall, Rees, and Williams, 1943). The moisture content was determined by drying to constant weight at 105°. The bone was extracted with a large excess of water at 100° for 24 hours in order to dissolve the collagen by converting it to gelatin. The residue, which consisted mainly of inorganic matter was filtered off, dried at 105°, and weighed. A small correction was applied, based on the total nitrogen content of the residue, for the residual pro-

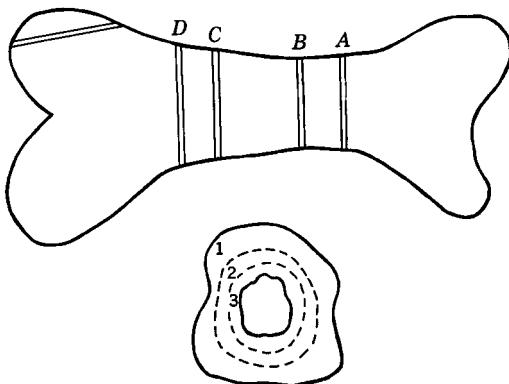


FIG. 2. Method of sampling ox femora (after Rogers *et al.*, 1952).

tein. The solution from the extraction was evaporated to dryness and the residue heated to constant weight at 105°; it was assumed to consist of degraded gelatin derived from the collagen. This material was finally ashed to obtain a value for the water-soluble inorganic matter. The percentage of protein-mucopolysaccharide complex, known from a separate lime-water extraction experiment, was subtracted from the collagen value. Extraction of a separate portion of powdered bone with ether showed that it contained no fat.

Ideally all the weight of bone should have been accounted for in the experiment, since the material was separated into several parts, each capable of being completely recovered and weighed. Actually the total of the material recovered was only approximately 99%. The remaining 1% could be accounted for by either:

- (i) mechanical losses occurring in the analysis,
- (ii) possible differences in the small amounts of moisture retained by "dried," bone powder, inorganic matter and gelatin at 105°.

This result does not conclusively prove that almost the whole weight of bone was accounted for as known constituents, since the methods employed were not sufficiently specific to preclude the possibility of an unidentified substance being included in the weight of one of the stated constituents. The analysis was, however, checked in two ways, each of which suggested that substantially all the bone was suitably accounted for.

- (i) The value for the inorganic matter agreed well with the ash value (Table V). The ash value definitely excluded any organic component and the small difference between the figures could be reasonably interpreted as due to a small amount of citrate

combined with the dried inorganic residue, together with water driven off between 105° and 450°.

- (ii) The total nitrogen content of bone tissue, when multiplied by a factor derived from the nitrogen content of demineralized bone, gave a value of 20.0% for the total nitrogenous organic matter of the bone. This is in good agreement with the figure of 19.9% for the total weight of collagen, resistant protein, and mucopolysaccharide complex (Table IV).

TABLE V
INORGANIC MATTER IN COMPACT BONE TISSUE
[OX FEMUR SHAFT]
(Eastoe and Eastoe, 1954)

	Per cent by weight
Total inorganic matter from (Table IV)	70.91
Bone ashed at 450°	70.1
Bone ashed at 500°	68.7
Bone ashed at 550°	67.9

II. The Chemistry of Bone Collagen

1. GENERAL

Collagen is a fibrous protein which appears in its most characteristic form in the mesodermal tissues of vertebrates. Bear (1952) has drawn up a list of proteins which have some claim to membership in the collagen group, on the basis of their X-ray diffraction diagrams. This list contains both vertebrate proteins and others from the phyla Mollusca, Echinodermata, Coelenterata, and Porifera. There is not a great deal of information about the amino acid composition of these invertebrate proteins, but that which exists suggests that they differ so sharply from vertebrate collagen that they cannot be considered as collagens in the commonly accepted sense. The vertebrate collagens, on the other hand, while showing some variation in properties and composition, form a more closely related group. Mammalian and avian collagens from various species differ little in their fibril structure (Randall *et al.*, 1952) and show only very slight variations in amino acid composition (Neuman, 1949). Fish collagens have lower contents of hydroxyproline and proline and more serine, threonine, and methionine than collagen from mammals and birds, Neuman, 1949; Eastoe, 1954). The scope of this chapter is restricted to mammalian collagen.

Fibrous proteins are the main constituents of animal fibers in contrast to plant fibers which are chiefly composed of carbohydrates. The fibrous

proteins include keratin, which is found in epidermal structures such as wool, fur, and feathers, myosin, a muscle protein, fibrin, the protein responsible for blood clotting, and the proteins of the mesoderm. The mesodermal or connective tissue proteins form a special group which includes elastin, reticulin, and collagen (Jacobson, 1953). Elastin occurs where very high elasticity is required, as in the walls of blood vessels; it differs in composition from collagen in having a much lower content of hydroxyproline and basic amino acids and a much higher content of valine (Bowes and Kenten, 1949). Reticulin is a relatively poorly characterized material that frequently occurs in conjunction with collagen and until recently has been identified only by its appearance under the light microscope together with its staining reactions. Kramer and Little (1953) have suggested, on the grounds of its appearance under the electron microscope and its strongly argyrophilic reactions, that it may consist of collagen in combination with a high proportion of carbohydrate. Collagen is the most abundant protein in connective tissues (skin, sinew, cartilage, bone, and interstitial connective tissue) where it occurs typically as striated fibrils visible with the electron microscope. A great deal of work on its chemical nature has been carried out (Borasky, 1950).

Collagen fibers are insoluble in cold water, but when heated with water they contract and slowly pass into solution as gelatin. Gelatin forms a clear colorless solution which, if of sufficiently high concentration, sets to a jelly on cooling. Collagen is more readily converted to gelatin if it is initially treated for a prolonged period with cold dilute alkali and then with warm water. The chemistry of the conversion of collagen to gelatin is not entirely understood (Ward, 1954a).

2. AMINO ACID COMPOSITION

When a protein is heated with a strong acid for a sufficient time the polypeptide chain is entirely broken down into its constituent amino acids, which undergo comparatively little decomposition. The quantitative determination of amino acids has, until lately, presented a difficult problem. The recent introduction of microbiological methods (Neuman, 1949) and more especially, of quantitative chromatographic techniques (Moore and Stein, 1951) has enabled almost the entire weight of proteins such as collagen to be accounted for in terms of amino acids. Moreover, complete analyses can now be carried out on a few milligrams of protein, whereas the classical methods required many grams.

In Table VI, values are given for the amino acid composition of collagen and gelatin from various mammalian sources, including bone.

TABLE VI
AMINO ACID COMPOSITION OF COLLAGENS, GELATIN AND OSSEOMUCOID
(Grams of amino acid in 100 g dry, ash-free, protein)

Reference	Gelatin	Ox hide Collagen	Cattle Bone Collagen	Collagen from Compact Bone		Osseoinucoid
				(ox femur)	(human femur)	
	Tristram (1953)		Neuman (1949)	Eastoe (1955)		Eastoe and Eastoe (1954)
Methods	Various		Micro-biological	Resin column chromatography		
Total nitrogen ^a	18.0	18.6	18.0	18.26	18.45	11.3
Alanine	9.3	9.5	—	10.5	10.9	3.70
Glycine	26.9	27.2	23.3	25.3	25.8	2.65
Valine	3.3	3.4	2.7	2.65	2.97	4.50
Leucine	3.4	} 5.6	3.4	3.93	3.60	7.27
Isoleucine	1.8		2.1	1.73	1.88	3.65
Proline	14.8	15.1	—	14.7	15.3	4.24
Phenylalanine	2.55	2.5	2.6	2.88	2.49	2.86
Tyrosine	1.0	1.0	0.98	0.56	0.86	1.98
Tryptophan	0.0	0.0	<0.01	—	—	—
Serine	3.18	3.37	3.1	4.24	4.06	3.61
Threonine	2.2	2.28	2.3	2.52	2.35	4.13
Cystine	0.0	0.0	0.11	0.0	0.0	1.13
Methionine	0.9	0.8	0.72	0.80	0.84	1.09
Arginine	8.55	8.59	9.0	9.2	8.8	3.87
Histidine	0.73	0.74	0.64	0.96	0.96	2.65
Lysine	4.60	4.47	5.5	4.11	4.40	4.26
Aspartic acid	6.7	6.8	—	7.1	6.7	9.66
Glutamic acid	11.2	11.3	11.2	11.9	11.4	11.67
Amide N ^a	0.07	0.66	—	0.63	0.56	1.07
Hydroxyproline	14.5	14.0	14.1	14.1	14.1	0.00
Hydroxylysine	1.2	1.1	^b	1.12	0.62	0.00
Glucosamine	—	—	—	0.00	0.00	1.23
Galactosamine	—	—	—	0.00	0.00	7.67
Total	116.81	117.25	—	118.3	118.0	81.82
% Recovery by weight	97.6	98.0	—	99.0	98.6	circa 70

^a Not included in total.

^b Probably included in "lysine" figure.

Collagen is unique in possessing a high proportion (13–14% by weight) of hydroxyproline; the only other protein to contain this amino acid is elastin which has 1.5–2.0%. Hydroxylysine occurs in no protein other than collagen, apart from traces found in elastin, which may be attributable

to collagen occurring as an impurity. Collagen also has very large amounts of the short side-chain residues, glycine and alanine, and comparatively small amounts of the leucines, with their long aliphatic side chains and the aromatic amino acids, phenylalanine and tyrosine. Bone collagen appears to be almost identical with collagen from skin in amino acid composition (Table VI); the low value for glycine, obtained by microbiological analysis (Neuman, 1949), is probably erroneous, since it has not been confirmed by chromatographic methods (Eastoe, 1955).

3. CHARACTERISTICS OF THE INTACT MACROMOLECULE

a. *Molecular Weight*

The molecular weight of collagen is likely to be very high. Bowes and Moss (1953) were unable to detect any α -amino groups in native collagen by the fluorodinitrobenzene method of Sanger. The sensitivity of their method would suggest that the molecular weight must exceed ten million, unless the terminal amino groups of the chain were blocked by combination with other molecules (e.g. mucopolysaccharides) or alternatively if the polypeptide chain formed a closed ring. M'Ewan and Pratt (1953) used a different approach and determined the molecular weight by light-scattering measurements on a solution of collagen in acetic acid. They obtained results ranging from ten to twenty million for tendon collagen. The number average molecular weight of gelatin, as determined by end group studies (Courts, 1954), is very much lower, being of the order of 60,000. It is clear, however, that molecules of gelatin with molecular weights of the order of 300,000 can readily be obtained (Ward, 1954b).

b. *Functional Groups*

The characteristic functional groups of the individual amino acids are detectable in the intact collagen molecule, where they appear as side chains attached to the much longer polypeptide chain. The properties of the groups are usually only slightly modified by the incorporation of the amino acid into the protein. Thus, collagen has marked acid-base properties due to the presence of free carboxyl, amino, and guanidino groups in the side chains of the acidic and basic amino acid residues. The acid-base characteristics of collagen have been determined and represented by a titration curve (Bowes and Kenten, 1948). The dissociation constants of the various groups in collagen vary only slightly from those of the free amino acids.

Below pH 1.5 the collagen molecule has only positively charged groups, the free carboxyl groups being un-ionized. In this region the net positive charge on the molecule is clearly at a maximum. With

increase in pH the net positive charge gradually decreases to zero at the isoionic point, the pH at which the molecule has zero net charge, further increase of pH results in the molecule acquiring a gradually increasing negative net charge which rises to a maximum in the neighborhood of pH 14. Thus over the range pH 1.5 to 6.5, the uncharged carboxyl groups dissociate and acquire negative charges through the loss of a proton; between pH 6.5 and pH 8.5 the imidazole groups of histidine and the terminal α -amino groups lose their positive charges. The same thing occurs with the ϵ -amino groups of lysine and hydroxylysine from pH 8.5–11.5 and finally with the strongly basic guanidino groups of arginine between pH 11.5 and 14. At the isoionic point, both positively and negatively charged groups are present, but in exactly equal numbers. The position of the isoionic point clearly depends on the exact composition of the protein as regards ionizable groups.

The pH at which a substance in solution or suspension does not migrate in either direction in an electric field can be observed experimentally and is known as the isoelectric point. Where no foreign ions are present the isoelectric point is identical with the isoionic point. In the presence of a dissolved salt, however, they may no longer coincide owing to preferential adsorption of either the salt anion or cation by the protein molecule. The presence of a salt in solution is essential for the measurement of the isoelectric point, to render the solution conducting. The value found is therefore unavoidably a function of the type of salt present and its concentration, so that the experimentally determined isoelectric point is not so fundamental a property as the isoionic point determined in the absence of small ions.

When collagen is converted to gelatin by a method involving substantially no loss of amide groups and the gelatin is deionized by treatment with mixed-bed ion-exchange resins, the resulting solution contains only gelatin at pH 9.4, the isoionic point of the gelatin and presumably also that of the parent collagen (Janus, Kenchington and Ward, 1951). Brown and Kelly (1953) found that extracted skin collagen in acetate buffer of ionic strength 0.05 had an isoelectric point at pH 5.8. There is some evidence to suggest that extracted collagen may have a slightly lower amide content than insoluble collagen, but this could only account for a small part of the large observed difference, clearly the reduced value of the isoelectric point can only result from a marked preferential adsorption of anions.

Collagen, *in vivo*, may generally be assumed to be in an environment whose pH is below that of the isoionic point (pH 9.4), probably closer to pH 7. The collagen molecule itself, therefore, carries a net positive charge and to maintain electrical neutrality must be associated with other negatively charged ions. In most tissues the predominating chloride

ion probably fulfils this role, the considerable weight of collagen in the body forming a reservoir for chloride. In bone, however, the collagen may perhaps be associated with the ionic lattice of the hydroxyapatite, the positively charged groups possibly replacing surface calcium ions. The possibility of interaction with ionized sulfate groups of acidic polysaccharides having a cementing function must also be taken into account (See Section IV. 2).

The more reactive functional groups can combine with various reagents to form derivatives, e.g. the ϵ -amino groups of the lysine residue reacts with fluorodinitrobenzene. Amino acids such as tyrosine which give rise to ultraviolet absorption in the free state, show almost identical absorption in the collagen molecule (Goodwin and Morton, 1946).

The properties of the side chain groups, taking into account their relative spatial distribution, are mainly responsible for the characteristic physical and chemical properties of individual proteins. One of the more disappointing features of recent advances in our knowledge of the amino acid composition of proteins is that it does not enable the properties of a protein to be predicted or even explained (except perhaps such simple considerations as solubility), so important are the stereochemical relationships (in the widest sense) governing the relative positions and hence the interaction of the side chain groups.

c. Amino Acid Sequence

Methods have recently been developed for the determination of the amino acid sequence in proteins. The sequence in insulin, a protein of relatively simple composition and low molecular weight, has been completely worked out (Sanger and Tuppy, 1951). Attempts to determine the amino acid sequence in collagen (Kroner, Tabroff and McGarr, 1953; Schroeder, Honnen and Green, 1953) are only to be regarded as of a preliminary nature, owing to the complexity of the problem. It seems possible, in the light of electron microscope and X-ray diffraction evidence, that collagen has a partially ordered and partially random sequence. The sequence, proline (or hydroxyproline)—glycine—any other residue, suggested by Astbury (1940), which is based mainly on early amino acid analyses, is neither in agreement with recent analytical data nor with the results of chemical studies of the amino acid sequence. See also Chapter 5.

d. Stereochemical Structure

Pauling and Corey (1951) have suggested, on the basis of X-ray diffraction data, that the native collagen molecule consists of a three-chain helix with a sequence of two *cis* and one *trans* (proline or hydroxyproline) residues. Later, this detailed hypothesis was withdrawn because it was not in agreement with all the experimental data. The idea that the

collagen molecule may be some sort of helix still persists, however, although no very definite suggestions have been put forward to date (Kendrew, 1954). Exact knowledge of collagen structure at this order of size would go far towards bridging the gap between chemical structure and structure capable of being rendered "visible" with the electron microscope. Fibrils could conceivably be made up of large numbers of overlapping helices, laid side by side. The observed periodicity would still require explanation.

4. SOLUBLE COLLAGENS

Orekhovitch, Toustanovski, and Plotnikova (1948) have extracted a soluble form of collagen from the skins of various animals, by using citrate buffers of pH 3-4. The citrate extract was dialyzed against distilled water, when the soluble collagen was thrown out of solution as needle-shaped crystals. Since the skins of young animals contain larger amounts of soluble collagen than those of older ones, the soluble form was considered to be a precursor of collagen in the organism and was named procollagen by the Russian workers. Isotope studies on the incorporation of glycine labelled with C¹⁴ into collagen (Harkness *et al.*, 1953) have largely confirmed the idea that procollagen is converted to collagen in the organism. It is possible that osteoid tissue (Weinmann and Sicher, 1947) which appears in developing bones contains a high proportion of procollagen. The material resembling gelatin, reported as being present in hydrochloric acid (Rogers *et al.*, 1952) and water (Eastoe and Eastoe, 1954) extracts of bone may be some form of soluble collagen; alternatively it may be collagen which has become degraded by heat during the preparation of the sample.

Bowes, Elliot, and Moss (1953) have recently investigated the amino acid composition of citrate-extracted soluble collagen and find that it resembles gelatin in containing slightly more hydroxyproline and less tyrosine and hexosamines than native collagen. They have suggested that this could be explained by assuming that procollagen is converted to collagen by the addition of a fraction relatively rich in tyrosine and hexosamines and poor in hydroxyproline. The main difficulty in the way of a complete elucidation of this point would appear to be the preparation of a sample of collagen free from adventitious impurities (e.g. the resistant protein) which represent larger structures than the collagen macromolecules. Such impurities would confuse the data regarding the relation of procollagen to collagen.

Jackson and Ball (1953) have precipitated collagen from acetic acid solution in fibrous form by the addition of sodium chloride. Adjustment of the concentration of sodium chloride enabled the process of fiber formation to be observed.

Recently, an alkali-soluble collagen has been discovered and there are an increasing number of reports indicating that collagen can go partially into solution under a variety of conditions. The exploration of the properties of the various soluble collagens may eventually throw more light on the complete fibrous structure of collagen itself.

III. Chemical Nature of the Other Constituents of the Matrix

1. ISOLATION OF A PROTEIN-POLYSACCHARIDE COMPLEX

In 1901, Hawk and Gies isolated from ox femora and ribs, a protein-polysaccharide complex which they designated "osseomucoid." The bone was first slowly demineralized in dilute hydrochloric acid and shavings of "ossein" were scraped off. The demineralized bone shavings were finely chopped, washed, and extracted for 48 hours with half-saturated lime water. A slight excess of dilute acid was added to the filtered extract, which resulted in the separation of a bulky, flocculent precipitate of osseomucoid. This was purified by redissolving in lime water and again precipitating with acid. Hawk and Gies reported that the acid filtrate from the original precipitation contained gelatin and a substance related to, if not identical with, the separated mucoid. This method of isolating polysaccharide material from bone has been subsequently used by several workers. Sodium hydroxide solution is a more efficient extracting agent than lime water for mucopolysaccharides, but causes rapid degradation resulting in a decrease in molecular weight (Partridge, 1948).

The complex nature of osseomucoid was realized by Hawk and Gies, although at that time suitable techniques were not available for its characterization. It gave the standard tests for protein, but neutral solutions did not coagulate on boiling. After hydrolysis with hydrochloric acid, sulfate and a substance with reducing properties were shown to be present. This carbohydrate gave an osazone with phenylhydrazine but it was not identified. Considerable variation was found in the elementary composition of osseomucoid from different preparations (C, 49.08–46.40; H, 7.42–6.53; N, 14.44–11.72; S, 1.40–2.55; O, 27.92–32.65) and it was concluded that two or more "glucoproteids" were present.

2. SEPARATION OF THE CONSTITUENTS OF THE COMPLEX AND COMPOSITION OF THE CARBOHYDRATES

Hisamura (1938b) separated osseomucoid into two components, fraction a, which was soluble in 1.2% acetic acid and an insoluble fraction b. These were separated by repeatedly precipitating the acid-insoluble portion in the presence of an excess of kaolin. From 6.8 g of

osseomucoid, 0.95 g of fraction "a" and 1.8 g of fraction "b" were obtained. The analysis of the original material and the fractions gave the results shown in Table VII.

TABLE VII
COMPOSITION OF THE MUCOPOLYSACCHARIDE FRACTIONS
(Hisamura, 1938b)

	Entire complex	Fraction a	Fraction b
Nitrogen	13.28	3.06	12.55
Ash	2.22	23.56	1.71
Sulfate sulfur	1.07	5.85	0.56
Total reduction (as glucose)	—	43.54	—
Carbohydrate reduction (as glucose)	19.9	—	14.59
Chondrosamine	11.2	26.81	7.53
Acetyl	2.75	6.35	1.96
Glucuronic acid	6.6	26.85	0.54
Galactose	4.8	—	6.9
Non-amino sugar (as glucuronic acid)	—	36.8	—

The composition of each fraction was almost identical with the corresponding fraction obtained from the mucopolysaccharide of ox trachea cartilage (Hisamura, 1938a). The cartilage complex, however, contained a higher proportion of fraction "a" than did osseomucoid. Fraction "a" in both preparations was identified as chondroitin sulfate, the hexosamine being shown to be chondrosamine (galactosamine) from the optical rotation of its benzoate. The hexosamine constituent of fraction "b" was also identified as chondrosamine while indirect evidence was put forward that the hexose present was galactose. The high nitrogen figure for fraction "b" was compatible with the presence of a protein constituent, while the small amounts of sulfate sulfur and glucuronic acid found, were attributed to contamination by the polysaccharide of fraction "a." Hisamura considered that equimolecular proportions of galactose and acetyl-chondrosamine were present in fraction "b."

Rogers (1951) isolated a polysaccharide from demineralized bone, after destroying the protein constituents with trypsin, by repeated extraction of the residual non-dialyzable material with alkaline ethanol in the presence of potassium acetate. This polysaccharide contained approximately equimolecular amounts of a hexosamine, a hexuronic acid, and sulfur. It seems reasonably certain that this polysaccharide was identical with the "fraction a" of Hisamura (1938b) and consisted of chondroitin sulfate. Rogers noted that the amount of this polysaccharide

accounted for only a small part of the total hexosamine of ox shaft bone as determined colorimetrically.

Recently, attempts have been made to separate the component monosaccharides from a complete hydrolyzate of osseomucoid by paper chromatography. Masamune *et al.* (1951) confirmed the presence of galactosamine, glucuronic acid, and galactose, already noted by Hisamura (1938b) and in addition found glucosamine (chitosamine) to be present. Eastoe and Eastoe (1954) also noted the occurrence in osseomucoid hydrolyzates of a small amount of mannose in addition to the residues already demonstrated by Masamune *et al.* (1951). They confirmed the presence of glucosamine and galactosamine by separation with ion-exchange resin and determined the amounts present colorimetrically (Table VI). The presence of two additional residues, glucosamine and mannose, suggests that osseomucoid contains polysaccharides besides the two separated by Hisamura, which accounted for only part of the weight of the material.

3. COMPOSITION OF THE PROTEIN

Eastoe and Eastoe (1954) analyzed a hydrolyzate of osseomucoid by the ion-exchange chromatography method of Moore and Stein (1951) and were able to account for the whole of the nitrogen of the complex in terms of amino acids and hexosamines. The results of the analysis are shown in Table VI. The composition of the protein portion of osseomucoid is different from that of collagen. It contains no hydroxyproline, greatly diminished amounts of glycine, alanine, and proline and large amounts of leucine and tyrosine compared with collagen. The overall amino acid composition of the protein part of osseomucoid is somewhat similar to that of the blood serum proteins (Tristram, 1953).

4. RELATIONSHIP BETWEEN THE PROTEIN AND POLYSACCHARIDES

In the past, the tacit assumption has sometimes been made that hyaluronic acid, which notably occurs in skin, and chondroitin sulfate, one of the major constituents of cartilage, are the only characteristic mucopolysaccharides of connective tissue. The protein portion of osseomucoid has been assumed to be degraded collagen co-precipitated with the mucopolysaccharides. The fact that this protein has been shown to be not collagen but a protein of entirely different composition, which can only be present in limited quantities in bone, suggests that it is not an accidental contaminant in loose combination with the polysaccharide. This, together with the apparent stability of Hisamura's fraction "b," which contained galactose, galactosamine, and a high proportion of nitrogenous material, implies that this fraction is a true mucoprotein in

which protein and carbohydrate residues are firmly bound to form a macromolecule. The simultaneous precipitation of this mucoprotein together with chondroitin sulfate and other mucopolysaccharides may, however, be the result of chance similarities in the properties of molecules that are entirely unrelated in the living bone. On the other hand, there may be some kind of loose chemical association between these mucoids that exist as a complex in the bone; this association is broken down during the alkaline extraction and reformed on addition of acid.

Chondroitin sulfate and the supposed mucoprotein are the two best characterized components of the osseomucoid complex. The same two constituents occur in cartilage (Hisamura, 1938a). The existence of these polysaccharides in both tissues is not surprising in view of the formation of bone by calcification of cartilage.

The demonstration of other sugar residues, glucosamine and mannose in osseomucoid, suggests that further mucopolysaccharides (or possibly mucoproteins) are present in bone. Consden (1953) has recently found galactose, glucose, mannose, and glucosamine residues in the mucopolysaccharides of human subcutaneous connective tissue.

5. THE "RESISTANT" PROTEIN

The presence in bone and dentin of a protein which is more highly resistant than collagen to solution in hot water, has been reported by several workers (Stack, 1951; Rogers *et al.*, 1952; Eastoe and Eastoe, 1954). The residue from bone consists partly of a mass of tubules, visible to the naked eye and partly of an amorphous powder. The resistant protein passes gradually into solution on prolonged boiling, which explains the rather erratic variations in the apparent percentage found in various bone specimens (Table II). Eastoe and Eastoe (1954) have examined the amino acid composition of the entire heterogeneous insoluble fraction by paper chromatography and found differences from both collagen and elastin. No hydroxyproline was present and the proline and glycine contents were much less than are found in collagen. The valine and alanine contents were much less than those of elastin and the tyrosine content was higher than in either protein.

Le Gros Clark (1945) has described the existence of vascular canals in cartilage which resemble in appearance the vascular portion of the residue from autoclaved demineralized bone. Cartilage canals contain blood vessels and if the residual material from bone is of an analogous type it probably contains elastin. In any event these vascular inclusions may be regarded as adventitious to true bone tissue and therefore without bearing on its microstructure.

The apparently amorphous powder may result from the disruption of

smaller vessels. There is a slight possibility, however, that it may be derived from the lining to the osteocyte cavities since this has been stated to differ in composition from the rest of the bone and to be more resistant to hydrochloric acid (Carleton and Leach, 1949) and alkali (Weinmann and Sicher, 1947). Weinmann and Sicher also state that the osteocyte capsule contains no argyrophilic fibers and can be stained with hematoxylin.

IV. The Place of the "Organic Matrix" in Bone Structure

In the foregoing three sections of this chapter, the structure of bone has been examined from an "analytical" point of view, in terms of its structural and chemical components. These two types of structural unit which intrinsically differ only in their order of size, have hitherto been considered separately.

The object of this final section is to show how the various structural units already described may fit together. In order to do this it is also necessary to be able to relate the chemical constituents as closely as possible to the tissue elements visible with the electron and optical microscopes. Since our knowledge of the fine structure and chemical make up of connective tissues generally is rudimentary, there will be, of necessity, large gaps in our complete picture of bone structure. These point the way for future research.

1. RELATIONSHIP BETWEEN THE CHEMICAL AND VISUALIZABLE COMPONENTS

More than 50% of the weight of bone consists of inorganic matter, which is almost certainly the material making up the minute crystallites recently demonstrated with the electron microscope (Robinson and Watson, 1952) and which give rise to definite X-ray diffraction patterns. It seems likely that the crystallites account for all the inorganic matter of the tissue, other than dissolved salts.

The protein, collagen, is the second most abundant constituent of bone. Collagen has been well characterized by its amino acid composition in bone and other connective tissues and seems to be free from carbohydrate residues bound up as an essential part of the molecule, although it frequently occurs in association with a variable quantity of mucopolysaccharide. Collagen, considered purely as a protein, is clearly the main constituent of the banded fibrils observed with the electron microscope.

The mucopolysaccharides and mucoproteins of bone fall into a less definite category. Comparatively little is known of the chemistry of

these compounds, although it seems certain that at least one of them, chondroitin sulfate, is a major component of cartilage and a minor constituent of other connective tissues. There is good reason to believe that the other compounds of this class, already described, are not confined to bone. The mucopolysaccharides are associated less definitely with structural units visible with the electron microscope.

Water is always found in bone tissue and probably occurs in the calcified matrix as well as in the cells. The recrystallization of bone crystals requires the presence of water, while collagen is normally associated with a quantity of firmly bound water. In addition it may be supposed that water fills up any small spaces in the structure.

The water-resistant protein would appear to be an adventitious constituent as far as the structure of true bone is concerned. Some of it is undoubtedly derived from small vessels penetrating the bone.

2. RELATION BETWEEN COLLAGEN AND POLYSACCHARIDE

Collagen and mucopolysaccharide occur in all mammalian connective tissues which have been investigated, although the proportion of the latter varies a good deal. The collagen fibril forms the central element of connective tissue structure. Since the length of the fibrils is many times their thickness, they can readily form an interlaced feltwork like a rather randomized fabric, which has both strength and flexibility. The fact that the fibrils tend to run parallel to one another in bundles enables the fabric to have anisotropic mechanical properties. Some cohesion must exist between neighboring or crossing fibrils due to electrostatic forces between oppositely charged ionized groups. Hydrogen bonding will also produce a similar effect, giving a collagen network a degree of cohesion in itself. The cohesion will be somewhat enhanced by residual forces and dipole interaction.

It seems likely, however, that one of the functions of polysaccharide is to link together neighboring fibrils. In this sense, the mucopolysaccharides may form a type of cementing substance. Since collagen would carry a net positive charge in the region pH 7-8, which is somewhat below its isoionic point, it could readily form polar bonds with, for example, the negatively charged sulfate groups of chondroitin sulfate. It is difficult, however, to assess the total contribution of this relatively small proportion of polar bonds to the cohesion of the whole system and its importance compared with the more numerous hydrogen bonds. The very small quantity of mucopolysaccharide in bone would be sufficient to carry out this cementing function in view of the comparatively small number of positively charged groups on the collagen at this pH. It is possible that in some tissues the polysaccharides form a true ground

substance filling up spaces between fibrils. Thus cartilage, which contains much chondroitin sulfate, has an obvious organic ground substance. Bone, on the other hand, would appear to have too little mucopolysaccharide to form an all pervading ground substance.

Local variations in the distribution of polysaccharides occur as shown by the examination of collagen specimens treated by silver impregnation. This implies, that in some places, bundles of collagen fibers firmly bound together with polysaccharide occur, while in other regions the fibers run singly or in smaller bundles.

It is possible that some mucopolysaccharide is included within the framework of a single fibril and is necessary for fibril formation. Bowes *et al.* (1953) have shown that soluble collagen contains little polysaccharide and have postulated that the formation of collagen fibers may result from combination between smaller pure protein molecules of the "procollagen" type and polysaccharide molecules. This could explain the existence of more than one type of mucoid in bone, one being present within the fibril and the other acting as an interfibrillar cementing substance. On the other hand, Bowes' results could be explained by the difficulty of removing all the cementing polysaccharide to leave insoluble fibrils of collagen (considered as a pure protein) without them becoming dispersed in solution. Without further evidence, it seems impossible to decide whether the collagen fibril contains carbohydrate as an essential constituent.

Collagen and polysaccharide occur together in the uncalcified precursors of bone—cartilage and membranous connective tissue. Considered together they constitute the organic matrix, the calcification of which results in bone tissue. Unfortunately, this statement is oversimplified for it makes no reference to the particular manner in which protein and polysaccharide are combined, which possibly varies from one type of connective tissue to another and gives each its own characteristics.

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CHAPTER V

COLLAGEN FIBERS OF CONNECTIVE TISSUE

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I. Introduction

Connective tissues are derived from the mesoderm. They may be classified into four main types: connective tissue proper, bone, cartilage and the hemopoietic tissues. The first is subdivided into loose, dense, regular (tendinous, lamellar) and specialized (adipose, mucous). All these tissues are composed of cells, fibers and ground substance. The fibers may be collagen, reticular, or elastic.

The present chapter deals essentially with the collagen fibers. In recent years, a vast amount of literature has been accumulating on this subject. Renewed interest in collagen was largely the result of technical progress in the fields of X-ray diffraction and of electron microscopy; and the new concept of "collagen diseases" opened further pathways for research in pathology.

Reticular fibers will be considered from the point of view of their relation to collagen. Elastic fibers will be omitted from this study, since they bear few direct relations to bone tissue.

II. Methods of Investigation

The study of collagen fibers relies essentially upon the following methods: histologic stains and silver impregnations, polarization microscopy, electron microscopy, and X-ray diffraction.

The latter will be dealt with in Chapter VI. Of the other techniques, two will be here considered in greater detail: *polarization microscopy* (method of choice for the study of collagen fibers in bone tissue) and *electron microscopy*.

1. POLARIZATION MICROSCOPY

If the refractive index of an object remains constant, independent of the direction of the incident light, such object is called *optically isotropic*. If the refractive index varies with the plane of vibration of the incident light, the object is *optically anisotropic* or *birefringent*, the term referring to the existence of *two* refractive indices.

A birefringent object, such that a light beam is transmitted without double refraction in one given direction, is called *uniaxial*, and the direction as above defined is the *optical axis*. To this category belong the fibers of animal and plant tissues, in which the optical axis coincides with the longitudinal axis of the fibers. A fiber is called *positive uniaxial* if the refractive power is greater in the directions parallel to the long axis than in those perpendicular to the same. It is called *negative uniaxial* in the opposite case.

Optical anisotropy, as observed in biological objects, falls into one—or more—of three types of birefringency:

(a) *Intrinsic or crystalline birefringency*, due to the regular array of atoms and molecules in the object. This type is characteristic for crystals, with the exception of those belonging to the cubic system.¹ The structural components of animal cells and tissues which are ordinarily of protein or lipid nature and which exhibit some degree of crystallinity show, as a rule, intrinsic uniaxial positive birefringence, the optic axis indicating the orientation of the long axis of the molecules.

(b) *Photoelastic birefringence*, which appears whenever a solid capable of elastic deformation is submitted to pressure or tension.

(c) *Form birefringence* (Frey, 1924). The latter manifests itself when particles of non-spherical shape, and of small size as compared to the wave length of light, are oriented in a preferential fashion. Such particles or micelles must be separated from the intermicellar spaces by definite phase boundaries (Frey-Wyssling, 1953). Form birefringence is not constant; it varies with the optical properties of the intermicellar phase. It falls to zero when the latter has a refractive index identical to that of the particles. In histologic sections, the intermicellar substance corresponds to the mounting medium. This variability is a distinctive feature of form birefringence; intrinsic birefringence, on the contrary, remains constant independent of the medium in which the crystal is placed.

If an object is composed of oriented birefringent rodlets (e.g. collagen), the intrinsic birefringence of the latter will be superimposed upon the form birefringence. The relative importance of each type of double refraction may be gauged by submerging the object in media of different refractive index (Ambroon, 1916, 1917; Frey-Wyssling, 1953). Thus double refraction cannot be reduced to zero; the remaining double refraction is due to the intrinsic birefringence of the particles.

The study of tissues by polarized light yields information concerning the presence and disposition of crystalline structures and of oriented submicroscopic particles. Furthermore, it may provide useful clues as to the chemical structure of tissue components (Schmitt, 1939).

2. ELECTRON MICROSCOPY

The electron microscope is the only device permitting direct and adequate observation of single collagen fibrils. The possibility of direct observation is certainly an advantage over X-ray diffraction studies, by which the structural character of the fibrils must be deduced on the basis of diffraction patterns. Moreover, the size of the object may be much smaller and submicroscopic structures may be detected even if the

¹ Another exception occurs when the actual size of the crystalline particles is beneath the resolving power of the optical microscope (Meyer, 1950a, b).

spatial arrangement of the particles is not sufficiently orderly for obtaining useful X-ray diffraction patterns.

On the other hand, electron microscopy is handicapped by a few limitations:

(a) Objects of average density, e.g. organic compounds, appear completely opaque if thicker than 0.3μ , because of supervening electron scattering which interferes with the formation of an image. Thus only very thin preparations are suitable; but these are obtained through a series of manipulations which inevitably entail some alteration of the material. This difficulty is minimized in X-ray diffraction studies, which are performed on larger specimens, requiring fewer preparatory manipulations.

(b) Objects must be observed under conditions of extreme vacuum, so that only dehydrated specimens can be utilized.

(c) While under observation, the specimen undergoes considerable electronic bombardment. The energy carried by the electrons is partly absorbed, causing heat production and ionization. Organic substances are thus disintegrated and give rise to compounds similar to graphite.

(d) Theoretically, the resolving power of the electron microscope should be 100,000 times greater than that of the optical microscope. Practically, it is only 100 times greater; the aberrations of the lenses cannot be corrected beyond certain limits set by the very nature of the electric and magnetic fields and by the necessity of obtaining proper contrasts. In biological materials, an electron microscope built according to classical concepts cannot reveal structural details of dimensions smaller than approximately 20 Å. These dimensions lie well above the limits of wide-angle X-ray diffraction, which is capable of providing information on structures of the order of 2 Å (e.g. amino acids).

Nevertheless, all these drawbacks are not as important as they may appear at first sight. Electron microscope studies on collagen have proved that the necessary manipulations (teasing, blending, embedding, etc.) do not entail major distortions, and that structural details of the order of magnitude of 100 Å are comparable with those deduced from the X-ray diffraction patterns.

The transformations caused by the electronic bombardment, on the structural level, are minimal. The aspect of the fibril is not modified; apparently, the ill effects of ionization are counterbalanced by the simultaneous ill effects of heating.

In concluding, the electron microscope provides us with means of exploring dimensions which lie—at present—between 20 Å and the domain of the optical microscope. Its range is that of the macromolecules; it is sufficiently wide to include the fundamental features of

biological structures. The specific features of crystalline bodies, ranging beneath 20 Å, can only be detected by means of wide-angle X-ray diffraction.

For the study of collagen-rich tissues three methods are available, all designed to provide very thin preparations (order of magnitude: 1/20 μ).

The first method involves the preparation of a *suspension of fibrils*, either by teasing, grinding, blending, or exposure to ultrasonics. A drop of this suspension is then placed on a supporting film. This method is useful for the study of the internal structure of the collagen fibrils.

The second technique is that of the imprint or *replica*, which is particularly suitable for the study of hard tissues (bone, teeth). Following this procedure, one does not observe the object itself, but an imprint of its natural surface, or of a surface obtained artificially (e.g. by polishing). If the imprint is obtained on the surface of a very thin sheet of material, as collodion film, the latter—once detached from the surface of the object—may be observed directly with the electron microscope. This procedure is referred to as *simple replica*. If the imprint is taken on thick material, it cannot be examined directly; a second and thinner casting is molded on its surface, so that the final observation is carried out on the “imprint of the imprint.” This technique is named *double replica*.

A type of double replica technique especially suited to the study of collagen fibrils in bone is that of Kellenberger (1948), utilizing celluloid and silicon monoxide. A bone surface is polished, moistened with acetone, and covered with a sheet of celluloid 0.3 mm in thickness, equally moistened with acetone. The solvent penetrates throughout the most minute asperities of the bony surface; and as it evaporates through the celluloid sheet, it is gradually replaced by the dissolved plastic. Within a few hours the celluloid is dry and can be easily detached from the object. Starting from this negative imprint, two positive imprints will be prepared: one of silicon monoxide, to be used for electron microscopy, and a thicker one of *n*-methylmethacrylate for use with the optical microscope (Fig. 2) * (Rouiller *et al.*, 1953).

The third method is that of *thin sectioning*. A number of observations have been performed on parenchymatous organs, but very few on connective tissues. A remarkable exception is the work of Robinson and Watson (1952), who succeeded in adapting the technique to calcified and decalcified bone.

* Figs. 1–3 appear in Plate I, Figs. 4–9 in Plate II.

III. Structure of Collagen Fibers

Collagen fibers appear under the optical microscope—especially if suitably stained or impregnated—as cords, bundles or ribbons of indefinite length, and 20 to 200 μ in width. They may be loosely woven and unoriented (loose or areolar connective tissue), more tightly interlaced (fibrous connective tissue, e.g. skin dermis, intestinal submucosa), arranged in planes (fasciae, aponeuroses, etc.) or in parallel bundles (ligaments, tendons).

The *primitive fibers*, 2 to 10 μ in diameter, are the thinnest components visible with the aid of the optical microscope (Kuntzel, 1929, 1941). Early dark-field studies showed that they could be further subdivided into thinner elements, a finding which was later confirmed and extended by electron microscopy and X-ray diffraction. These primitive fibers are actually bundles of *fibrils*, ranging in diameter between 2000 Å (the lower limit of resolution for the optical microscope) and 200 Å. Somewhat schematically, the primitive fibers and the fibril can be defined as the collagen *units* pertinent to the optical and electron microscope, respectively.

But the possibilities of longitudinal cleavage do not cease with the fibril. Wide-angle X-ray diffraction studies suggest the existence of the *protofibril*, an entity which lies beyond the resolving power of modern electron microscopes (Bear, 1952).

1. THE COLLAGEN FIBRIL (Fig. 1)

Collagen fibrils exhibit an axial repeating pattern or “overperiod” which measures, on dried material, 640 Å in length. This regular pattern was first discovered indirectly, through analysis of small-angle X-ray diffraction diagrams (Bear, 1942, 1944; Kratky and Sékora, 1943), and shortly thereafter photographed through the electron microscope (Schmitt, Hall and Jakus, 1942, 1945; Wolpers, 1943, 1944). It is characteristic of all mature collagen fibrils in the vertebrates and invertebrates so far examined.²

Whereas the figure of 640 Å is practically constant if derived from X-ray diffraction, electron micrographs show that it represents a mean value. In fact, though constant within a given fibril, the period may vary considerably from one fibril to another. Schmitt, Hall, and Jakus found values ranging from 400 to 1000 Å, with an average of 640. Such differ-

² It was further encountered in Echinodermata (e.g. starfish), Coelenterata, and Porifera (Marks, Bear, and Blake, 1949), in the earthworm, in the toad, the frog, and the eel (Randall *et al.*, 1952), and even in prehistoric material—mammoth tusks (Bear, 1944; Randall *et al.*, 1952).

ences are partially interpreted as artifacts caused by the preliminary manipulations.

Wyckoff (1952) has described a larger repeating unit or superperiod measuring 1950 Å, and determined by the regular alternation of two wider and one thinner (or "weak") period.

The pattern so far described is characteristic of the adult collagen fiber, but several authors have described collagen fibrils with a smaller period, measuring almost exactly one third the normal period (210 Å). They are thinner than average, and can be found in the connective tissue surrounding muscle fibers (Wyckoff, 1952), in cartilage (Randall *et al.*, 1952), and in tissue cultures (Porter, 1951). Wyckoff and Porter believe that they are not truly immature fibrils, but rather the expression of particular conditions existing during the early stages of fibrillogenesis. Porter speculates that the periodicity of 210 Å might represent a fundamental unit for biological fibers, since it has been described in collagen, fibrin and myosin.

On electron micrographs of shaded collagen fibrils, the overperiod can be subdivided into two bands, named *A* and *B* (Schmitt *et al.*, 1942) or *D* and *H* (Wolpers, 1944). The *A-band* is broader and more opaque. Its greater width indicates greater concentration of non-volatile material, thus proving that the greater opacity is not entirely due to the presence of atoms more opaque to electrons. It can be further broken down into two transversal bands separated by a shallow groove (dense bands *d*/1 and *d*/2 of Wolpers).

The *B-band* is narrower and more transparent to electrons. In some preparations it is crossed transversally by an elevated stripe. When the latter and the two stripes of the *A-band* are very marked, the fibril looks as if it had an axial periodicity of 640 Å, viz. 215 Å.

3

In electron micrographs of fibrils pretreated with phosphotungstic acid (PTA) it is possible to recognize further details. Collagen fibrils from rat tail tendons show five transversal bands within each period: *bands a, b, c, d and e* (Schmitt, Hall, and Jakus, 1945). The first four are in the denser *A-band*, the fifth in the *B-band*, all are slightly raised above the surface (Nutting and Borasky, 1948). Some may be split in two: *b*-1 and *b*-2 (observed in fibrils of chromium-tanned hides), *e*-1 and *e*-2 (Schmitt and Gross, 1948). When this occurs, the period is composed of seven bands (rat epineurium). The latter structures also can be distinguished on fibrils not treated with PTA, but the contrast is not as sharp. It may be concluded that the treatment with PTA merely accentuates a pre-existing structural feature, characterized by a slightly higher opacity to electrons.

On shaded preparations bands *a* and *d* are broader than the others. Thus *the overperiod is asymmetric* and can be assigned an anterior and a posterior end, conventionally oriented towards *a* and *e*.

Evidence drawn from small-angle X-ray diffraction studies of dried fibrils led Bear and collaborators (Bear, 1951, 1952; Bear, Bolduan, and Salo, 1951) to conclude that the overperiod of 640 Å is subdivided into two zones which differ as to the degree of perfection with which the polypeptide chains are aligned. In the zone corresponding to the A-band visible with the electron microscope, the polypeptide chains are distorted; in the zone between the bands, corresponding to the interband, they are regularly packed with a degree of regularity approaching that of crystalline perfection. The alternation of bands and interbands is thought to be determined by the distribution of amino-acid residues along the polypeptide chain (Bolduan, Salo, and Bear, 1951).

2. THE PROTOFIBRIL

Collagen fibrils are susceptible to further longitudinal cleavage, either as an effect of particular treatments, or even spontaneously in certain tissues. The subfibrillar ramifications maintain the striation characteristic of the fibril, and, if they rejoin, their periods match transversally.

In collagen obtained from human skin, washed in water and chromium-shaded, Schmitt and Gross (1948) could distinguish 3 to 10 filaments within each fibril. These observations led Schmitt and collaborators (1942, 1944, 1948) to postulate the existence of subfibrillar units, extremely thin (approx. 20 Å) but exhibiting nevertheless all the chemical and morphological characteristics of collagen. These units or *protofibrils* are thought to be formed by lateral aggregation of a small number of polypeptide chains. Porter (1951) admits as protofibrils the minute filaments, close to the limit of resolving power for the electron microscope, which he detected in cultures of fibroblasts. Bear (1952) prefers to reserve the name of protofibril for the ultimate (theoretical) unit beyond which no further cleavage is possible without loss of the specific properties of collagen. Otherwise stated, the protofibril should be identified with the polypeptide chain. Its width is probably that of a collagen molecule; it lies therefore beyond the reach of modern electron microscopes and can only be studied by wide-angle X-ray diffraction.

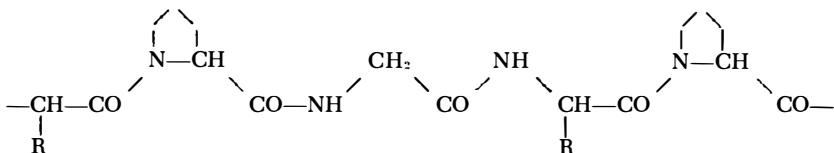
Mathematical analysis of such diffraction patterns yields three characteristic repeating units. The first measures 2.86 Å in length and is oriented along the longitudinal axis of the fibers; the two others are oriented perpendicular to this axis, and measure respectively 11-15 and 4-5 Å. A detailed description and analysis of the diffraction patterns is beyond the scope of the present article; the reader is referred to the

fundamental papers by Astbury (1938, 1940, 1950) and Bear (1951, 1952).

The diffraction pattern of collagen is obtained from bundles of macroscopic dimensions. It arises as the summation of the elementary patterns corresponding to each individual fibril. Significant results can be obtained only if the fibrils display a certain degree of orientation in parallel bundles. Both diffraction and electron microscope studies show that adjacent fibrils are juxtaposed in such a way that regions of corresponding structure fall in register. It is therefore reasonable to assume that the same topographic coincidence occurs between protofibrils. Accordingly, the morphologic characteristics displayed by the fibrils are determined by the addition of specific chemical structures existing along the protofibrils. We will therefore consider more closely the chemical structure and the mutual relations of the protofibrils.

Numerous models have been suggested as tentative reconstructions of the collagen polypeptide chain, striving to reconcile the data accumulated through X-ray diffraction, electron microscopy, infrared studies, and other physical methods, as well as through chemical analyses.³

The amino acid links of the polypeptide chain measure 2.86 Å in length. In order to explain this figure, which is smaller than that usually found in stretched polypeptide chains (3.4 Å), Astbury (1940) introduced *cis*-amide groups in the structure of the chain, and held the numerous and large proline and hydroxyproline molecules responsible for the mixed *cis* and *trans* configuration. Since the amino acid composition of collagen is approximately consistent with an amino acid sequence such as -P-G-R-P-G-R-P-G-R-, where P represents proline or hydroxyproline, G glycine and R other amino acids (alanine, lysine, serine, etc.), the structural formula of chain may be outlined as follows:



³ The main chemical characteristics of collagen are the following: unusually high content of proline (15–16%), hydroxyproline (13%) and glycine (27–29%); on the other hand, very low percentage of aromatic amino and sulfur-containing acids (Neuman, 1949; Gustavson, 1949). This chemical composition is constant for all collagens of one or of related species. Significant differences in the amino acid composition can be demonstrated only between collagens of species very remote from one another. As a rule, collagens from more primitive species show higher sulfur levels.

This model as proposed by Astbury encountered numerous objections. The existence of *cis* and *trans* configurations has never been proved, and according to Pauling, Corey, and Branson (1951) such structures are unstable. Furthermore, it cannot account for more than a 28% increase in length through stretching (Bear, 1952) whereas Schmitt and Gross (1948) have shown that the collagen fibril (not the fiber) can stretch well beyond that limit. Finally, it does not explain the infrared dichroism observed by Ambrose and Elliot (1951) and by Randall (1952).

Huggins (1943) suggests that the polypeptide chain is not stretched but coiled or folded. For Pauling and others (1951) the collagen molecule is formed of three polypeptide chains, intertwined and held together by hydrogen bonds. Ambrose and Elliott (1951) and Randall (1952) adopt the model proposed by Huggins, with some modifications suggested by diffraction and infrared studies and concerning particularly the position of the C=O groups.

For Bear (1952), the various models suggested by Astbury, Huggins, and Ambrose, and Elliot are not acceptable, on the basis of their very limited capacity to stretch. In order to explain the extensibility of the protofibril (see page 136), Bear was led to postulate that the polypeptide chain is helicoidal. Taking into consideration the cross-sectional surface of the protofibril, the mean molecular weight of the amino acid residues, and the density, Bear concludes that the axial projection of 2.86 Å includes not one but three residues, the projection of each one averaging between 0.91 and 0.95 Å. Such a short projection cannot be explained except by admitting that the polypeptide chain is helicoidal. This spiral structure is stiffened by inter-turn hydrogen bonds provided by the imino and carbonyl groups of the main polypeptide chain. The latter can be stretched to approximately four times its original length.

The relationships between the main chains depend on the various types of *side chains*. Non-polar side groups are provided by glycine, proline, and alanine, which represent approximately 64% of the axial length. Such groups are chemically inert, and contribute to cohesion within the fibril only through electrostatic (or van der Waals-type) forces. Most of the hydroxyl-containing side chains belong to hydroxyproline and serine, which account for 16% of the axial length. Their share in cohesion consists in the formation of hydrogen bonds. The lateral chains provided by acid groups (glutamic and aspartic acids) and basic groups (lysine, arginine) make up for the remaining 20% of the axial length; they are positively or negatively charged and form salt-like links.

In concluding, the links between polypeptide chains and therefore the cohesion of the collagen fibril involve two types of bonds: (a) the *direct valence bonds*, including electrovalency bonds (e.g. the salt-like

links) and coordinate valence bonds, which occur between carbonyl and amino groups of adjacent chains. The latter are rather weak if considered individually, but may add up to considerable importance as a factor of cohesion, on account of their great numbers (Schubert, 1952). (b) *Indirect valence bonds*, of electrostatic nature, or of van der Waals forces.

According to Astbury (1940) and Huggins (1943), the main chains are placed side by side, and held together by hydrogen bonds resulting in the formation of sheets. The axes of adjacent chain are spaced 4 to 5 Å apart. Adjacent sheets are separated by a distance of 10.4 Å (measured in the dry state) which is determined by the length of the lateral chains. For Bear the fibril is cylindrical in shape; longitudinal cleavage should give rise to cylindrical and not to laminar filaments, and a cross-sectional surface of the interband, where the protofibrils are very closely packed, should demonstrate a hexagonal pattern.

The transversal measurement of 4.5 Å identifies either the distance between main chains of the same sheet (Astbury, 1938, 1940, 1950; Huggins, 1943), or the width of the backbone of the main chain, or the distances between atoms across the main chain coil (Bear, 1942, 1944, 1951, 1952). The measurement of 10.4 Å (dry fibrils) to 15 Å (wet fibrils) corresponds either to the space between adjacent sheets (Astbury, Huggins) or to the gap between adjacent protofibrils (Bear).

The *collagen molecule*, according to Bear (1952), includes only a portion of the protofibril. Its width is equal to the cross-sectional diameter of a single coiled polypeptide chain and its length is that of the overperiod (640 Å). It is very unlikely to be shorter, since electron micrographs have proved that the overperiod is asymmetrical. The boundary between molecules aligned in a protofibril lies probably between the *e* and the *a* bands; at this level the fibrils show a marked tendency to undergo sharply transversal breaks.

Granted that the collagen molecule measures 640 Å in length, each molecule should consist of 670 to 700 amino acid residues with an average molecular weight of 93. The *molecular weight of a collagen molecule* should therefore lie between 63 and 65,000. These figures are in good agreement with those obtained by Scatchard *et al.* (1944) and Pouradier, Roman and Venet (1949).

The occurrence of intraperiodic structures, as demonstrated by X-ray diffraction and by electron microscopy, has been ascribed to a specific distribution of amino acids along the main chain. The acid and basic amino acids carry side chains of considerable length, which could scarcely fit between the main chains since the latter, at the level of the interband, are as close as 4.6 Å. Bear suggests that the amino acids

bearing these side chains are grouped within the region defined as band, which is wider, more irregular and more opaque to the electron beam.

If this view is accepted, one may conclude that side chains with acid or basic polar groups prevail in the bands, whereas side chains carrying hydroxyls prevail at the interbands. As for non-polar residues, they are thought to be uniformly distributed throughout the entire length of the chain.

3. RETICULAR FIBERS

The relationship between collagen and reticular fibers has been the subject of debate for well over half a century.

We will first consider the evidence which tends to prove that the two types of fibers are fundamentally different. Arguments are drawn from their morphological aspect and affinity for stains, as well as from their physical and chemical properties.

Reticular fibers are thinner; they show true branching, usually at right angles. Collagen fibers, though apparently branching, rather subdivide in bundles. Reticular fibers do not stain well with acid fuchsin, unless the latter solution is strongly acid and concentrated (Lillie, 1952). They react positively with the PAS technique, and stronger so than collagen. They appear black with silver impregnation (collagen fibers appear brown). They are isotropic, as opposed to the birefringency of collagen. Chemical analysis of reticulin fibers shows a higher carbohydrate (Glegg, Eidinger, and Leblond, 1953) and a lower proline-hydroxyproline content (Bowes and Kenten, 1948). Antigenicity, admitted for reticular fibers (Cruickshank and Hill, 1953), has been consistently denied for collagen until very recently (Watson, Rothbard, and Vanamee, 1954). The present evidence may be interpreted as indicating that reticular fibers (or, more generally, "basement membranes") are endowed with higher antigenic capacity than collagen.

Other differences become apparent in the behavior towards enzymes. According to Pearse (1953) reticular fibers are more sensitive to trypsin. Pectinases abolish their argyrophilia without altering that of collagen. Aikat and Dible (1953) described a reticulinase present in cultures of *Clostridium histolyticum*, which destroys reticular fibers without attacking the collagen.

The major arguments which tend to prove the identity of collagen and reticular fibers may be summarized as follows. Their fine structure as studied by electron microscopy and X-ray diffraction, is identical (Gross, 1950, 1953; Bairati, Massari and Marsico, 1952a, b; Porter, 1951; Little and Kramer, 1952; Tomlin, 1953). No differences have been detected as to the conditions of formation. In a study of experimental

hepatic cirrhosis, Morrione (1951) found that all quantitative variations of collagen, as established by chemical analysis, were accompanied by parallel variations in the number of reticular fibers visible by silver impregnation. Finally, it is possible to demonstrate fibers with adjacent segments that behave as reticular (argyrophilic) and collagenic (argyrophobic). This was shown by Lillie (1952) in bullae of artificial edema, obtained by intracutaneous injection of fluids.

The question is not entirely settled. It is reasonable to assume that the two types of fibers are not fundamentally different, since collagen and reticular fibers may be continuous. The evidence derived from electron microscopy is entirely in favor of this view. Differences in argyrophilia are not conclusive, because this property does not involve a true combination with silver, but adsorption of the metal in the form of colloidal micellae (as demonstrated by Tomlin (1953) with the electron microscope). Hence surface phenomena play the major role, and the properties peculiar to reticulin may depend upon the conditions of its surface (e.g., surface/volume ratio, relation of surface to ground substance, etc.) rather than upon a specific chemical composition. More significant differences are found with chemical and immunological means. Recent findings point to the carbohydrate component as a differential characteristic. Hydrolysis of collagen always yields a small amount of carbohydrates, even if it is highly purified; in reticular fibers this component is more conspicuous (Glegg, Eidinger, and Leblond, 1953). This observation may well be related to the histochemical properties demonstrated with the PAS technique: collagen fibers become faintly pink, whereas reticular fibers are strongly positive (Lillie, 1947).

4. FINE STRUCTURE OF COLLAGEN FIBERS IN BONE

Electron microscopy of collagen fibrils in bone has been carried out with the aid of several techniques: blending (Wolpers, 1949); Robinson, 1951, 1952; Martin, 1953; Schwarz and Pahlke, 1953), the replica methods (Rutishauser *et al.*, 1950; Kellenberger and Rouiller, 1950; Rouiller *et al.*, 1952, 1953), and thin sectioning (Robinson and Watson, 1952).

The fibers appear identical to those observed in noncalcified connective tissues. The overperiod measures 610 to 640 Å, and consists of A- and B-bands. The A-band measures 400 Å in length; it is more opaque and often contains two transversal striations, best observed after decalcification with buffered sodium versenate (Robinson and Watson, 1952). Pretreatment with phosphotungstic acid causes the appearance, within each period, of five bands each approximately 120 Å in length.

Age and species differences can be demonstrated. In young individuals, or in bone of recent formation, the diameter of the collagen

fibrils is smaller (Huber and Rouiller, 1951; Schwarz and Pahlke, 1953). Fibers of different species vary in their behavior towards acids. Those of birds (hen, pheasant) are not visibly altered by nitric or trichlor-acetic acid (Martin, 1953), whereas those of mammalian origin are very sensitive to nitric acid, less so to hydrochloric acid (Rouiller *et al.*, 1953). Trypsin and papain do not alter the aspect of collagen fibers in human bone; in the same material, pepsin destroys the axial periodicity quite rapidly (Rouiller, unpublished data). In necrotic bone tissue we were able to demonstrate collagen fibers with no morphologic sign of degradation.

In bone tissue, the collagen fibrils are surrounded by an organic ground substance (which probably contains mucopolysaccharides) and by inorganic salts. On a bone surface studied by the replica technique, the fibers are masked by both these components. They can be unmasked if the surface is treated with an acid capable of removing the salts and with an enzyme capable of digesting the cement (Fig. 3), the second procedure is effective only as a complement of the first. The degree of masking varies from one bone to another, and also according to the age and species. It is almost nil when the bone has been laid down very recently; e.g. in the study of a young bone callus, no treatment of the polished surface is necessary in order to demonstrate the fibrils. Such is not the case with an older callus. These observations show that both ground substance and inorganic salts are laid down (or continue to be laid down) after the formation of fibrils has taken place. A similar conclusion was reached by previous X-ray diffraction studies (Henschen, Strauman, and Bucher, 1932; Henschen, 1933): during the formation of a callus, the diffraction pattern of collagen appears earlier than that of hydroxyapatite.

The spatial relations between fibers and crystals of inorganic salts were first investigated by Stuehler (1937) (with X-ray diffraction) and by Schmidt (1934) (with polarized light). Both authors concluded that the *c*-axes of the single crystal units lie parallel to the longitudinal axis of the collagen fibers. More recent investigations with the electron microscope (Robinson, 1952; Robinson and Watson, 1952) carried out on bone tissue without previous decalcification, succeeded in detecting minute bodies appearing as plaques, 25 to 50 Å in thickness, 180 to 400 Å in diameter (extreme values: 90 and 600 Å). These plaques were identified as crystals of bone salts; in fact, treatment with a decalcifying agent causes them to disappear. As for their relation to the collagen, the present evidence is that they lie against the surface of the fibers, with the *c*-axis parallel to the axis of the latter, and preferentially against the *A*-bands. Thus, in a system of parallel fibrils, the crystals form

transversal "ribbons" spaced approximately 630 Å apart. There is some indication (Robinson and Watson, 1955) that bone salts are also present *within* the collagen fibers.

A mechanism by which the collagen fibers may be causally related to the orientation of the inorganic salts has been suggested by Robinson. The molecules of mucopolysaccharides which lie in the interfibrillar spaces may possess some degree of orientation, on account of chemical bonds with the collagen; and thus be capable of directing the formation of hydroxyapatite crystals (see Chapter VI).

5. SPATIAL ARRANGEMENT OF COLLAGEN FIBERS IN BONE

The single collagen fibrils are not anastomosed. They form bundles, in which the axial periodicity of contiguous fibrils is shown to match with great regularity (Rutishauser *et al.*, 1950; Rouiller *et al.*, 1952, 1953) (Fig. 3). These bundles may run parallel, join or interlace. They correspond to the ill-defined fibers visible with the optical microscope.

With reference to the spatial arrangement of the collagen fibers, which in turn determines the shape and distribution of the osteocytes and their canaliculi, two main types of bone tissue may be distinguished: coarse-fibered or primary bone, and fine-fibered or lamellar bone (see also Chapter I).

a. Coarse-fibered Bone

(Faserknochen of Koelliker, 1886; and Weidenreich, 1930; geflechttartiger Knochen of von Ebner, 1874, 1875) (Figs. 4 and 5). This type of bone, which is the most primitive, is characterized by the presence of very coarse collagen bundles, lacking any orderly arrangement. As a rule, the bundles are seen to interweave in all directions; this *disorderly pattern* is well demonstrated in sections prepared by silver impregnation.

In one exceptional situation, the collagen fibers exhibit an almost orderly pattern of parallel bundles. This occurs when direct ossification takes place in a type of connective tissue which contains oriented fibers. Such is the case for the ossified tendons of birds, and for the bone at the site of tendon insertions. In mammalian embryos, the bulk of bone tissue is of the coarse-fibered type. This is gradually replaced by the lamellar type, and disappears completely between the 2nd and the 4th year; traces remain only at the insertions of tendons, of fasciae and of articular capsules, in the labyrinth,⁴ and in the alveolar walls of the maxilla. Furthermore, almost all pathologic ossifications in the adult develop through an initial stage of coarse-fibered bone. Such is the case for the

⁴ Weidenreich classifies the latter bone with the lamellar type (parallel-faseriger Schalenknochen).

process of repair occurring after trauma, for the osteogenesis induced by circulatory or inflammatory disturbances, etc. Later, this immature bone is slowly resorbed and replaced by the more mature lamellar type. An exception to the general rule occurs when the abnormal stimulation is very slight, and entails a mere exaggeration of the normal growth process (lamellar apposition; e.g. effect of mild mechanical stress).

Bone tissue may also contain fibers which do not belong, strictly speaking, to its own structures. Such is the case for the Sharpey fibers (Fig. 5), which are destroyed when the coarse-fibered bone is replaced by the lamellar type.

b. *Fine-fibered or Lamellar Bone*

(Von Ebner, 1875; Jaffe, 1929; Schalenknochen of Weidenreich, 1923) (Figs. 6 and 7). This type of bone differs from the preceding not only on account of its thinner fibers, but also because of its orderly arrangement. Weidenreich recognizes a further subdivision in "parallelfaseriger Schalenknochen" and "lamellärer Schalenknochen," in acknowledgment of the fact that not all fine-fibered bone tissue is lamellar. The first fine-fibered, non lamellar bone is present in embryonal Haversian systems; it consists of a single layer of parallel fibrils, with no subdivision in lamellae. It also appears in the course of endochondral ossification, as the bony layer of the primary trabeculae. Similarly to the coarse-fibered type, this variety of bone is later replaced by *fine-fibered lamellar bone*. The latter (which commonly referred to as lamellar bone) is the most highly differentiated type of bone tissue. It is organized in "lamellar systems," such as the osteons (in the post-embryonic stage), the outer and inner "ground lamellae."

On cross section, a Haversian system appears as a succession of concentric lamellae. Two theories have been advanced in order to explain this stratification. According to von Ebner (1887), Gebhardt (1906), and Weidenreich (1930), the appearance of superimposed lamellae is brought about by a different orientation of the fibers in adjacent lamellae. For Ranzier (1875), Ziegler (1908), and Ruth (1947), the stratification occurs because there exist two types of lamellae of different composition: some contain more collagen, others more cementing substance (including organic ground substance and inorganic salts). The first hypothesis held the ground until recent years. It certainly appears very likely, particularly because it implies a fibrillar pattern which recalls analogous patterns in other connective tissues (e.g. substantia propria of the cornea). Nevertheless, a mere variation in the spatial arrangement of the fibers cannot account for all the differences described between contiguous lamellae (Ziegler, 1908; Ruth, 1947).

A study based on several techniques, including optical and electron microscope study of imprints, led us to the following conclusions (Rouil-

ler *et al.*, 1952). All lamellar systems are composed of *two different types of lamellae*, regularly alternating. A striking difference is manifest, in the first place, on imprints of polished surfaces. Here the lamellae (presumably on account of varying hardness, and therefore varying resistance to polishing) appear as a succession of ridges and grooves. If the polished surface is submitted to a very brief treatment with dilute HCl ("etching"), the corresponding imprint shows that the bottom of the groove is lined by a system of parallel fibrils. The ridges appear as irregular elevations, intersected by few transversal bundles of collagen fibrils; many more fibrils appear after treatment with papain, crossing over the ridge obliquely or transversally, and seemingly flowing from one groove to the next. The lamellae which appear as *grooves* are very rich in collagen; they are birefringent; we termed them "fibrillar lamellae." The *ridges* contain more cementing substance; they are isotropic, and were correspondingly termed "cementing lamellae."

In summary, grids of collagen fibrils (fibrillar lamellae) are held together by bridging fibrils, which pass from one grid to the next through the intervening cementing lamellae. We concluded from these data that the stratified appearance of lamellar bone is not explained by the classical hypothesis layers of fibrils, identical in structure, differing in orientation, but by the existence of two types of lamellae: one type containing more collagen, the other containing more inorganic salts and cementing substance. The existence of quantitative differences in the composition of contiguous lamellae has been confirmed recently by Engström and Engfeldt with the aid of microradiographs (1953).

In higher vertebrates, the trend of physiological metabolism is such that coarse-fibered bone tends to be replaced by lamellar bone. A transformation in the opposite direction never takes place under normal conditions, but may be observed in pathological states. The most interesting example is provided by Paget's disease (*osteitis deformans*), in which lamellar bone is replaced by coarser bone of very disorderly structure (Figs. 8 and 9) (Engfeldt *et al.*, 1952).

6. THE BIREFRINGENCY OF BONE

Bone tissue is anisotropic; its total birefringency is the sum of several birefringencies (v. Ebner, 1874; Schmidt, 1934; Picken, 1940). This has been proved by the study in polarized light (and in media of different refractive indexes) of various materials: ground sections of fresh bone, decalcified or mineralized sections. The latter have been deprived of all organic components by one of several methods, including calcination, Gabriel's glycerol-potash, etc.

Collagen fibers account for the larger share of total birefringence. In fact, the anisotropy of decalcified bone is close to that of fresh bone.

Collagen fibrils display a positive uniaxial birefringence, which is the sum of two components: a "form" and an intrinsic birefringence. The first persists in bone deprived of its organic components, but it is then due to the presence of minute canals which arise through the disappearance of the fibrils.

The crystals of hydroxyapatite show a negative intrinsic birefringence; and the ground substance a positive form birefringency on account of its steric properties (subdivision in minute layers between fibrils and crystals).

IV. The Formation of Collagen Fibers

1. FIBRILLOGENESIS *in Vitro*

Nageotte (1927 to 1934) was the first to demonstrate that a solution of collagen in dilute acids, upon the addition of NaCl or the neutral salt of a monovalent metal, yields a fibrillar precipitate which has the optical and staining properties of natural collagen.

This fundamental discovery became the starting point of a great number of investigations. The X-ray diffraction pattern of the precipitated fibrils was found to be identical with that of the original fibrils (Wyckoff and Corey, 1936), and the typical periodicity of collagen was demonstrated with the electron microscope (Schmitt *et al.*, 1942). Further investigations were concerned with the effect of varying the pH, as well as the dissolving and precipitating agents. Studies along this line led Bahr (1950, 1952) to discover that fibrils obtained with different precipitants were identical as to the length of the overperiod (640 Å) but differed in their intraperiodic structure. Such data may indicate that the different precipitants are capable of altering the sequence of amino acids along the polypeptide chain, without affecting the length of the molecule.

With the electron microscope it is possible to demonstrate that the acid solution of collagen contains filaments of varying diameter (from 200 Å down to the limit of resolving power, i.e. 30 Å), but rarely beyond 400 m_u in length (Vanamee and Porter, 1951). Vanamee and Porter studied the relation of fibril formation to pH, salt concentration, and duration of contact between salt and collagen solutions. One minute after the addition of 1% sodium chloride, fibrils appear in the shape of needle-like crystal, at least 5 microns in length and 1000 Å in diameter, with a periodicity of 210 Å. After 15 minutes the fibrils are thicker and their banding is more obvious, consisting of three distinct striations of which every third coincides with a slight constriction of the fibril. The overall periodicity therefore becomes 630 Å.

The number of fibrils formed increases with increasing salt concen-

trations. In precipitates obtained with 0.5% NaCl, the fibrils are long, smooth (i.e. with no evidence of striation), and their average diameter is 700 Å. Striated fibrils appear with salt concentration of 1% again to be replaced by the smooth type if the concentration is raised to 5%. The electron micrographs prove conclusively that the larger fibrils are formed by lateral aggregation of the smaller.

The hydrogen ion concentration is of primary importance: fibrils may be obtained from an acetic acid solution merely by adjusting its pH to a suitable level. Those formed between pH 4.8 and 5.8 are broad and their overperiod measures 640 Å. More acid solutions yield thinner fibrils and the periodicity tends to disappear.

The process of fibrillogenesis evolves through the same stages, independent of the causative agent (shift in pH, precipitating action of salts). Protofibrils gather in larger units, which in turn give rise to fibrils. A comparable mechanism underlies the transformation of fibrinogen to fibrin, in the presence of thrombin (Porter and Hawn, 1949).

The fine structure of all collagen fibrils obtained by the aforesaid techniques is similar to that of natural collagen. However it is also possible to obtain fibrils of a very different structure. Orekhovich and his colleagues (1948) observed that dense connective tissues such as skin chorion, which are not easily dissolved by dilute acids (by the technique of Nageotte), may be extracted with organic acid buffers, and especially with citrates. Dialysis of such extracts against water causes the appearance of a fibrous crystalline material which was given the name "procollagen." This substance, under the electron microscope (Highberger, Gross, and Schmitt, 1950, 1951), contains two types of fibrils. Some exhibit an average periodicity of 640 Å, others represent an entirely new entity; their period measures 2000 to 3000 Å, hence the name of "long-spacing (LS) fibrils," and their banding pattern is symmetrical, contrary to that of collagen, which is polarized (see page 114). It is unlikely that such fibrils pre-exist in the original tissue. They are interpreted rather as an artificial product obtained from "precursors" present in the connective tissue extracts. Highberger *et al.*, thought that the connective tissue extracts might contain some material, possibly a mucoprotein, capable of reacting with collagen and of producing LS fibrils. In testing this hypothesis, they prepared an acetic acid solution of tendon collagen; and to the filtrates of this they added a purified plasma mucoprotein. Dialysis against water produced fibrils which were almost all of the LS type.

Proceeding along the same lines, Morrione (1952) attempted to correlate the formation of collagen fibrils with another glycoprotein-heparin—and therefore with the mast cells, which are thought to secrete heparin or a closely related substance. In fact, the addition of heparin

to a solution of collagen in acetic acid succeeded in precipitating collagen fibrils. Chemically related compounds, such as hyaluronic acid, sodium and potassium hyaluronate, and chondroitin sulfate, are also capable of precipitating fibrils, but only of the LS type (chondroitin sulfate) or else deprived of periodic structure.

Another type of precipitate, the "segment long spacing" (SLS) was recently obtained in the same laboratory, by dialyzing a phosphate extract against citrate buffer.

It is interesting to note that the SLS material, as well as normal collagen and LS fibrils, may be redissolved and with suitable methods converted into either of the other types of precipitate.

In summary the experimental models of fibrillogenesis support the conception that collagen originates—on a molecular level—by lateral and longitudinal aggregation of elongated, submicroscopic particles (the protofibrils). Other modes of growth, such as interstitial accretion or "intussusception" (suggested by Nageotte and Guyon, 1934), and aggregation of globular particles, are in conflict with electron microscopic findings. As for the mechanism of fibrillogenesis, the study of collagen solutions led to the conclusion that the phenomenon of fibril precipitation is non-specific. At the outset of these studies, the crucial factor was thought to be a specific collagen precipitant; the interest has now shifted to the *conditions* of precipitation. Overwhelming evidence indicates that the formation of fibrils is largely dependent upon the medium. It can be determined by a wide variety of substances, as well as by a mere variation in the pH or in the concentration of the solution. These are the determining factors in fibrillogenesis. They determine the formation of the fibrils, as well as their nature, presumably by affecting the sequential arrangement of the amino acids in the polypeptide chain. They decide whether the final product will be collagen—which occurs in well-defined optimal conditions—or another type of fibril not encountered *in vivo*. The process is reversible; thus the various types of fibers are susceptible to reciprocal transformation.

2. FIBRILLOGENESIS *in Vivo*

Collagen formation *in vivo* has been investigated using a variety of materials and techniques, including tissue cultures (Maximow, 1928; Doljanski and Roulet, 1933; Porter, 1951, etc.), the transparent ear-chamber (Stearns, 1940), healing wounds (Wolbach, 1933; Penney and Balfour, 1949; Buck, 1953) and foreign body granulation tissue (Curran, 1953). The main sequence of events of collagen formation appears to be the same in all cases.

Two to four days after the appearance of fibroblasts, fibrils begin to

develop, embedded in a strongly metachromatic ground substance. The first fibrils visible with the electron microscope have a periodicity of 210 Å, and a diameter of 200 to 300 Å which rapidly increases. It is thus demonstrated that fibrils may undergo extracellular growth. Porter (1951) explains this phenomenon by assuming that fibroblasts secrete collagen in a non polymerized form, capable of condensing from the intercellular spaces onto the surface of the fibrils. Later the fibrils acquire a period of 640 Å, defined by three bands of 210 Å, two thick and one thin.

The straining properties are initially those of reticular fibers, superseded after 8–10 days by those of collagen. At the same time the metachromasia of the ground substance disappears.

As a rule, the collagen fibers of connective tissue tend to be arranged in layers of parallel bundles. The bundles of each layer are oriented at right angles with those of both contiguous layers (Stearns, 1940). An entirely different structure is found in fibrin clots, where the orientation of the fibrils is haphazard. It may be inferred that mere extracellular polymerization does not account for the orderly stratification of collagen bundles (Porter, 1951).

Once formed, collagen is relatively stable. Yet its maintenance may follow either of two patterns. A dynamic equilibrium between formation and destruction occurs in bone tissue, and in the connective tissue of the organs which undergo cyclic modifications (mostly related to menstruation and pregnancy—the uterus, the pubic symphysis and in certain cases also the endosteum). The other pattern consists in very slow and constant anabolism, not paralleled by appreciable catabolism. Isotope studies show this to be the rule for the collagen of most organs (Neuberger and Perrone, 1951).

The aging of collagen fibers is reflected primarily in their number and size; less obvious changes have been described in their chemical and histological properties. In loose connective tissue, the fibers become more tightly packed while the ground substance decreases (Gross, 1952). Within a given species (and with the exception of rat tail tendons), the solubility of collagen in dilute acids decreases and may even disappear with age (rabbit and human tendon (Nageotte and Guyon, 1934), human dura mater (Morrione, 1952)).

3. THE MECHANISM OF COLLAGEN FORMATION:

PRIMARY AND ACCESSORY FACTORS⁵

a. *Cytological Factors*

Few doubt that the formation of collagen is related to the presence of cells defined as fibroblasts. Statements to the contrary are usually

based upon observations prior to the electron microscopic era. For Bait-sell (1916) and von Möllendorff (1932) fibrogenesis can occur in the absence of fibroblasts, and these play an insignificant role.

The present consensus of opinion is that the fibroblasts are indispensable for the genesis of collagen. The next and most debated question is whether the fibers arise within the fibroblasts, or freely in the intercellular spaces. Fleming (1897) and Lagesse (1921) supported the first hypothesis, stating that collagen fibers arise in the cytoplasm and are then "pulled out" with the pseudopodia. The two views are not mutually exclusive, and in fact recent evidence tends to prove that there is some truth in both.

If the extracellular theory is accepted, then the role of the fibroblasts may be interpreted in several ways: the synthetic process requires building blocks and presumably enzymes; the fibroblasts may produce either or both.

That fibroblasts secrete substances involved in fibrogenesis is clearly indicated by optical and electron microscopic investigations. Utilizing the ear-chamber technique, Stearns (1940) showed that the cytoplasm of fibroblasts contains numerous granules; the extrusion of these is followed by the appearance of fibrils. These observations are in agreement with the theory of Wolbach (1933) holding that fibroblasts secrete a collagen precursor—or precollagen—which is further transformed, possibly through polymerization, into fibrillar collagen. Porter (1951) studied the process of fibrillogenesis with the electron microscope, in cultures of chick embryo skin. He concludes that the cellular membrane and the whole ectoplasmic layer of the fibroblast are involved in the process of fibrillogenesis. The ectoplasm (or layer of cytoplasm immediately underlying the cell membrane) becomes organized into fibrils; the latter reach the surface of the cell, where they are polymerized into mature collagen fibrils. The presence of fibrils within the cytoplasm of fibroblasts was also observed by Wassermann (1954), in regenerating rat tendons.

In concluding, the present status of the debate over the origin of collagen fibrils—whether it is intra- or extracellular—may be summarized as follows. There is good evidence for the intracellular origin of the building blocks (e.g. the presence of fibrils within the cell). The building blocks—presumably the protofibrils—may aggregate into fibrils on the surface of the fibroblasts and apparently even "on their way out," in the ectoplasm. This evidence supports the intracellular theory.

⁵ The roles of circulatory disturbances, of inflammation and of constitutional factors are not considered pertinent to the present study and therefore are not discussed.

On the other hand, the young collagen fibrils increase in diameter, thus proving that the deposition of collagen may occur also in the extracellular spaces. The process may be visualized as a crystallization of secreted protofibrils, by a mechanism analogous to that occurring *in vitro*, though this is mere speculation.

Another function has been attributed to the fibroblast, namely the secretion of ground substance. This hypothesis (Meyer, 1947; Gersh and Catchpole, 1949; Curran, 1953) is based on observations which are highly suggestive, but not entirely conclusive. The possible transformation of fibrin into collagen has been another traditional subject of debate (Baitsell, 1915, 1925; Baitsell and Mason, 1934; Nageotte 1931). The question now seems to be almost entirely settled. According to Nageotte (1931), during the process of wound healing the fibrin network may be directly transformed into collagen and this process, called "metamorphosis," is brought about by enzymes secreted by fibroblasts. Its occurrence is highly unlikely. Angevine (1950) has shown that collagen appears in tissue cultures in the absence of plasma. In the transparent ear chamber collagen fibrils develop within the fibrin framework, which remains unchanged and acts merely as a support (Stearns, 1940; Huzella, 1929; Weiss, 1929, and Buck, 1953) conclude that fibrin acts as an orientating framework for the fibroblasts.

In addition to fibroblasts, other cells have been related to collagen formation, namely the mast cells (see page 125) and the macrophages (Maximow, 1928, 1929).

b. *Mechanical Factors*

Pressure and tension exert a twofold action, by stimulating the fibroblasts and by determining the orientation of the fibrils (Wolbach, 1933; Doljanski and Roulet, 1933; Buck, 1953). During the early stages of fibril formation, the latter effect is mediated through an orientation of the cells; later, it depends upon a direct action of the forces on the fibers.

c. *Chemical Factors*

The chemical factors capable of inducing precipitation of fibrils from appropriate solutions have been considered in a previous section.

As for the chemical compounds serving as building blocks, our present knowledge is extremely limited. It can be said, for instance, that plasma contains substances which are utilizable for the synthesis of collagen (Gerarde and Jones, 1953). The study of wound healing in hypoproteinaemic animals led to the same conclusions (Cameron, 1952).

It is suggested by an increasing number of authors (Sylvén, 1941; Meyer, 1947; Partridge, 1948; Penney and Balfour, 1949; Gersh and

Catchpole, 1949; Holmgren, 1949; Campani and Reggianini, 1950; Randall, 1952; Morrione, 1952; Curran, 1953; Buck, 1953, etc.) that the mucopolysaccharides are linked to fibrogenesis. The spatial and chronologic relationships between collagen and ground substance are in favor of such a view, but the crucial point of this interrelation remains obscure. Tentative mechanisms have been suggested. According to Meyer (1947, 1950) a native protein (precollagen) is precipitated over the elongated polysaccharide molecule as the result of the local production of acid, possibly by cellular glycolysis. Thus, "the regularly spaced acidic groups of the polysaccharide chain would form the template on which the fibrous proteins are built up" (Meyer, in Angevine, 1950). The "template" or matrix capable of shaping both reticular and collagen fibrils is hyaluronic acid. The same acid has been thought to be responsible for the maturation of collagen (Curran, 1953). Another acid mucopolysaccharide, chondroitin sulfate, is said to be involved in the formation of collagen. Because of its numerous anionic groups, chondroitin sulfate may be capable of binding together the fibrous macromolecules and perhaps the bundles of fibers, acting therefore as a cement (Partridge, 1948). It has already been mentioned that heparin, hyaluronic acid, and chondroitin sulfate are capable of precipitating fibrils from acid solutions of collagen (see page 125). Nevertheless, specific roles cannot be ascribed as yet to the single mucopolysaccharides. The problem is further complicated by the fact that certain mucopolysaccharides which are known as fibrogenic factors are at the same time inhibitors of fibroplastic functions (Fischer, 1930; Balazs and Holmgren, 1950).

d. *Enzymatic Factors*

Alkaline phosphatase enjoys a well-established reputation as a factor in collagen synthesis (Bourne, 1943-44; Fell and Danielli, 1943; Danielli, Fell, and Kodicek, 1945; Dempsey, 1950; Talmage, 1950; Gould and Gold, 1951; Frieden and Hisaw, 1951, 1953; Buck, 1953). The major argument lies in its increased activity at the sites of enhanced collagen formation healing. Whether the enzyme acts directly on the formation of collagen, or indirectly by enhancing the elaboration of mucopolysaccharides (Moog and Wenger, 1952; Kroon, 1952) is not known.

e. *Role of Vitamins*

Vitamin C is essential to the formation of fibrils; in its absence, neither reticular nor collagen fibers are formed (Wolbach, 1933; Lauber, 1933; von Jeney and Törö, 1936; Mazoué, 1937, 1939; Hunt, 1941; Reid, 1948; Robertson and Schwarz, 1953; Buck, 1953). On the other hand, it is not indispensable for the maintenance of the same fibers once they have been laid down (Ham and Elliot, 1938; Hunt, 1941; Lightfoot and

Coolidge, 1948; Elster, 1950; Robertson, 1950; Robertson and Schwarz, 1953), with the exception of the most recently formed (Hunt, 1941). It has been shown that the young collagen of recent scars disappears as the result of supervening ascorbic acid deficiency. Robertson and Schwarz, (1953) state that the collagen of recent formation reverts to procollagen, whereas the older collagen at the margins of the scar persists. (The bone lesions of scurvy are discussed in Chapter XVIII.)

The mechanism by which vitamin C affects collagen formation is unknown. Wolbach and Howe (1926) were of the opinion that the ascorbic acid acts on a fluid intercellular matrix secreted by the fibroblasts, converting it into a gel; in scurvy, the inhibition of fibrogenesis follows the lack of such gelification. For Ham and Elliot (1938) the sites of action is intracellular: ascorbic acid is involved in cellular respiration, and the defective fibrogenesis of scurvy is related to a metabolic disturbance of the specific cells. Others maintain that ascorbic acid is a factor in cellular differentiation; in avitaminosis C the cells tend to lose the specific functions pertaining to their degree of differentiation. In this connection it is interesting to recall that in the scorbutic organism osteoblasts revert to the stage of fibroblasts, and (not accepted by all workers) odontoblasts are replaced by osteoblasts (Weinmann and Sicher, 1947). Meyer (1946) believes that vitamin C is incorporated into the molecule of chondroitin sulfuric acid, which is further utilized in the skeleton of collagen. A more direct action is suggested by Robertson and Schwarz (1953): vitamin C favors either the synthesis of hydroxyproline, or its incorporation into the macromolecule of collagen.

Vitamin A affects essentially the ectodermal and endodermal tissues, but is also related to the formation of collagen (see Chapter XVII).

f. Role of Hormones

Two groups of hormones are known to affect the formation of collagen fibers: the *adrenal cortical steroids* (and therefore, indirectly, the corticotrophic hormone of the pituitary) and the *sex hormones*.

The spectacular results obtained in recent years by treating the diseases of connective tissue with cortisone and ACTH touched off an endless series of experimental investigations. In animals artificially maintained in a state of hyperadrenocorticism by cortisone or ACTH, the growth of connective tissue is reduced. This inhibiting effect concerns the cells, the ground substance, and the collagen fibers.

The most obvious inhibitory effect on cells concerns multiplication. It has been observed in macrophages (Ragan *et al.*, 1949; Spain, Molomut, and Haber, 1950), fibroblasts (Ragan *et al.*, 1949; Plotz *et al.*, 1950; Spain *et al.*, 1950; Baker and Whitaker, 1950; Castor and Baker, 1950; Creditor *et al.*, 1950; Schneebeli, 1950; Bangham, 1951; Barber and

Nothaker, 1952; Dorfman, 1953, etc.), reticular cells (Baker, Ingle, and Li, 1951), chondroblasts and osteoblasts (Baker and Ingle, 1948; Ragan, 1950; Sissons and Hadfield, 1951; Asling, Reinhard, and Li, 1951; Baker, 1952) and capillary endothelial cells (Sprague, 1951; Baker and Whitaker, 1950; Baker, 1952).

The ground substance is decreased or modified, as shown by decreased metachromasia (Ragan, 1950; Lattes *et al.*, 1953), more rapid destruction by hyaluronidase (Hayes, Reed, and Baker, 1950) and decreased uptake of radioactive sulfate. The latter finding is interpreted as indicating a decreased synthesis of the sulfate (Layton, 1951; Gerarde and Jones, 1953).

In the course of wound healing, and more generally in the development of granulation tissue, treatment with cortisone or ACTH does not cause a delay in the appearance of collagen fibers but a decrease in their number (Curran, 1953). Embryonic tissues of hen's eggs injected with cortisone have a higher titer of free hydroxyproline, possibly indicating a decreased synthesis of collagen (Roberts, Karnofsky, and Frankel, 1951).

The same inhibitory effects are observed following topical application of cortisone (Baker and Whitaker, 1950).

In bones, cortisone inhibits both osteo- and chondrogenesis, to a degree which varies from bone to bone; e.g. long bones are more severely affected than vertebrae (Baker and Ingle, 1948). (See Chapter XX).

The inhibitory effects of cortisone are proportional to the dose administered and further subject to species differences. Moreover, the inhibitory effects are temporary. They are not observable in the early stages of the repair of a wound, and though the healing is deferred, it does not fail to occur. The brief duration of the hormonal effect is not related to a decrease in sensitivity of the animal as a whole, but to local phenomena, occurring within the tissue. A possible explanation is suggested by Ragan (1950): it may be that as the result of hyperadrenocorticism the tissue becomes incapable of utilizing some material, until its concentration rises to a level at which it can again be utilized.

On a chemical level, the mechanism of action of cortisone and ACTH remains obscure. Most of the structural changes of hyperadrenocorticism are related to their antianabolic action on the metabolism of protein. Nevertheless, similar changes occur at the site of topical application, indicating that the disturbance of protein metabolism is not a general one. All the known effects may be explained as the result of the inhibition of fibroblasts and related cells. The inhibition of fibroblastic proliferation is the result of inhibition at the level of a fundamental chemical reaction necessary for the multiplication and differentiation of the fibroblasts

(Dorfman, 1953). This reaction is not specific for mesenchymal cells; the marked effect on connective tissue cells is an artificial result produced by experimental situations in which the effect on connective tissues is merely the most obvious. Dorfman thus adopts the opinion of Green (1950), who maintains that cortisone is an inhibitor of mitosis, independent of the type of cell.

The effects of *sex hormones* on collagen formation varies with species, sex, age, localization, dose, duration of treatment, and hormonal status of the treated animal. It should be noted that in trying to define the structural differences related to sex it is often difficult to extricate the effects of different hormonal pattern from those genetically determined. In the rat, for instance, testosterone, and even more estrogens, inhibit the growth of fibroblasts and the genesis of fibers in granulation tissue (Taubinhaus and Amromin, 1949). In the same animal, and in the mouse, estrogens enhance the development of collagen fibers in the stroma of the female sex organs (Loeb, Suntzeff, and Burns, 1939; Wolff *et al.*, 1942).

The effects of these hormones on the skeleton have been extensively investigated by Gardner and Pfeiffer (1943). (See Chapter XX.)

It can be stated in summary that both adrenal and sex steroids are related to collagen in a manner similar to that of vitamin C: they affect the formation of collagen, not its maintenance. In other words, a modification of established collagen does not occur. It is known to occur, however, with other hormones; at least in well-defined areas of connective tissue. A clear example is the relaxation produced in the pubic symphysis of certain mammals (guinea pig, mouse) by relaxin.

The pelvic relaxation is a two-stage phenomenon: it requires a preparation with estrogens, following which several steroids are capable of inducing the secretion of relaxin.

During the preparatory phase, the fibro-cartilage is replaced by loose and strongly metachromatic connective tissue. This process is relatively slow; in the castrated animal, at least 3-6 days of estrogenic treatment are required (Frieden and Hisaw, 1953). The second phase, comprising the secretion of relaxin, is non-specifically initiated by several steroids: progesterone, desoxycorticosterone, pregnenolone (Courrier, 1941; Zarow, 1948). It occurs very rapidly: the first histologic modifications appear 4 hours after the injection, and reach a maximum between the 6th and the 12th hour. The cycle is concluded by a reparative process, already apparent at the 18th hour.

Histologically, during the phase of relaxation the collagen fibers break down longitudinally and transversally, and eventually appear as thin filaments which are dissolved. Chemical analysis shows that collagen is

transformed in degradation products (Frieden and Hisaw, 1951; Joseph, Engel, and Catchpole, 1952). During the same phase, mucopolysaccharides are said to undergo depolymerization (Perl and Catchpole, 1950). It is not known whether the alterations of the ground substance are responsible for the breakdown of the fibers.

The phenomenon of pelvic relaxation is unique in its effect on collagen. Other localized areas of connective tissue are affected by the sex hormones, but mainly through modification of the ground substance and the degree of hydration. We will quote as examples the turgescence produced by the androgens in the comb of castrated cocks, and the changes occurring in the sexual skin of estrogen-treated monkeys.

V. Relation of Collagen to its Environment

1. THE ALTERATIONS OF COLLAGEN *in Vivo* AND *in Vitro*

Of the various definitions of collagen—histological, chemical, etc.—the most satisfactory arises from the X-ray diffraction and electron-microscopic data. Nevertheless, if one admits that the characteristics of the diffraction pattern or the periodicity of 640 Å seen with the electron microscope are necessary and sufficient criteria for the definition of collagen, several difficulties are encountered. In the first place, the definition would exclude a certain number of fibrils which obviously belong to the family of fibrous proteins referred to as "collagen." On the other hand, it would include other fibrils which histologists would refuse to accept as collagen. And finally, this definition does not take into account the differences which exist between collagens of different sources.

For example, reticular fibers would appear under the heading of collagen, together with such fibrous proteins as elastoidin and spongin of porifera. The structural definition based upon X-ray diffraction would even extend to certain epithelial secretions, or "secreted collagens" (Bear, 1952) such as ovokeratin and byssokeratin found in a hypotrem and a mollusk respectively (Champetier and Fauré-Frémiel, 1938; Brown, 1947). On the other hand, it has been shown that under proper conditions connective tissue may produce fibrils which are certainly collagenic but exhibit a periodicity of approximately 210 Å.

The regular structure demonstrated by X-ray diffraction and electron microscopy merely indicates that the sequence of amino acids in the polypeptide chain follows a regular pattern. This finding does not and cannot imply a constant chemical composition. Certain amino acids may be replaced by others, without affecting the basic structural pattern of the fibril.

It is concluded that neither X-ray diffraction nor electron microscopy

are capable of providing a complete definition of collagen. A closer definition must take into account the relation of collagen to its physical and chemical environment. We will therefore review, in the following pages, the main physical and chemical characteristics of collagen.

a. *The Swelling Property of Collagen*

Placed in acid or basic media, or in solutions of neutral salts, collagen fibers undergo considerable swelling. Different mechanisms are postulated (for a detailed description see Bear, 1952). The swelling in acids or bases (pH below 5 or above 8) entails a shortening of the fiber. Admittedly the salt-like links between acid and basic groups of contiguous polypeptide chains are opened, and swelling occurs as the result of a Donnan effect.

The swelling in salt solutions at neutrality depends on a lyophilic effect (of Hofmeister). In this case no shortening occurs; in fact, the fiber may become slightly longer. Bear offers the following explanation: water penetrates in the bands and interbands and separates the main chains as widely as the hydrogen bonds will permit. More space is made available to the charged lateral chains, and the result is a straightening of the main chains.

b. *The Transformation of Collagen Into Gelatin*

If suitably heated in water, collagen—an insoluble protein—is transformed into a hydrosoluble degradation product or *gelatin* (hence the name of collagen, "generator of colla" or gelatin). This is a rather unusual property, since the solubility of most proteins is decreased by heating. Rupture of hydrogen bonds connecting the main chains is seemingly an important factor in this transformation.

c. *The Shrinkage Temperature*

A curious phenomenon is observed when collagen is submitted to gradual heating. As the temperature reaches 63 to 65° C, the fibers shrink suddenly to about two thirds the original length. In this condition their periodicity falls to 400 Å. The shrinkage temperature rises if the fibers are placed under tension, or if the moisture content is lowered (Lennox, 1949). It is pH-dependent, and decreases to 30–40° C in acid or alkaline media (below 4 or above 11).

The shrinkage is reversible, and so is the concomitant loss of birefringence. The cyclic character of the phenomenon proves, according to Bear (1952) that the mechanism involves a structural characteristic of the protofibril. Within physiological temperatures, the condition of the protofibril is not the amorphous state of contraction, but the crystalline state of extension.

d. Extensibility of Collagen: Fibers as Opposed to Fibrils

It is a well-known fact that collagen fibers, contrary to elastic fibers, cannot be stretched. Nevertheless, electron microscope observations have shown that individual collagen fibrils are capable of considerable extension. Moderate stretching causes an elongation of the axial period: if the latter does not exceed 1300 Å, the banding pattern of the fibril is retained. This indicates that within such limits an increase in length is not related to the rupture of some protofibrils, or to the fact that single protofibrils slide past each other. It is logical to assume a true stretching of the molecules. This is one of the major arguments in support of Bear's helicoidal model of the polypeptide chains. For greater elongations, reaching e.g. 4000 Å, it becomes necessary to admit the occurrence of ruptures and/or reciprocal sliding of the polypeptide chains. This is consistent with the finding that bands and interbands become indistinct in such fibrils (Schmitt *et al.*, 1942; Schmitt and Gross, 1948; Bear, 1952).

e. Effect of Enzymes on Collagen

Of all known proteolytic enzymes, only one—collagenase—is capable of attacking native collagen; another—reticulinase—has been said to attack reticular fibers selectively. All the other “non-specific” proteolytic enzymes are capable of hydrolyzing collagen only if it has been denatured by physical or chemical means.

As a rule, collagen is highly resistant to enzymatic hydrolysis. Factors such as the low solubility, the diameter and compactness of the fibers certainly contribute to this property (Bear, 1952). Collagen is attacked by pepsin in acid media (Sizer, 1949), but if purified with special precautions or if tanned (French and Edsall, 1945), it is resistant to trypsin, to chymotrypsin, and to papain (Neumann and Tytell, 1950). However, fragmentation of the fibers and higher temperatures facilitate the hydrolysis of collagen by trypsin, in neutral or slightly alkaline media (Sizer, 1942).

An enzyme of great interest on account of its substrate-specificity is *collagenase* (Maschmann, 1937, 1938a, b; McFarlane and McLennan, 1945; Oakley, Warrack and von Heyningen, 1946; Bidwell and von Heyningen, 1948, and Bidwell, 1950). It is a bacterial product, originating from *Clostridium welchii* type A, and identical with the “k-toxin” described by Oakley, Warrack, and von Heyningen (1946) and by Bidwell and von Heyningen (1948). Its pH optimum lies between 6.0 and 7.5. *Clostridium welchii* type B produces a λ-toxin capable of hydrolyzing gelatin, casein, and hemoglobin, but not collagen (Bidwell, 1950). There is no acceptable evidence proving that enzymes similar to collagenase

described the presence of collagenase in the pubic symphysis of the relaxin-treated guinea pig.

An enzyme of similar properties, *reticulinase*, was recently discovered by Aikat and Dible (1953) in culture filtrates of *Cl. histolyticum*. It is said to destroy reticular fibers without affecting the collagen.

f. Effects of Vitamins and Hormones on Collagen

Several vitamins and hormones bear known relations to collagen. However, their effect on *mature collagen* is little or none (a notable exception is the hormone relaxin). It is *fibrogenesis* which is most obviously affected. The subject was discussed in Section IV, on the formation of collagen.

VI. Alterations of Collagen Fibers Observed by X-ray Diffraction and Electron Microscopy

The swelling of collagen fibers has been investigated by X-ray diffraction (Küntzel and Prakke, 1933). It was found that the distance between adjacent protofibrils increases from 10.4 Å (normal value) to 15 Å.

The effect of various chemical agents and enzymes was investigated by Gross (1953) with the electron microscope. Hydrochloric acid causes the fibrils to swell and their periodicity to become distorted. Sodium hydrate produces transversal splitting or transformation into an amorphous mass. Trypsin has no observable effect on the structure of the fibrils, whereas pepsin affects the axial periodicity and eventually destroys the fibrils. The first observable effect of collagenase is an obvious tapering, a loss of substance at the free ends of the fibrils. Peculiar fibrils appearing as strings of beads, with a periodicity of 600–650 Å, are often encountered.

Pathological modifications of collagen have been investigated by several authors. Fibrils lacking axial periodicity were described by Wolpers (1950) in lesions due to hypersensitivity of the anaphylactic type. The disappearance of the periodicity is interpreted as a sign of "necrosis" of the fibril. In areas of fibrinoid necrosis, Kellgren *et al.* (1951) found small amorphous masses, but no alterations of the collagen fibers. In anaphylactic lesions due to the "Arthus" phenomenon, Rich, Voisin, and Bang (1953) detected smooth, swollen fibrils, with a "nibbled" appearance, which had lost their periodicity. Since they can be found in non-necrotic areas as well, they should not be interpreted as "necrotic" fibrils. They are presumably the electron-microscopic counterpart of the "hyalin fibers" visible with the optical microscope.

exist in animal tissues. Nevertheless, Gersh and Catchpole (1949) Thus the alteration of collagen in lesions of anaphylaxis, already noted by Arthus in 1906, is confirmed on a submicroscopic level.

VII. Destruction of Collagen Fibers

Destruction of collagen occurs physiologically (e.g. during the resorption of bone) and in many pathological conditions (e.g. regression of liver cirrhosis and late stages of wound healing, etc.). We are faced thus with the anomalous situation of a protein being broken down on a large scale, whereas no known tissue enzyme is capable of attacking it. It may be noted in passing that a similar situation exists for the ground substance: mucopolysaccharides are present but not the corresponding enzymes (hyaluronidases).

It is generally believed that the destruction of collagen is enzymatic; but, as earlier stated, no enzymes of the collagenase type can be demonstrated in connective tissues, or for that matter, in any animal tissue. A histological approach was attempted by Ungar and Feldman (1953) who studied the formation and regression of the reactive tissue in rat liver following implantation of autogenous tendon fragments, and of surgical gut. Neutrophils appear first, followed by histiocytes and newly-formed capillaries. All these elements take part in the breakdown of the implanted material; giant cells appear, and their cytoplasm contains birefringent, argyrophilic fragments of collagen fibers. As soon as the resorption of the foreign material is completed, the granulation tissue disappears, together with its collagen component. The authors favor the opinion that collagen is dissolved through an enzymatic mechanism.

It is possible that collagen is destroyed through the cooperation of several factors, none of which is specific. Trypsin-like enzymes are known to exist in tissues. As earlier mentioned, collagen is not sensitive to trypsin unless prepared by chemical or physical denaturation. An unknown variation of the extracellular fluids may be capable of sensitizing the collagen to these otherwise ineffective proteolytic enzymes.

The role of polymorphonuclears and histiocytes in the destruction of collagen is still obscure. According to Ungar and Feldman (1953), it is unlikely that leukocytes secrete an enzyme capable of degrading collagen, because (under their experimental conditions) the lysis of collagen is initiated much later than the arrival of these cells.

Giant cells are obviously capable of destroying collagenic debris, but whether these are in the "native" or in a denatured state cannot be decided. They cannot be very profoundly denatured, since they retain both argyrophilia and birefringence.

The occurrence of giant cells at the site of collagen destruction leads

us to discuss very briefly the role of osteoclasts in bone resorption. An extensive discussion of this topic will be found in Chapter VIII.

Granted that the osteoclast is endowed with lytic capacities, it may take part in the lysis of bone minerals, of the bone matrix, or of both. Several observations tend to prove that they are related to the dissolution of minerals. Thus McLean and Bloom (1941) have shown that the edge of the Howship lacuna is less calcified whenever in contact with an osteoclast. Other observations support the secretion of proteolytic enzymes. Hancox (1949) derived such evidence from tissue cultures. Rutishauser, Veyrat, and Rouiller (unpublished data), in decalcified sections of Paget's disease and of *ostitis fibrosa*, detected numerous argyrophilic debris within the osteoclast. Furthermore, the collagen fibers underlying the osteoclasts are often modified: they show a tendency to cluster and their argyrophilia is increased, with respect to the collagen fibers of the underlying bone. These findings suggest that osteoclasts are capable of altering the structure of collagen fibers, thus contributing to their destruction. However no direct evidence can be quoted in support of this hypothesis.

We are indebted to Miss Ann W. Hibbard for help in the translation of this chapter.

PLATE I

FIG. 1. Electron micrograph of a collagen fibril from the periosteum of a human rib (shaded preparation). Sequence of overperiods formed by alternating A-bands (elevated) and B-bands (depressed). 83,000 \times .

FIG. 2. Positive replica on plexiglas of a polished bone surface "etched" with dilute HCl (human femoral diaphysis. Shaded preparation). Microphotograph demonstrating a succession of ridges (cementing lamellae) and Grooves (fibrillar lamellae). Top right: osteocytic lacuna. 1,500 \times .

FIG. 3. Positive replica of a polished surface etched with HCl and papain (silicon, monoxide replica, shaded). Electron micrograph. Top left and lower right: depressions corresponding to osteocytic canaliculi. 27,000 \times .

PLATE II

FIG. 4. Coarse-fibered bone. Microphotograph of a decalcified section; silver impregnation by the method of Urechia and Nagy. (Osseous metaplasia, subperiosteal, from the tibia of a dog.) 100 \times .

FIG. 5. Same material under a higher magnification (900 \times), showing the disorderly pattern of the collagen fibers. In the center: two Sharpey fibers.

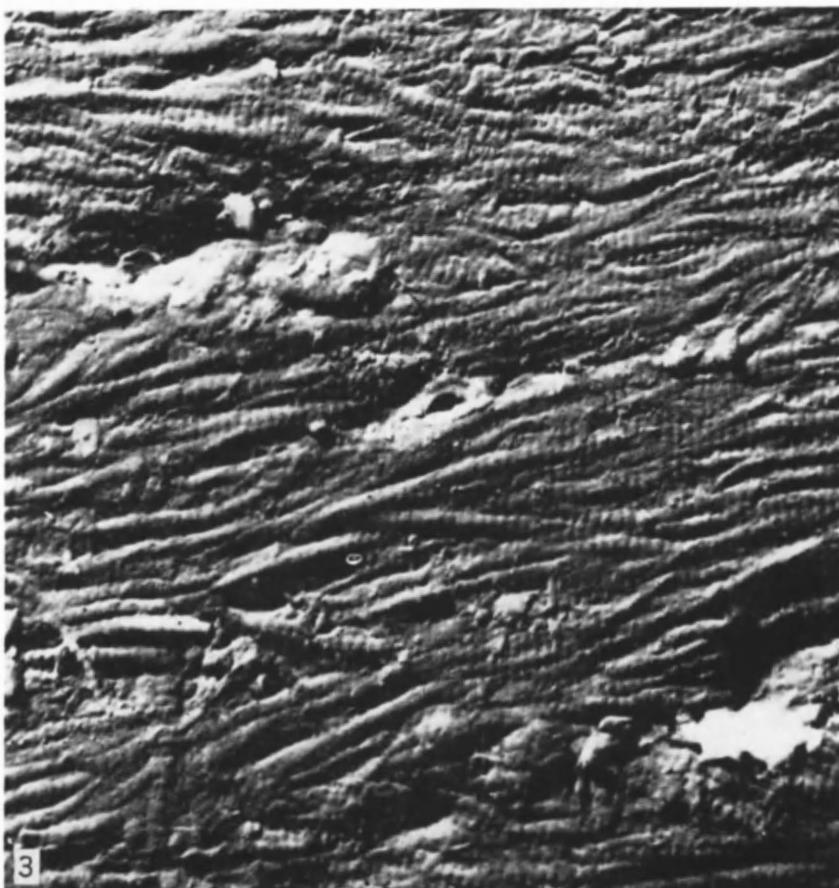
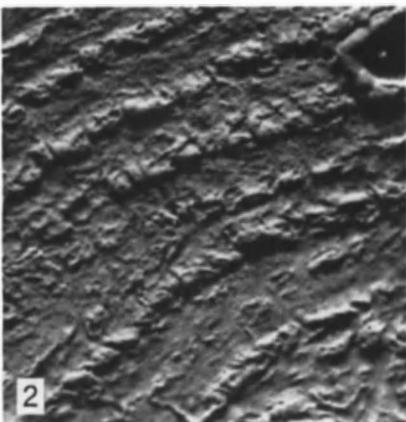
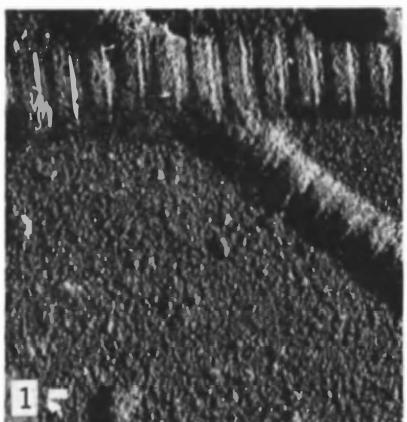
FIG. 6. Lamellar fine-fibered bone in a Haversian system, from the cortex of a human tibia. Silver impregnation. 100 \times .

FIG. 7. Same material viewed under higher magnification (900 \times). Note the orderly arrangement of the collagen fibers. The darker lamellae are the *fibrillar*; the lighter are the *cementing*. It is possible to observe collagen fibers connecting two fibrillar lamellae across the intervening cementing lamella.

FIG. 8. Paget's disease (osteitis deformans) in a human skull. Silver impregnation. 100 \times .

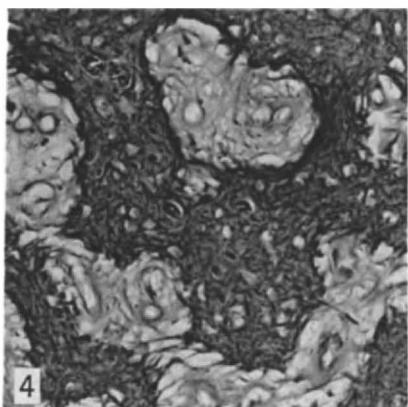
FIG. 9. Same material under a higher magnification (900 \times). Note the irregular pattern of the collagen fibrils.

PLATE I

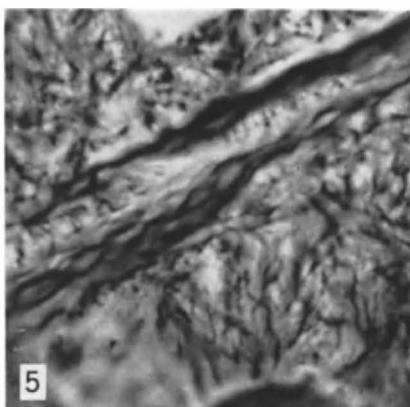


For explanation, see pp. 112, 120, 121.

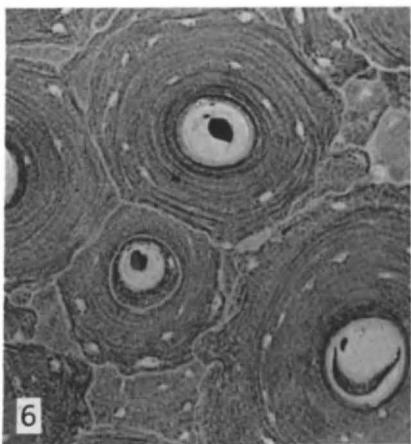
PLATE II



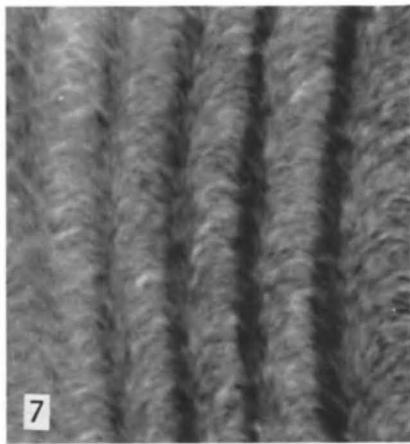
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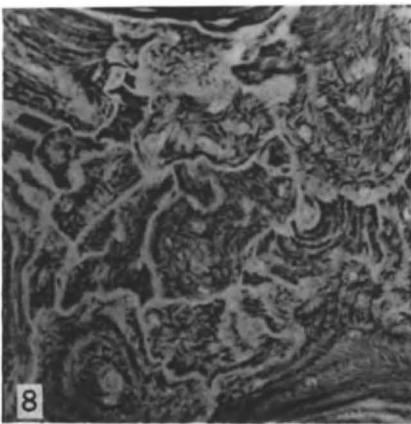
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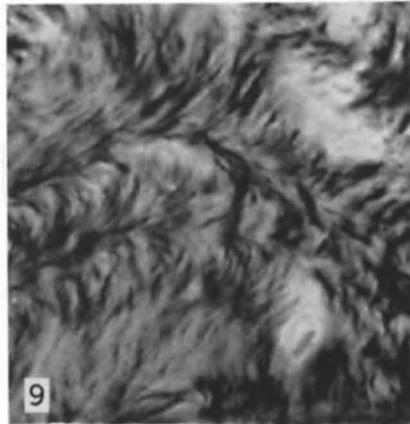
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7



8



9

For explanation, see pp. 121 to 123.

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CHAPTER VI

ULTRASTRUCTURE AND DISTRIBUTION OF MINERAL SALTS IN BONE TISSUE

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I. Introduction

The skeleton was for a long time considered to be of relatively little physiological interest as its main function was considered to serve as a supporting organ. The introduction of new biophysical techniques such as X-ray diffraction, electron microscopy, and the use of radioactive isotopes to the study of bone tissue has given us a new point of view on the structure and possible physiological function of bone tissue. The organic and inorganic components of bone together form a marvellous construction on the ultrastructural level, a construction that has good mechanical properties but also has the ability to incorporate and exchange various ions at a very rapid rate. The last-mentioned mechanism probably has a profound physiological meaning; it serves to regulate part of the ionic milieu in the body.

This characteristic property of bone tissue—the quick pick up and

release of ions by some surface exchange phenomena or recrystallization—means that a number of radioactive isotopes introduced into the body become localized in the skeleton at specific "hot spots" which may cause radiation sickness. In our time when each individual risks contamination with "bone seeking" radioisotopes, such as products from uranium or plutonium fission, it seems extremely important to study bone structure and physiology from the molecular to the anatomical level. A coordinated work on bone may help to find specifics or principles for the detoxication of the skeleton with respect to radioisotopes. This seems to be an immediate goal for the work on bone today but a clarification of the biophysical and biochemical properties of bone tissue will undoubtedly also reveal that the skeleton is a hard-working organ of great physiological significance which also functions as a supporting organ.

Bone tissue is, as is well known, composed of inorganic and organic material. The proportions between these two fractions vary from one part of the skeleton to another and also from one microscopic structure to another. Water-free bone tissue contains about two thirds inorganic and one third organic material. About half of the organic fraction consists of collagen or a substance very close to collagen. It is the purpose of the present survey to describe the nature of the inorganic part of bone tissue—the bone salt—and show its relations to the organic material and particularly to the collagen. Our present knowledge does not permit us to give the complete structure of the organic part (collagen, mucopolysaccharides, etc.), but there are some main structural features of the organic components which are well established and which can be correlated readily with the structure of the inorganic fraction.

II. Distribution of the Inorganic Salts in Bone Tissue

Before discussing the molecular structure of the bone tissue it is of interest to collect some information about the distribution of the chemical fractions of bone. The microscopic distribution of the inorganic part (the bone salt) can be studied by quantitative microradiography (X-ray absorption measurements) as developed by Engström (1946, 1950, 1953) and Engström and Wegstedt (1951).

The technique for the microradiographic demonstration of the bone salt is as follows: A thin-ground section of bone tissue is placed in close contact with a special fine-grained photographic emulsion and exposed with X-rays of suitable wave length. After processing, an image of the sample (unit magnification) is obtained and this microradiographic image is enlarged by photomicrography. The distribution of the photographic density within this microradiogram can be interpreted in terms

of distribution of mineral salts, as is evident from the following reasoning. The X-rays are absorbed in the thin bone sample according to following expression:

$$E = \ln \frac{I_0}{I} = \frac{\mu}{\rho} \cdot m_i + \frac{\mu}{\rho} \cdot m_o \quad (1)$$

In this equation E is the X-ray extinction. I_0 the intensity of the incident and I the intensity of the transmitted X-rays, $\frac{\mu}{\rho}$ is the mass absorption coefficient ($\text{cm}^2 \cdot \text{g}^{-1}$) and m the mass in $\text{g} \cdot \text{cm}^{-2}$. The subscript i stands for the inorganic and subscript o for the organic fractions of the bone. The numerical value of the mass absorption coefficients for the inorganic fraction of the bone (apatite) and for the organic fraction which can be represented by nitrogen, are given in Table I for various wave lengths. The wave lengths commonly used for micro-radiography of thin bone sections are in the region 0.5 to 3 Å. If we assume that the water-free bone tissue contains one third organic and two thirds inorganic substances the influence of the "organic" absorption can be estimated; it becomes apparent that for the X-rays used the absorption of the organic component can be neglected in comparison with that of the inorganic.

TABLE I
MASS ABSORPTION COEFFICIENTS FOR APATITE AND NITROGEN

$\lambda \text{ A}$	Mass absorption coefficients, $\frac{\mu}{\rho}$, $\text{cm}^2 \cdot \text{g}^{-1}$	
	apatite	organic (N)
0.5	~ 3.5	~ 0.4
1.0	26	2.1
1.5	81	7.7
2.0	178	19
2.5	330	29
3.0	545	57

The resolution in the microradiographic technique depends mainly on the granularity of the fine-grained photographic emulsions used to record the X-ray shadow image. The best fine-grained photographic emulsions, Lippmann-film, Eastman Kodak Spectroscopic Plates 548 or 649, and Kodak Maximum Resolution Plate, have a resolution which under favorable circumstances is better than 1000 lines per millimeter. The geometry of the X-ray image can easily be arranged in such a way that the blurring is below 0.5 micron. Resolution tests have shown that

objects which give good contrast and as small as 0.5 microns can be imaged by soft X-rays (Combée and Engström, 1954).

The distribution of organic material can be studied by microradiographic examination of very thin sections of decalcified bone. In this case the technique for the determination of the weight of cytological structures introduced by Engström and Lindström (1949, 1950) has to be used. This type of microradiography requires extremely soft X-rays, which are generated in a specially built continuously pumped X-ray tube. Recently, new and very simple equipment for such analysis has been developed (Combée and Engström, 1954).

Microradiographic examinations of the distribution of mineral salts in the bone tissue have given results of great interest. In a thin cross section from the middle of the diaphysis of a human femur the microradiogram reveals that the Haversian systems (osteons) have a varying degree of mineralization (Plate I, 1). From the general topography it can be concluded that the young Haversian systems are less mineralized than the older ones. A high concentration of mineral salts are found in the cementing lines. Within each Haversian system and especially in young systems the mineralization seems to be greater towards the lumen (the Haversian canal) than at the periphery of the system. The osteocytes contain no bone salts, they appear as empty holes. By combining microradiography with autoradiography on thin bone slices from animals which had received radioactive phosphate, Engfeldt, Engström, and Zetterström (1952) could demonstrate that the bone structures with a low content of mineral salts had a high uptake of radiophosphate. The same general patterns of isotope uptake could be demonstrated for radiocalcium and radiosulfate (Engfeldt, Engström, and Boström, 1954), and for other isotopes.

The pattern of radioisotope distribution obtained with *in vivo* labelling can also be produced by labelling *in vitro* (Amprino, 1952a, b, 1953; Engfeldt, 1953). The ability of certain bone structures quickly to reach ionic equilibrium with the body fluids is a mechanism that probably serves the regulation of certain ions in the body. A more detailed discussion of this principle is found in the papers by Amprino and Engström (1952) and Engfeldt and Zetterström (1954a).

A great number of pathological bone tissues have been examined by microradiography in order to study the distribution of mineral salts, and for details the reader is referred to the original papers. Among others the following diseases (some microradiograms are given in Plate I) have been investigated: osteogenesis imperfecta (Engfeldt and Engström and Zetterström, 1954a), Plate I, 5; marble bone disease (Engfeldt, Engström, and Zetterström, 1954b), Plate I, 4; bone tumors (Engfeldt, 1954); Paget's disease (Engfeldt *et al.*, 1952), Plate I, 2; hyper-

parathyroidism (Engfeldt and Zetterström, 1954b); rickets (Engfeldt and Zetterström, 1954c). A new bone disease of the early childhood was discovered on the basis of microradiography, Plate I, 3, and was named osteodysmetamorphosis fetalis (Engfeldt and Zetterström, 1954a).

The results obtained on normal bone tissue by microradiography have recently been further confirmed by Davies and Engström (1954) using microinterferometry. The theory for the quantitative evaluation of bone constituents by microinterferometry was given by Davies and Engström (1954). For microinterferometry in general see Davies *et al.* (1954).

The principle for microinterferometry can be briefly described in the following way. The mass of material other than water per unit area, m , in a homogeneous object, of thickness t , situated in water is given by

$$m = \frac{\phi_w}{\chi} \quad (2)$$

where ϕ_w is the optical path difference (o.p.d.) in water, i.e. $(\mu_o - \mu_w) \cdot t$ where μ_o and μ_w are the refractive indices of the object and water respectively. For solutions χ is 100α where α , the specific refractive increment, is given by $(\mu_o - \mu_w)/c$ and c is the concentration in g per 100 cm³. The use of the Dyson interference microscope permitted the determination of o.p.d. of sections of bone tissue and the sum of inorganic and organic material could be calculated. After decalcification only the organic part was left and the amount was easily measured by the interference microscope. Tables II and III are taken from the paper by Davies and Engström (1954) and show the evaluation of the composition of single Haversian systems. In the experiment the phase shift (optical path difference, o.p.d.) was measured in water and paraffin (Plate I, 6) for a thin-ground section of bone. These measurements permitted the calculation of the thickness of each Haversian system and also its density. It is seen that the mean value of the densities for various Haversian systems is in good agreement with the density value of 2, which has been obtained on macroscopic powdered samples. Interference microscopy of decalcified sections showed that the content of organic material did not vary much from one Haversian system to another. The microinterferometric measurements were in full agreement with the results obtained by microradiography (Davies and Engström, 1954).

III. Survey of the Methods for the Study of the Ultrastructure of Bone

1. X-RAY DIFFRACTION TECHNIQUES

X-ray diffraction techniques have been extensively applied to the study of the crystalline structure of bone tissue. In this communication only such X-ray diffraction techniques will be discussed that have been of direct value for bone structure work. The following general textbooks

TABLE II. MICROINTERFEROMETRIC MEASUREMENTS ON BONE
From Davies and Engström (1954)

Haversian system	Position	o.p.d. in water before decalcification	o.p.d. in water after decalcification	m_o g/cm ² ($\chi = 0.17$) $\times 10^{-4}$	Difference in o.p.d. before and after decalcification	m_i g/cm ² $\times 10^{-4}$	m_i/m_o	X-ray extinction arbitrary units	X-ray extinction o.p.d. difference
A	1 Y	2.70	1.20	3.9	1.50	8.2	2	23	15
	2 O	3.40	1.30	4.2	2.10	11.5	2.7	29	14
	3 O	3.00	1.15	3.7	1.85	10.1	2.7	28	15
	4 Y	2.35	1.15	3.7	1.20	6.5	1.8	20	17
	5 Y	2.75	1.25	4.0	1.50	8.2	2.0	23	15
	6 Y	2.35	1.20	3.9	1.15	6.3	1.6	23	20
	7 Y	2.25	1.25	4.0	1.50	8.2	2.0	25	17
	8 O	3.05	1.35	4.3	1.70	9.3	2.2	27	16
	9 O	2.70	1.15	3.7	1.55	8.5	2.3	26	17
	10 Y	2.70	1.15	3.7	1.25	6.8	1.8	24	19
	11 Y	2.70	1.35	4.3	1.35	7.4	1.7	24	18
	12 O	2.60	0.90	2.7	1.70	6.3	3.4	25	15
B	1 O	4.40	1.80	5.8	2.6	14.2	2.4	39	15
	2 Y	3.50	1.80	5.8	1.7	9.3	1.6	29	17
	3 O	4.75	1.80	5.8	2.15	16.1	2.8	41	14
	4 Y	3.85	1.80	5.8	2.05	11.2	1.9	34	16
C	1 O	3.25	1.30	5.2	1.95	10.7	2.5	33	17
	2 O	3.75	1.60	5.1	2.15	11.8	2.3	30	14
	3 O	3.70	1.40	4.5	2.30	12.6	2.8	32	14
	4 O	3.60	1.30	4.2	2.20	12.6	3.0	30	13
D	1 Y	4.15	2.0	6.4	2.15	11.8	1.8	33	15
	2 Y	4.40	2.0	6.4	2.40	13.1	2.0	35	15
	3 O	4.80	2.0	6.4	2.80	15.4	2.4	37	13
	4 O	5.0	2.0	6.4	3.00	16.4	2.6	41	14

The following symbols are used in this table and in Table III:
Y and O stand for young and old Haversian systems, respectively. o.p.d. is the optical path difference. m_o = mass (weight) of the organic fraction.
 m_i = mass (weight) of the inorganic fraction. (Reprinted with the permission of *Experimental Cell Research* and Academic Press Inc.)

TABLE III
 MICROINTERFEROMETRIC MEASUREMENTS OF THE DENSITY OF VARIOUS BONE STRUCTURES (HAVERSIAN SYSTEMS)
 From Davies and Engström (1954)

Haversian system	Position	o.p.d. in paraffin intact bone	Difference between o.p.d. of intact bone in water and paraffin	Effective thickness $t(l-f)$ μ	$m_i + m_s$ g/cm ²	Density g/cc
A	1 Y	0.86	1.85	6.5	12.1	1.8
	2 O	1.25	2.25	8.0	15.7	1.9
	av. 5, 4 Y	0.85	1.70	6.5	11.0	1.75
	av. 6, 7 Y	0.80	1.70	6.5	11.0	1.8
	8 O	1.15	1.90	7.0	13.6	2.0
	10 Y	0.75	1.60	6.0	10.5	1.8
B	2 Y	1.20	2.95	8.5	15.8	1.8
	3 O	1.75	2.30	11.0	21.9	2.0
C	1 O	1.30	1.95	7.0	14.9	2.1
	av. 2, 3 O	1.40	2.30	8.5	17.0	2.0
D	1 Y	1.60	2.55	9.5	18.2	1.95
	2 Y	1.10	2.80	10.5	19.5	1.9
	3 O	1.80	3.0	11.0	21.5	2.0
	4 O	1.85	3.15	11.5	22.8	2.0

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 See footnotes to Table II.

on X-ray crystallography are recommended for technique and interpretation of the diagrams: Bunn (1946), Henry, Lipson and Wooster (1951), Guinier (1952), Wyckoff (1951), Klug and Alexander (1954).

Crystalline compounds such as sodium chloride give well defined X-ray reflections at large angles. A liquid gives diffuse X-ray reflections, and in case of water at an angle corresponding to an interplanar distance of i.a. 3.1 Å. The gas-type of X-ray diffraction pattern consists of a diffuse scatter close to the central beam. The gas-type solid also gives a scatter close to the central beam.

It is customary to distinguish between low-angle and high-angle X-ray scattering and the limit is assumed to be at about $2^\circ \theta$ where θ is defined according to Bragg's law:

$$n \cdot \lambda = 2 d \cdot \sin \theta \quad (3)$$

λ = wave length of radiation

d = interplanar distance

n = order of reflection

θ = glancing angle

X-ray reflections can occur both at high (wide) and low angles. In bone, for example, the apatite component gives a high-angle pattern with many lines and the collagen gives in addition to a wide-angle pattern a low-angle pattern with several orders of a fundamental repeating period of about 660 Å. Bone in addition gives a low-angle diffuse pattern of a "gas-type solid" appearance.

(a) High-Angle Diffraction

The wide-angle pattern as examined in a powder camera gives information about the size of the unit cell. The powder diffraction technique has been developed to a method of extremely high precision and in favorable cases the errors in the determination of the lattice parameters may be no more than ± 0.00002 Å. Wide-angle X-ray diffraction studies of single crystals yield information that allows the solution of the positions of the atoms in the unit cell. In bone, however, the scattering particles (crystallites) are so small that it has not been possible to obtain single crystal patterns.

If the crystals are oriented in the samples this can be determined by either rotating an oriented piece of the specimen in a cylindrical camera or taking a flat film photograph, fiber diagram.

The sharpness of the wide-angle X-ray reflections in a powder diagram depends partly on the size of the crystallites that constitute the sample. If the crystallites are large the lines are sharp. With decreasing crystallite size the reflections become more and more diffuse and when the crystallites are very small no reflections at all can be detected in the

pattern. This circumstance is used to estimate the size of the crystallites; it is done by measuring the width of the diffraction lines from the substance investigated and comparing the results with a standard. The accuracy of this way of determining the particle size is not great. Recently, however, improved methods have been described that allow careful measurements of the line profiles.

For biological work the sample very often is small. It is therefore necessary to develop microdiffraction techniques which can be of use in histo- and cytochemistry. Microdiffraction methods for the study of wide-angle patterns have been developed by several authors, Chesley (1947), Klein *et al.* (1951), Fournier (1948), Kratky (1931), Kreger (1951), Spark (1953), Engström and Finean (1953). Carlström (1954) has reviewed microdiffraction techniques for use in histochemistry and described the specialized techniques. These newly developed microdiffraction procedures have been of great use when studying the crystallographic properties of various microscopic structures of bone tissue (Engström and Zetterström, 1951; Amprino and Engström, 1952, Carlström, 1954).

(b) Low-Angle Diffraction

Low-angle diffraction requires a refined collimation system so that reflections close to the central beam can be resolved and accurately measured. The theory for such collimation systems has been given by Bolduan and Bear (1949), among others, who also describe low-angle cameras with high resolution. Finean (1953) has published the description of a simple versatile low-angle camera, with which some of the low-angle patterns discussed in this communication have been recorded. The low-angle *reflections* as they appear from collagen arise from a series of equidistant reflecting planes and in principle these reflections arise in the same way as the wide-angle patterns. The low-angle "particle scatter," however, approximates a phenomenon observed by visible light. It is known that when a luminous beam traverses a medium containing opaque particles there is a halo of diffracted light around the incident beam. The intensity of the diffracted light diminishes with the angle of diffraction and is zero for an angle ε given approximately by the relation (Guinier, 1952),

$$\varepsilon = \frac{\lambda}{d} \quad (4)$$

where λ is the wave length and d the mean diameter of the particles. In order to observe the phenomenon the particle diameter must be 10 to 500 times the wave length of the radiation. The low-angle particle scatter can be interpreted according to Guinier:

$$\log I = \log K - \frac{4\pi^2}{3\lambda^2} \log e \cdot R^2 \cdot \varepsilon^2 \quad (5)$$

In this formula I is the intensity of the scattered X-rays at an angle ε from the central beam, R is the radius of gyration, defined in ordinary mechanical sense, and K is a constant. The expression above can be simply written

$$R = 0.644 \cdot \sqrt{\alpha} \quad (6)$$

where α is the slope of a curve relating $\log I$ versus ε^2 . CuK α radiation with the wave length of 1.54 Å is assumed. In practice the fall off of the X-ray energy is measured and a diagram relating log X-ray intensity to the square of the distance from the primary beam is constructed. The slope of this curve gives the radius of gyration of the particles. If the particles are anisodiametric and randomly oriented, the dimension of the particles can be determined only if the axial ratio is known. When a system of well oriented elongated particles is arranged with their axes perpendicular to a symmetrically collimated X-ray beam the resulting shape of the low-angle scatter will be similar to that of the particle, but turned through 90°. If the axes of the scatter corresponding to the axes a and $v \cdot a$ of the particle are x and y , respectively, then the expression for the intensity of scattered radiation from one particle at any point on the film can be written,

$$\log I = \log K - \frac{4\pi^2}{5\lambda^2 S^2} \log [a^2 x^2 + (v \cdot a)^2 y^2] \quad (7)$$

where S is the sample to film distance (and the expression given by Jellinek and Fankuchen, 1945, is taken into account). *Thus the low-angle particle scatter from a well oriented system can give information about the dimensions of the particles and their orientation.*

(c) Preparation of Bone Tissue: for X-ray Diffraction

Bone tissue can be prepared in several ways for the X-ray examination. Orientation of the crystallites is studied in thin-ground sections of known orientation in the bone. In general the thickness, t , of the sample for X-ray diffraction analysis should not exceed $1/\mu$ where μ is the linear absorption coefficient. For copper K α -radiation, t is about 0.1 mm for normal bone, thinner for enamel and thicker for less calcified tissues. Soft tissues such as collagen have little X-ray absorption and therefore t has to be 1 mm or even more if maximum scattered intensity is required (CuK α -radiation).

In the powder camera a fine powder of bone is filled on thin capillaries and rotated. It is preferable to have capillaries less than 0.25 mm

in diameter in order to reduce the absorption corrections. For high precision work a known standard such as rocksalt can be admixed to the specimen.

2. ELECTRON MICROSCOPY AND ELECTRON DIFFRACTION

Electron diffraction in the electron microscope can be used to identify the inorganic part of bone. The disadvantage of electron diffraction is that the wave length associated with the electrons in motion is short and therefore only relatively short interplanar spacings can be measured. The calibration of the electron diffraction unit in the electron microscope offers certain difficulties. The determination of the unit cell from such electron diffraction measurements is therefore not very exact. However, orientation can be studied effectively by the electron diffraction method and the main advantage over X-rays is that very small samples can be studied and identified. The exposure-times are short as the scattering power is considerably higher for electrons than for X-rays. There is, however, the disadvantage that the specimen becomes heated during the exposure, which may cause growth and chemical changes of the crystallites.

The successful application of the electron microscopic imaging technique to the study of bone tissue is mainly dependent upon the possibility of making intact preparations of bone of such minute thickness that they transmit the electrons. Early attempts to apply electron microscopy to bone studies were based on the examination of fragmented or powdered bone and on decalcified bone. Replica techniques have also been used. General electron microscope references and sample preparation techniques are found in the monographs by Hall (1953), Wyckoff (1949), and in the review articles by Bretschneider (1952), Dalton (1953), and others. Decalcifying the bone yields samples which can be successfully sectioned in modern ultramicrotomes such as the one designed by Sjöstrand (1954). Sectioning of intact (non-decalcified) bone is very difficult, but new types of knives for the ultramicrotome make this possible. Particularly the diamond knife, introduced by Fernández-Morán (1953), is of great use. The fragmentation technique seems to be of less value for the study of the inorganic part of bone. Since the apatite crystals grow on heating it is important that the specimen receives as little bombardment with electrons as possible.

3. POLARIZED LIGHT

The polarizing microscope is the classical tool for ultrastructure research. The principles are too well known to be described here and general information can be found in Burri (1950), Bunn (1946), Frey-

Wyssling (1953). Especially in the hands of Prof. W. J. Schmidt in Giessen has the polarization microscope given us new and valuable information about ultrastructural organization of calcified tissues, among others. A complete list of the works of Prof. Schmidt appeared 1954 on his 70th birthday. Many of his papers are devoted to the fine structure of calcified tissues.

IV. Structure of the Inorganic Constituent of Bone

Since bone is a living tissue in dynamic equilibrium with the body fluids, its chemical composition varies not only from one bone to another, but also within its microscopic structures. The overall chemical composition of calcified tissues have been known for a long time and many fruitless efforts have been made to find the real chemical structure of the bone salts. These efforts have resulted in more or less monstrous formulas of which none could explain all facets of the complex behavior of the bone salt. The chief constituents, calcium, phosphate, carbonate, and water form a basic calcium phosphate of varying composition in which the carbonate plays an intricate role.

Our knowledge of the basic calcium phosphates has come from many fields, and long before the era of X-ray diffraction it was realized that the most basic calcium phosphates belonged to the apatite group.

In order to establish a basis for the discussion of the structure of the bone salt it seems justified first briefly to discuss the general crystallographic properties of apatite.

1. STRUCTURE OF APATITE

Apatite (later called fluorapatite to avoid confusion with other members of the apatite group) got its strange name *ἀπατᾶω* (= I deceive) because it often was taken for such minerals as olivine and aquamarine. In pure form it occurs in transparent, often sea-green crystals belonging to the hexagonal-dipyramidal group. The structure of this mineral was determined by Náray-Szabó (1930) and Mehmel (1930) independently. Their interpretations differ only slightly and mainly regarding the position of the fluorine ions. Both found the fluorapatite unit cell to contain one molecule $\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$ and showed that it belonged to the space group $\text{C}_{6h}^2(\text{C}6_3/m)$. They determined the size of the unit cell to be $a = 9.37 \pm 0.01 \text{ \AA}$, $c = 6.88 \pm 0.01 \text{ \AA}$, and $a = 9.36 \pm 0.02 \text{ \AA}$, and $c = 6.85 \pm 0.02 \text{ \AA}$, respectively, which is in good agreement with later investigations.

The unit cell has two equal edges (a) inclined at 120° to one another and a third (c) perpendicular to these. The drawing in Fig. 1 shows

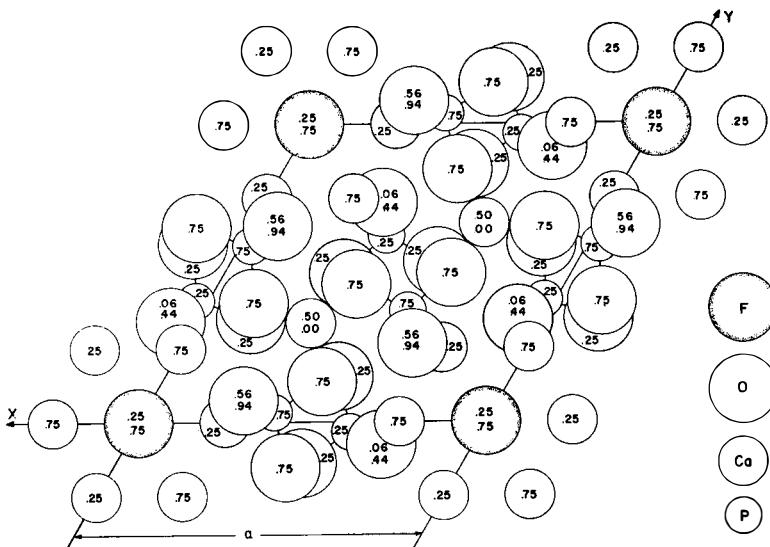


FIG. 1. Projection on 0001 of the atomic arrangement in fluorapatite. The figures are fractions of the height of the unit cell.

the three-dimensional arrangement of the atoms in the fluorapatite unit cell projected on 0001 (the basal plane).

In the structure of the closely related chlorapatite ($\text{Ca}_{10}(\text{PO}_4)_6\text{Cl}_2$) the chlor-ions in the corners of the unit cell seem to be situated at 0,0,0 and 0,0,0.5 (instead of 0,0,0.25 and 0,0,0.75) because of their bigger ionic radius, which also has a marked influence on the lengths of the axes of the unit cell. For a detailed description of the apatite structure see Náray-Szabó (1930), Mehmel (1930), Bale (1940), and Beevers and McIntyre (1946).

In fluorapatite (which can be considered as the prototype of the apatites) the ions can be fully substituted by other ions of close physico-chemical relationship. Ca can be replaced by Sr, Ba, and Pb, the PO_4 group by AsO_4 or VO_4 , and F by Cl or OH, forming a wide range of solid solutions. Mg, Na, K, and other elements, such as the rare earth metals can also substitute for Ca, but to a lesser extent. The possibility of CO_3 entering the lattice will be discussed later.

2. BONE — AN APATITE

The first time X-ray crystallographic methods were applied to bone tissue was in 1926. In this year de Jong could demonstrate that the mineral phase of bone had a crystalline structure and that X-ray diffraction of recent and fossil bone gave patterns almost identical with that of

fluorapatite. Independently of de Jong (1926), Gross (1926) demonstrated that tooth enamel gave a typical apatite pattern. Later investigators have not been able to detect any other inorganic crystalline substances than apatite in bone, although the X-ray diffraction technique has been much improved during the last 25 years. Another extremely important property of the bone salts was also stressed by de Jong (1926). He found that the diffraction lines were broadened and drew the correct conclusion that the apatite crystallites were small. He estimated each crystallite to contain only some hundred apatite molecules.

3. APATITES SUPPOSED TO OCCUR IN BONE

Comparing bone with known minerals and chemical compounds gave the result that there were mainly three substances, "carbonate-apatite," tricalcium phosphate hydrate, and hydroxyapatite, which showed great physicochemical similarities with bone (Armstrong, 1950). All gave an X-ray diffraction pattern almost indistinguishable from that of bone and they had a composition that did not deviate too much from that of bone ash. Unfortunately, there has been much confusion regarding these substances, mainly about their microcrystalline nature (crystallite size about 10^{-5} — 10^{-7} cm). Important crystallographic data are still missing; single-crystal diagrams never have been obtained. Ordinary powder patterns are often so diffuse that accurate measurements cannot be made. Because these substances have such a small particle size the chemical composition given by the analytical chemist may also be questioned. The crystallites have a large surface area and this can attract enough other ions to cause a different chemical composition of the surface and the interior. It is therefore doubtful if anyone ever has had a chemically pure sample *in sensu stricto*.

(a) *The "Carbonate-Apatites" and the Bone Carbonate*

Before apatite structure was deduced, carbonate-bearing apatites, such as dahlite, francolite, or staffelite, were supposed to have the formula $\text{Ca}_{10}(\text{PO}_4)_6\text{CO}_3$. The carbonate group should correspond to the fluorine in the fluorapatite. It was therefore natural that early investigators, for example, Andresen and Goldschmidt (1927), Roseberry, Hastings, and Morse (1931) thought that the carbonate in bone occurred in the same way as it was thought to do in the carbonate-bearing rock phosphates. However, Gruner and McConnell (1937) and Gruner, McConnell, and Armstrong (1937) could show that the CO_3 group is too large to fit into the F positions in fluorapatite without an expansion of the crystal lattice for which there is no X-ray evidence, and proved

that there is no compound of the composition given by the formula: $\text{Ca}_{10}(\text{PO}_4)\text{CO}_3$, a fact that often has been neglected. They suggested that CO_3 could substitute for P up to 10 atomic per cent but also that Ca might be replaced by CO_3 , and they thought that calcified tissues had this structure. McConnell (1952) has modified this earlier opinion and assumes that the CO_3 groups replace some PO_4 groups and that $(\text{OH})_4$ groups replace other PO_4 groups. Some calcium is replaced by neutral water. The discussion concerning the carbonate-bearing apatites is not yet finished and definite evidence of their existence has not yet been presented (Hendricks, 1952). Some recent publications on this matter are given by Silverman, Fuyat, and Weiser (1952), Geiger (1950), McConnell (1952), Carlström (1955) and Trautz (1955).

The type of substitution in carbonate-apatites suggested by McConnell and coworkers seems highly improbable to many crystallographers, who instead suggest that the carbonate in both minerals and bone either occurs as a second phase (CaCO_3) or is adsorbed at the surface of the apatite crystallites. Although the carbonate content of bone and of many carbonate apatites is up to 10% by weight (counted as CaCO_3), no CaCO_3 lines can be detected in the X-ray diffraction patterns. Niggli and Brandenberger (1934) and Thewlis (1939) found that CaCO_3 in a mixture of apatite could not be detected by means of X-ray diffraction unless the amount was higher than 10% by weight and this statement was thought to explain why no carbonate reflections were found in bone. Tovborg-Jensen and Møller (1944) demonstrated, however, that they with an improved technique could detect as little as 1% CaCO_3 in the form of calcite and 2% in the form of aragonite in a mixture with apatite, and they concluded that the carbonate present in bone and teeth does not exist as crystalline CaCO_3 . One explanation of this finding may be that the carbonate does not enter the lattice but is adsorptively bound to the surface of the apatite crystallites. In fact this seems to be an explanation that can account for most of the experimental data hitherto presented. The size of the surface area of the crystallites in bone and carbonate-apatites seems to be quite sufficient to explain the existence of surface-bound carbonate. There is indeed much evidence that points in this direction, especially the results of the experiments with differential rates of solubility. It has been shown by many workers (Dallemande, 1942; Dallemande and Brasseur, 1942; Dallemande and Melon, 1950; Klement, 1929; Logan and Taylor, 1938) that weak acids remove the carbon dioxide in bone at a more rapid rate than that of the phosphates. Recently, Neuman and Neuman (1953) showed, at least in experiments with synthetic apatites, that the carbon dioxide is reversibly bound at the surface of the crystallites.

(b) *Tricalcium Phosphate Hydrate, β -tricalcium Phosphate and Hydroxyapatite*

Tricalcium phosphate hydrate (TCPH), (called α -tricalcium phosphate by Dallemagne and coworkers) and hydroxyapatite are two closely related calcium phosphates with the formulas $\text{Ca}_9(\text{PO}_4)_6\text{H}_2(\text{OH})_2$ and $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, respectively. Taylor and Sheard (1929) were the first to show that tricalcium phosphate and bone gave very similar X-ray diffraction patterns, which also was shown by Shirakawa (1931), and Rosebery *et al.* (1931). In order to explain why this substance gave an apatite pattern, Hendricks *et al.* (1931) suggested the formula $\text{Ca}_9(\text{PO}_4)_6 \cdot (\text{H}_2\text{O})_2$, with two water molecules replacing the fluorine in fluorapatite and one Ca atom missing.

The overwhelming evidence that hydroxyapatite was the salt of choice among those considered as "the bone salt" threw TCPH into the shadow for a long time, but it was picked up again by Dallemagne and coworkers in order to explain the differences between the analytical composition of the bone salt and hydroxyapatite. Dallemagne and co-workers made the assumption that the carbonate in bone was present as a separate phase (CaCO_3) and their analyses therefore give a decreased Ca/P ratio of the remaining other phase, the calcium phosphate. In order to explain the apatite structure of TCPH, a calcium phosphate with a molar Ca/P ratio of 1.5, they put two hydrogens instead of the missing Ca and OH in the fluorine position, with the resulting formula $\text{Ca}_9(\text{PO}_4)_6\text{H}_2(\text{OH})_2$. In a series of papers from Dallemagne's laboratory they try to prove the existence of this compound as a well defined chemical entity, but really convincing evidence has not yet appeared.

The diffraction patterns obtained from precipitated calcium phosphates with the Ca/P ratio of 1.5 (TCPH) are always very diffuse because of the extremely small crystallite size and no accurate measurements can be made on these patterns.

To overcome this difficulty Posner and Stephenson (1952) and Brasseur (1953) have grown precipitated calcium phosphate with a Ca/P ratio of 1.5 (TCPH) in an hydrothermal bomb and could obtain crystals up to a few microns in length. These samples gave sharp diffraction patterns. The size of the unit cell calculated was given as

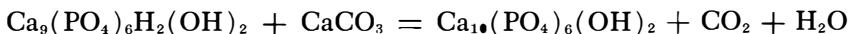
$$a = 9.42 - 9.44 \quad c = 6.87 - 6.88 \text{ \AA} \quad \text{Posner and Stephenson (1952)}$$

$$a = 9.41 \quad c = 6.86 \text{ \AA} \quad \text{Brasseur (1953)}$$

Posner and Stephenson, comparing TCPH with hydroxyapatite found that the powder patterns were very similar but with differences of about

0.01 Å in both axes of the unit cell. They suggested that TCPH "might be hydroxyapatite with phosphoric acid occluded in the ratio of 0.6 H_3PO_4 to one $Ca_{10}(PO_4)_6(OH)_2$." One of the strongest arguments in favor of the existence of TCPH should be that when heated to about 600°C it transforms to β -tricalcium phosphate (β -TCP). This compound is assumed to be anhydrous tricalcium phosphate $Ca_3(PO_4)_2$, identical with the mineral whitlockite (Dana, 1951). This substance gives a diffraction pattern quite different from that of apatites (See plate II). Beta-TCP is never found in normal bones or teeth but is in a few cases observed in some pathological calcifications such as testicular calcification (Frondel and Prien, 1946), concrements in the salivary glands (Tovborg-Jensen and Danø 1952), and calcified tuberculous hilus glands (Brandenberger and Schinz, 1945).

The formation of β -TCP from TCPH, however, is not a specific test for TCPH; a mixture of pure hydroxyapatite and phosphate (Na_2HPO_4) in the right proportions gives a perfect β -TCP-pattern after heating to 600°C. The presence of a β -TCP diffraction pattern in heated samples is merely a sign that the Ca/P ratio is below that of hydroxyapatite and it is even possible to obtain pure β -TCP-patterns from certain bones after heating, e.g. guinea-pig dentin (Carlström and Engfeldt, 1954) (See Plate II). On the other hand, a mixture of TCPH and lime or $CaCO_3$ in the right proportion gives a heat-stable apatite pattern, like hydroxyapatite, and it is this reaction Dallemande (1952) proposes when he explains the fact that bone ash (with a few exceptions) gives pure apatite patterns



The existence of TCPH was early doubted since it had been shown that the only calcium phosphate stable in water at or above the neutrality point was hydroxyapatite. $Ca_{10}(PO_4)_6(OH)_2$, Ca/P ratio 1.667, was already recognized as an apatite 80 years ago and no one except Dallemande (1952) questioned this substance as a chemical entity. It is rarely found as a mineral, but since every calcium phosphate by prolonged hydrolysis gives hydroxyapatite as an end product, it is easy to obtain in "pure" form. All preparations, however, yield micro-crystalline samples which give rather diffuse powder patterns. That heating causes the crystallites to grow was also first observed by de Jong (1926). Prolonged boiling in water gives a fairly pure product with crystallites up to microscopical dimensions. Fusion of Ca-pyrophosphate (obtained from heating pure dicalcium phosphate) and $CaCO_3$ at 1100°C in a water-saturated atmosphere gives a pure hydroxyapatite, according to Klement (1929).

Klement (1929) suggested that the chief constituent of bone was hydroxyapatite and in 1932 Klement and Trömel demonstrated the identity between the diffraction patterns of this compound and bone. The opinion that hydroxyapatite is the most important component of the bone salt has been widely accepted and thorough investigations into the chemical and physical properties of hydroxyapatite have been published.

The diffraction patterns of hydroxyapatite and fluorapatite are very similar but a calculation from careful measurement of the reflections gives a unit cell with a slightly longer a dimension. The values of the unit-cell dimensions in Table IV are for synthetic hydroxyapatites with the exception of those given by Burri *et al.* (1935), who investigated a mineral.

TABLE IV
LATTICE CONSTANTS OF HYDROXYAPATITE

Length of axes in Å		Reference
a	c	
9.42	6.94	Hendricks <i>et al.</i> (1931)
9.44	6.95	Burri <i>et al.</i> (1935)
9.43	6.88	Thewlis (1939)
9.450	6.871	Tovborg Jensen <i>et al.</i> (1944)
9.42	6.93	Müller (1947)
9.422	6.882	Wallaey <i>et al.</i> (1950)
9.45	6.89	Posner <i>et al.</i> (1952)
9.42 ₆	6.87 ₉	Trautz <i>et al.</i> (1952)
9.421	6.882	Carlström (1955)
9.421	6.881	Trautz (1955)

All values are converted to true Å (1 kX = 1.00202 Å)

The hydroxyapatite is like fluor- and chlorapatites, stable at high temperatures (up to ca. 1200°C). Between TCPH and hydroxyapatite there seems to be an infinite range of compounds, all of them giving an apatite pattern (Arnold, 1950).

4. X-RAY DIFFRACTION OF OSSEOUS TISSUES

As pointed out earlier, untreated bone gives a rather diffuse X-ray diffraction pattern depending on the small crystallite size, but it is without any doubt an apatite pattern (Plate II). Dentin gives the same general diffraction pattern as bone, but because none of them can be measured with any accuracy finer differences may escape the observer. Tooth enamel, on the other hand, gives rise to a pattern with rather well-defined reflections depending on a larger particle size (about 500 Å). See Plate II.

The size of the apatite unit cell of bone, dentin, and enamel has been measured by several authors but the values obtained show great differences because of the difficulties mentioned above. Calcined bone, dentin, and enamel give very sharp X-ray diffraction patterns and it is generally accepted that no change takes place in the unit cell dimensions during the heating (Table V).

TABLE V
LATTICE CONSTANTS OF OSSEOUS TISSUES

Bone or Dentin		Enamel		Reference
<i>a</i>	<i>c</i>	<i>a</i>	<i>c</i>	
9.29	6.96	—	—	Hendricks <i>et al.</i> (1931)
9.42	6.88	9.43	6.88	Gruner <i>et al.</i> (1937)
—	—	9.43	6.88	Thewlis (1939)
9.50	6.89	9.49	6.89	Bale (1940)
9.46	6.87	—	—	Lamarque (1943)
—	—	9.450	6.871	Tovborg Jensen <i>et al.</i> (1944)
—	—	9.44 ₀	6.88 ₆	Trautz <i>et al.</i> (1952)
9.42	6.88	9.440	6.881	Carlström (1955)
—	—	9.441	6.884	Trautz (1955)
After heating to 900°C for 2 hours				
9.421	6.882	9.429	6.884	Carlström (1955)

All values are converted to true A (1 kX = 1.00202 Å)

Precision measurements (Carlström, 1955) using a 19-cm evacuated powder camera and V-filtered Cr-radiation shows, however, that there is a shrinkage of the enamel apatite *a* axis, 0.01 Å, when heated to 900°C. It is possible that such a shrinkage occurs also in bone and dentin but no definite statement can be made. Calcined bone has an *a* axis 0.01 Å shorter than calcined tooth enamel, indicating that there is small structural differences. The *c* axis is not changed by heating (Table V).

The values found are in good agreement with those found for synthetic hydroxyapatite. The shift in the length of the *a* axis is not yet quite understood.

When discussing the chemistry and physical quality of the bone apatites one fundamental property, the extremely small particle size, is often overlooked. In the last years, however, a great number of new facts have appeared and the physiological role of the tremendous surface area of the bone apatite crystallites have been assembled. An up-to-date discussion is found in a recent review article by Neuman and Neuman (1953). In fact the small particle size is most suitable for various surface exchange phenomena. It can be demonstrated that about one half of the unit cells of one crystallite have sides at the surface of the crystallite.

When discussing the particle size (see next section) one would really like to give the *distribution* of particle sizes in different samples. The size of the "mature" crystallites which can be determined from low angle particle scatter and eventually from electron microscopy is only part of the story. Perhaps refined line profile measurements may help to find the distribution of particle sizes but this method may be of limited value as line broadening occurs both with decrease in particle size and with defects in the structures.

V. Relation between Mineral Salts and Collagen in Bone Tissue

Bone represents a complex system of collageneous fibers and an interfibrillar substance which is calcified. Optically bone behaves as a Wiener body.

Early investigations of thin sections of bone in the polarizing microscope disclosed that there was a relationship between the direction of the collageneous fibers and that of the crystallites of the mineral salts. W. J. Schmidt (1923, 1933, 1947) expressed the view that the collageneous fibers were formed first and that they had a directing influence on the deposition of the mineral salts "dass die (zuerst gebildeten) Kollagenfibrillen auf die (später) ausfallenden Erdsalze einem orientierenden Einfluss ausüben: die Krystallite werden von den Kollagenfibrillen orientiert adsorbiert." The bone salt crystallites appeared to be elongated and with their long axis parallel to the collagen.

The possibility of the existence of bonds between the ossein and the inorganic bone fraction was discussed by Ascenzi (1950). This author doubts the statement given by Dallemande and Melon (1946), that the organic and inorganic fractions of bone were physically mixed. They investigated the optical properties of ox bone. Ascenzi (1950), from his studies in the polarizing microscope, suggests that bone is really a complex system.

In the wide-angle X-ray diffraction patterns of bone it was early discovered that some lines were sharper than others. This sharpening was particularly clearly seen in the 002 reflection (Plate II). This observation means that the dimension of the crystallites is greater in the *c* direction than in the *a* direction. Measurements of line widths give some information about the average size of the crystallites. For example, Stühler (1938) found that the average size of the crystallites varied between 3.1×10^{-7} cm > *m* > $2.9 \cdot 10^{-6}$ cm. "Für die mittleren linearen Abmessungen (*m*) der Apatitkristalle des unbehandelten Knochens ergeben sich dem nach einige 10^{-7} cm." Bale, Hodge, and Warren (1934) and Tovborg-Jensen and Möller (1948) also deduced the size of the crystallites from other calcified tissues such as enamel and dentin.

In a series of papers dealing with the low-angle particle scatter of X-rays from thin sections of intact bone tissue, it was observed that the low-angle particle scatter was wing-shaped from longitudinal sections but symmetrical from cross sections of bone (Plate II, 9, 10, and 11). The direction of the wing was perpendicular to the fiber direction of the bone, which means that the crystallites are elongated and arranged in the direction of the bone (long bones). Measurement of the slope of the low-angle scatter (corresponding to the short dimension) gave the curve shown in Fig. 2. From this curve it could be deduced that the diameter of the particles was in the range 50–75 Å. The fact that the curve was a straight line indicated that the particles may have a fairly symmetrical cross section. An evaluation of the length of particles from the low-angle scatter gave a value of about 200 Å (Finean and Engström, 1953).

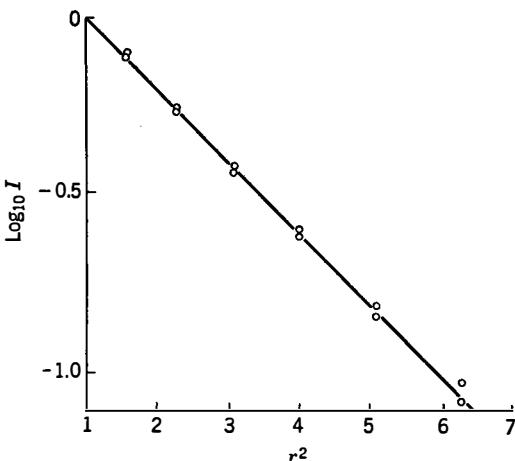


FIG. 2. Diagram relating log scattered X-ray intensity versus scattering angle measured as distance from the central beam (from Finean and Engström, 1953).

In an examination of fish bone where the fiber orientation is excellent as judged from phase contrast microscopy, beautifully oriented low-angle patterns were observed. Along the wing of the scatter a shoulder was superimposed and when treated to the first approximation of the Bragg-law this corresponded to a spacing of 68 Å, which is in good agreement with the value obtained from the low-angle particle scatter (Carlström and Finean, 1954). In the low-angle diagrams from fish bone a series of meridional low-angle reflections appeared. These could be interpreted of being orders of a fundamental period of 660 Å. Especially the third order (220 Å) was extremely strong, in fact the other orders were difficult to record. This strong 220 Å spacing was first believed to belong to the

collagen period (3rd order), but it was found also when the organic material had been removed with ethylenediamine. This reflection may represent a spacing in the inorganic part of the bone. Probably the reflection was the third order of a 660 Å period. Again the value found, 220 Å, is in good agreement with what was found from the low-angle particle scatter.

Bone collagen gives an X-ray period similar to that found in collagen from tendon (Bear, 1944; see Bear, 1952). The literature on the structure of collagen is too large to be reviewed here and the field of protein structure is almost daily being enriched with new information. For the structure of collagen the reader is referred to the excellent review given by Bear (1952) and to the printed proceedings of the Faraday Society collagen meeting (1953) edited by Randall.

Judged from electron microscopy the periodicity of bone collagen seems to be identical with collagen from other sources. However, this observation does not exclude significant differences between collagen from various sources. Martin (1953) examined fowl bone collagen and found a structure similar to that in tendon collagen. The bone from birds could be decalcified without too much disturbance of the collagen structure. Human bone, however, after decalcification, yields a product in which it is difficult to see the collagen structure. Therefore Kellenberger and Rouiller (1950), Rutishauser *et al.* (1950), and Huber and Rouiller (1951) used replica techniques to demonstrate the collagen structure. Robinson and Watson (1952) observed in the electron microscope that the collagen from decalcified human bone had a mean periodicity of 630 Å.

The low-angle pattern from bone of the combined system of collagen-mineral salts can readily be imagined as a system of particles with lengths varying slightly about an average of 220 Å, which are aligned along the collagen fibers in such a way as to produce precise crystallographic reflecting planes only where they coincide with the collagen 660 Å period. Such a picture appears to be capable of explaining the observed diffraction effects. After extraction of collagen (Carlström and Finean, 1954) the scattering curve corresponding to the short axis of the particles no longer shows the shoulder which in intact bone seemed to suggest a superimposed Bragg-reflection at 68 Å. Instead there is a regular decrease in intensity of scatter with increase in scattering angle, which could be treated as independent particle scatter and from which a particle diameter of 65 Å was deduced. It was assumed that the removal of collagen (pike bone) introduced sufficient disorder into the system to remove the diffuse reflection at 65 Å. It was mentioned above that the meridional reflection 220 Å was not affected by the removal of the organic fraction.

Electron microscopy of bone tissue has given results which in their main features are in agreement with the results obtained by the polarizing microscope and the X-ray diffraction techniques. It is only when giving the dimensions of the crystallites that there are some discrepancies. From an initial study of fragmented bone Robinson (1951) and more recently of thin sections of bone Robinson and Watson (1952) have suggested that the apatite is in the form of tabular crystals the most probable "mature" crystal length being 350 to 400 Å. The width of the same order as the length and the thickness was given a value between 25 and 50 Å. It was seen in the electron micrographs that the spacing of the crystals appeared to be associated with the periodic banding along the collagen fibers. In the work of Robinson and Watson (1952) it can be noted that the particle length most frequently found was about 180 Å, but these particles were assumed to be fragments of the larger particles. It may be suggested that the larger plaques frequently seen in electron micrographs may be formed by the association of a number of the more basic particles and reflect a higher stage of ultrastructural organization.

In Germany Schwarz and Pahlke (1953) made essentially the same observations as Robinson when studying bone by electron microscopy. Schwarz and Pahlke found that the apatite particles were related to the D-band of collagen (Wolpers) but they found dimensions varying from 150 to 1300 Å. Again these large fragments most probably are associations of the basic units. The periodicity of the bone collagen was measured to 640 Å.

Kellenberger and Rouiller, 1950, and Huber and Rouiller, 1951, studied bone by replica techniques and have described the structure of bone collagen and give the size of the apatite crystallites to be below 100 Å. Fernández-Morán (1954), who sectioned fish bone with a diamond knife, found that in longitudinal sections the apatite particles were like thin needles arranged parallel to the collagen and related to the periodicity. In cross sections it was seen that the apatite particles had an almost symmetrical cross section and were arranged in a hexagonal array. The width of the particles were about 50 Å.

In conclusion the following can be said about the ultrastructural organization of bone:

The basic structural components in all types of bone appear to be collagen and apatite. The collagen has the same essential structural characteristics (although chemical differences may occur) in all systems, and it would appear that the "mature" apatite particles are of approximately uniform size, the length in particular being remarkably constant in all types of bone studied. The probable length of the apatite particle corresponds roughly to one third of the fundamental repeating unit in the

structure of collagen, and X-ray diffraction and electron microscope studies have indicated that this may be an important repeating distance in the structure of collagen itself. There can be little doubt of the close relationship between the form of crystallization of the apatite component during the development of bone and the detailed structure along the collagen fiber, and it can be suggested that the structure is basically the same in all bone tissues, the observed differences in X-ray diffraction effects being associated with slight differences in degree of order among structural components and in their relative proportions.

PLATE I

DISTRIBUTION OF MINERAL SALTS WITHIN BONE TISSUE

Microradiograms of thin sections of bone (1-5). The pictures are exposed on Kodak Maximum Resolution Plate and enlarged by photomicrography. White areas contain large amounts of mineral salts. The organic material in the sections does not show up in comparison with the mineral salts.

1. Cross section (50μ) of normal bone. Femur of woman aged 51. Haversian systems have varying amounts of mineral salts, those which are formed most recently have the smallest amount (black in the picture). The spaces for the osteocytes are seen as X-ray transparent areas. In some Haversian systems it can be seen that there is a shift of the mineral salt content from the lumen towards the periphery of the system.

2. Cross section of a long bone from a case of Paget's disease. The normal pattern of mineral salt distribution seen in photomicrograph 1 is disturbed; there is increased resorption. The cementing lines are clearly seen and contain large amounts of mineral salts.

3. Microradiogram from an infant suffering from osteodysmetamorphosis fetalis (Engfeldt and Zetterström, 1954). The bone tissue is losing its rebuilding capacity. Only one Haversian system is seen in the picture. An examination of this section in polarized light reveals that organic substance is laid down in the resorption cavities in an almost normal way, but this organic material is not calcifying. The content of phosphatase in the bone is lowered. As this type of bone tissue lacks the rebuilding capacity a great deal of its ability to take part in the regulation of the ions in the body fluid is lost.

4. Marble bone disease (Albers-Schönbergs disease). The normal structure of bone is disturbed, increased density of the cementing lines and areas of extremely high mineralization.

5. Osteogenesis imperfecta. This mineral salt pattern is characteristic—a similar distribution has been found in experimentally produced ectopic bone (Engfeldt-Engström, 1954).

6. A thin-ground section photographed in the Dyson-interference microscope. From the shift of the interference bands the mineral salt content can be computed; after decalcification the amount of organic material is also obtained. In the upper part of the photomicrograph is a good demonstration that the Haversian system shifts the bands to a lesser extent than the surrounding tissue. This finding is in complete agreement with the distribution of mineral salts shown in 1.

PLATE I

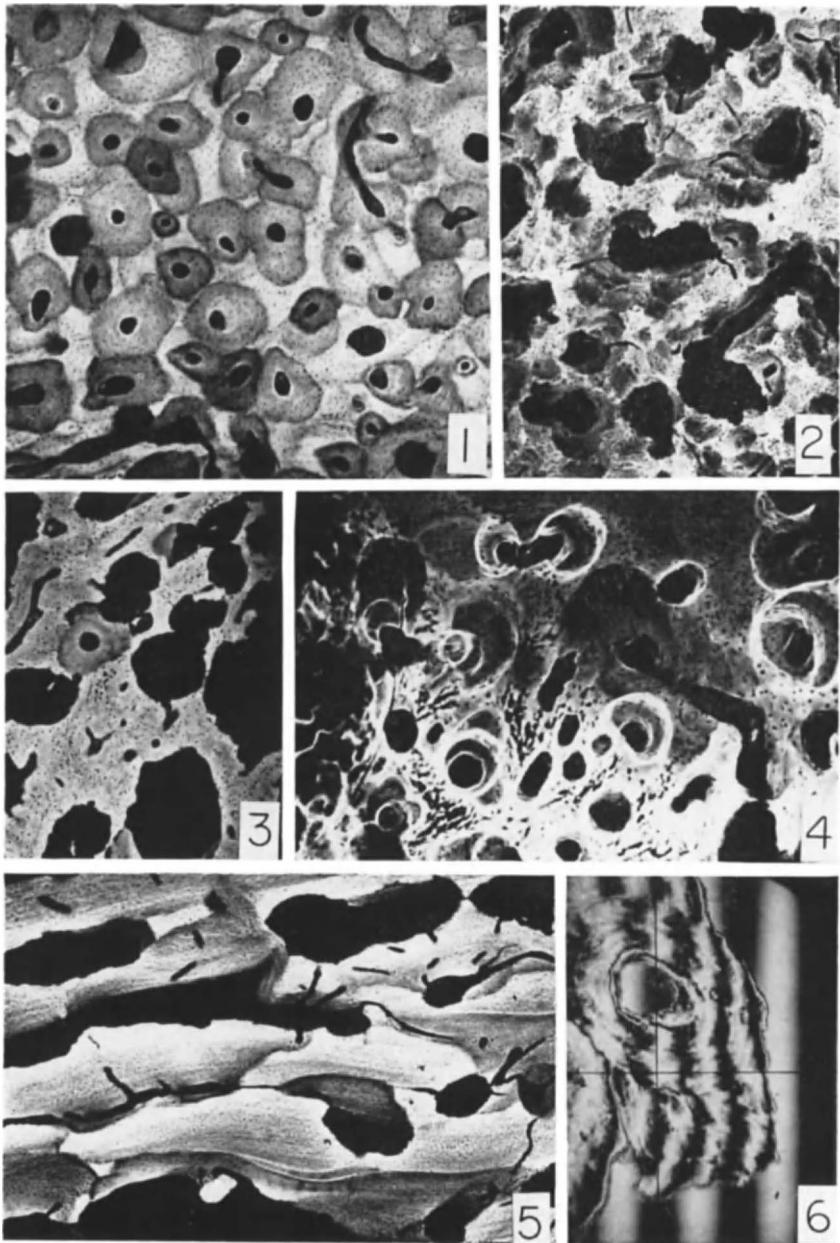
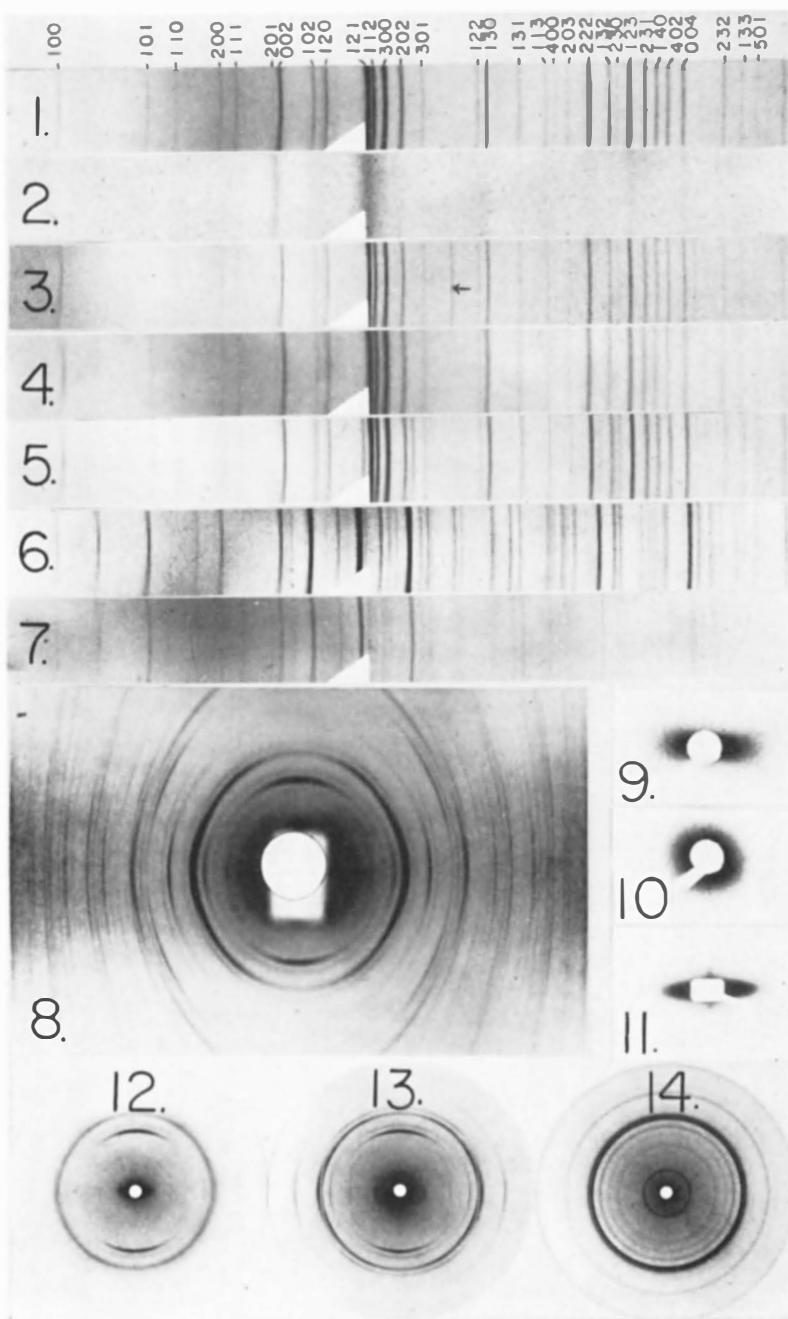


PLATE II

X-RAY DIFFRACTION PATTERNS OF BONE AND RELATED SUBSTANCES

- 1-7. Powder diagrams registered in a 19-cm camera with CuK α -radiation.
1. Apatite mineral with indexing.
2. Untreated bone.
3. Calcined bone. The line at the arrow belongs to CaO.
4. Enamel.
5. Calcined enamel. Partial transformation to a substance closely related to β -tricalcium phosphate.
6. β -tricalcium phosphate.
7. Calcined dentin from guinea pig, β -tricalcium phosphate.
8. Rotation diagram of calcined fish bone. Rotation and fiber axis is vertical.
9. Low-angle particle scatter from a longitudinal bone section. Note the wing which is perpendicular to the longitudinal direction of the bone.
10. Low-angle particle scatter from cross section of bone.
11. Low-angle diagram from calcified tendon, showing meridional reflections of a fundamental period of 660 Å. This diagram was registered at M.I.T., Boston, with Prof. R. Bears' equipment, and we thank Prof. Bear for permission to use this diagram.
12. Flat film diagram of a longitudinal section of fish bone. Note the orientation of the 002 reflection and also of the low angle scatter close to stop. This scatter consists of particle scatter and the equatorial 11 Å spacing of collagen. The fiber axis is vertical. Diffuse lines are due to small crystallites.
13. The bone in 12 heated to 900°C. Note the disappearance of the low-angle wing and the sharpening of the high-angle lines.
14. Flat film diagram from calcined cross section of fish bone.

PLATE II



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CHAPTER VII

THE OSTEOBLAST

J. J. PRITCHARD

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I. Introduction

1. HISTORICAL

In 1845 Goodsir wrote: "on the surface of young and vigorous bones I have observed numerous cells, flattened, elongated and more or less turgid, belonging doubtless to the system of Haversian cells" and he goes on to say: "that this cellular layer plays an important part in the economy of bone, appears probable from the prominent position it holds in its development, and from the intimate connection of the Haversian cells with all the morbid changes of bone. Its existence, great extent and probable powers, cannot be overlooked in any question regarding the economy of bone in health or disease."

Tomes and de Morgan (1853), in a classical paper on the structure

and development of bone, noticed similar cells on the surfaces of developing bone and called them, non-committally, "osteal cells." H. Muller (1858) also observed them but without naming them. It was Gegenbaur (1864, 1867), however, who first studied these cells in detail, and it was he who gave them the name "*osteoblasts*" as their distribution suggested that they were responsible for the manufacture of the bone matrix. He regarded them as a special race of bone-forming cells derived originally from the mesenchyme of the embryo. Later he erroneously supposed that they were of ectodermal origin.

In the past one hundred years vigorous controversy has raged regarding the role of osteoblasts in the manufacture of bone matrix, and their relationships with other cells. So far neither their functional role nor their morphological specificity has been settled, and very diverse opinions are held. And, as so often happens where knowledge is uncertain, these opinions tend to be expressed vigorously and categorically.

For Keith (1919) the osteoblasts are a specialized race of bone-forming cells which "seem to conduct the work of bone-building as if they had been given the training of expert and unerring engineers." Likewise Cartier (1951) expressed the view that "the osteoblasts preside over the formation and life of osseous tissue. This histological fact ought to represent the fount of all biochemical theories of ossification." Leriche and Pollicard (1926), however, regard the cells as "banal, reactionary fibroblasts" and deny them any morphological specificity, or special functional significance in bone manufacture. For these authors the so-called "osteoblasts" are merely swollen, degenerate forms of the ordinary connective tissue fibroblast which are in process of being engulfed by the spread of the bone matrix into the surrounding loose connective tissues, and, so far from promoting the deposition of the matrix, if anything, they attempt to resist it.

This conflict between those who support the idea that the formation and maintenance of the bone matrix are presided over by a special race of cells and those who believe that bone formation is governed solely by chemical changes in the intercellular fluids, quite independent of cellular activity, divides workers and writers on bone into two camps. On the whole histological investigators have supported the osteoblastic theory of bone formation, biochemical investigators the chemical or "humoral" theory. The result has been that morphological and chemical studies have gone on independently and the possibility of correlating results in the two fields into a unified theory of bone formation has seemed remote.

Recently, however, the advent of reliable histochemical methods has given rise to the hope that the two disciplines may be brought together,

for by locating specific chemical substances and processes at specific histological sites, morphological events have begun to be given a chemical meaning while chemical events have been given a habitation.

2. TERMINOLOGY

A great handicap in writing about the osteoblast is the lack of agreement as to what precisely is to be meant by the term. So far the osteoblast has been defined simply with reference to its position adjacent to bone matrix. There are, however, several morphological varieties of cell in this position, and opinions differ as to their relationship to one another and hence as to which of the cells the term "osteoblast" should apply. By some, all the mononuclear cells, whatever their size or shape, which lie in close contact with the surfaces of bone matrix, are called osteoblasts. Others would restrict the term to the larger mononuclear cells at sites where bone formation is active. It is generally agreed that the large multinucleated cells adjacent to bone matrix warrant separate designation as "osteoclasts," even by those who believe that they are derived from the fusion of a number of osteoblasts. From observations on developing bone the present author (Pritchard, 1952), concluded that no sharp dividing line could be drawn between the mononuclear cells, for all of them, from the very large rounded elements at sites of very active new bone formation, to the inconspicuous squamous cells lying against inactive bone surfaces, formed a continuous series and therefore warranted a common, generic name.

The variations in size, shape, polarity, and in cytological and cytochemical details within the series, are so marked, however, that the generic term "osteoblast" cannot usefully be employed without qualification. It is therefore proposed to call the larger members of the series *active osteoblasts* (Fig. 1) * and the flat inconspicuous ones *resting osteoblasts* (Fig. 10) because of the convenience and familiarity of the terms, but without defining at this stage what "active" and "resting" are meant to imply.

Where bone formation is particularly active, as in early centers of ossification, or where bone formation has been reawakened in adult life, incompletely differentiated osteoblasts may be found in the mesenchyme, periosteum or marrow cavity which appear to represent stages in the transformation of mesenchymal, periosteal or marrow-reticulum cells into osteoblasts. These will be termed *pre-osteoblasts* (Figs. 5 and 6).

* Figs. 1-6 appear in Plate I, Figs. 7-13 in Plate II, Figs. 14-19 in Plate III.

II. General Cytology

1. SIZE, SHAPE, AND ORIENTATION

Osteoblasts vary greatly in size. According to Koelliker (1889) active osteoblasts in man may be from $15-80\mu$ long, although most of them fall within the $20-30\mu$ range. They may also assume a wide variety of shapes depending on the way they are packed, the nature of the inter-cellular matrix and the position of the nucleus and cytoplasmic organelles. In general, however, the cellbody is more compact and squat (Fig. 6) than that of the ordinary connective tissue fibroblast, which tends to an elongated, fusiform shape.

The most characteristic arrangement of osteoblasts is a one-cell thick pseudo-epithelium carpeting the surfaces of newly-formed trabeculae of bone matrix (Figs. 1, 2 and 3). When so disposed osteoblasts tend to be columnar in shape with the nucleus at one end, and orientated perpendicularly to the bony surface (Fig. 6). Moreover the nucleus tends to be at the end of the cell furthest from the bone matrix (Koelliker, 1889). Such definite polarity is by no means the rule, however, for many osteoblasts lie with their long axes parallel to the bony surface, while others lie obliquely, overlapping like roofing tiles (Bidder, 1906). The nucleus, also, is not necessarily at the end of the cell furthest from the matrix (Disse, 1908, 1909; Lubosch, 1928). Nevertheless, from my own observations, I can confirm that where bone formation is particularly active practically all the osteoblasts present are highly polarized with respect to the bone matrix. Thus at the tips of rapidly growing trabeculae osteoblasts may be several layers thick, and characteristically arranged in the form of a rosette around the growing point. The cells are then pear-shaped with the nucleus occupying the blunt end which lies furthest from the matrix. (Figs. 5 and 6).

Inactive osteoblasts, on the other hand, are extremely thin, flattened, squamous elements, very closely applied to the bone, with a flattened nucleus occupying a more or less central position in the cell.

Osteoblasts can, in fact, be arranged in a continuous series, on the basis of shape variations, from the one extreme of pyriform cells with maximum eccentricity of nucleus and maximum polarity with respect to the adjacent matrix, to the other extreme of squamous cells with little or no evidence of nuclear eccentricity or cell polarity. These variations are closely correlatable with the rate of bone formation in the vicinity of the cells. As we shall see, there are other variations in the cytology of the osteoblast which correlate well with these changes in shape; which makes the conclusion irresistible that all these variations are significantly related to the rate of matrix production, although whether the cell

changes are indicative of an osteoblastic role in matrix formation or whether the matrix changes condition the cell changes, remains to be decided.

In their natural situation, on bony surfaces, it is difficult to determine how far the internal rearrangements of nucleus and cytoplasm, which are the reason for the variations in the shape of the osteoblasts, are self-induced, and how far they are due to mechanical pressures resulting from the mode of packing and the nature of the intercellular matrices. When osteoblasts are invading blood clot in bone repair (Pritchard, 1946a), or wandering freely at the periphery of an *in vitro* explant (Rodová, 1948), however, they are subject to a much more even distribution of mechanical forces and their "natural" shape can assert itself. Under these conditions osteoblasts are fusiform, like fibroblasts, only larger, plumper, and more irregular in outline. But when bone formation begins *in vitro* osteoblasts lose their fusiform appearance and assume the epithelioid form with eccentric nucleus characteristic of these cells *in vivo* under normal conditions (Fell, 1925; Hill, 1936).

2. PROCESSES

It has long been known that fine cytoplasmic processes extend from the cell body of an osteoblast to anastomose with those of its neighbors (Spuler, 1899). Some of these processes may also enter the superficially placed canaliculi of the bone matrix to connect with similar processes of the osteocytes, while others link up with those of the surrounding connective tissue cells. Whether these connections indicate protoplasmic continuity, or merely contiguity, is difficult to decide.

The processes are conspicuous in Mallory-stained preparations because of their affinity for acid fuchsin (Fig. 4). Some of them are surprisingly long, indicating that considerable displacements of the cells relative to one another could occur without the necessity of breaking the bonds between them. The migrations of osteoblasts *in vitro*, however, suggest that the cells are capable of withdrawing their processes and moving independently.

3. NUCLEUS (Figs. 6, 7, and 9).

According to Weidenreich (1928) the nucleus of the osteoblast is large, vesicular, spherical, or ovoid, poor in chromatin, and with one to three conspicuous nucleoli. The tendency for the nucleus to be eccentrically placed at one end of the cell has already been mentioned. Cappellin (1948), studying the maturation of the osteoblast in the chick embryo, found that the nucleoli became greatly hypertrophied and filled with ribonucleic acid as bone matrix began to be deposited

around the cells, but disappeared when the cells became converted into osteocytes.

All observers are agreed that definitive osteoblasts rarely show mitotic division. Nowikoff (1910) states that amitotic division may occasionally occur. Weidenreich (1928) concludes from the scarcity of dividing forms and the great increase in the number of osteoblasts at the beginning of bone formation that the greater number of cells must differentiate from other elements already present. I have also been impressed by the frequency of mitotic figures among pre-osteoblasts in the periosteum of growing bones, and in fracture callus, in contrast with the virtual absence of dividing forms among definitive osteoblasts in those situations (Pritchard and Ruzicka, 1950; Pritchard 1952).

4. THE JUXTA-NUCLEAR VACUOLE

In active osteoblasts, stained with ordinary dyes such as hematoxylin and eosin or methylene blue after formalin fixation, there is a large, round, clear area at the center of the cell, and about the size of the nucleus, which it adjoins (Figs. 3, 7, and 8). This was first pointed out by Spuler (1899), Askanazy (1902) and Sacerdotti and Frattin (1902). Schaffer (1922) termed it the juxta-nuclear vacuole. Maximow (1910) and Dubreuil (1913) stated that it corresponded to the "sphere" or "cell-center." Dubreuil (1913) reported that it contained a pair of centrioles. Fell (1925) showed that it harbored the Golgi apparatus, and appeared clear in ordinary preparations because the apparatus had been destroyed by fixation. Heller, McLean, and Bloom (1950) emphasize that the juxta-nuclear vacuole is only conspicuous in large, active osteoblasts. Like Fell, they regard it as the negative Golgi image (cf. Figs. 7 and 9).

5. THE GOLGI APPARATUS

Fell (1925) studied the Golgi apparatus of the osteoblast by da Fano's method in the developing long bones of the chick. She describes it as a compact network of argentophil filaments occupying the site of the juxta-nuclear vacuole. Hill (1936) studied the apparatus by the methods of Mann-Kopsch, Kolatchev, and da Fano in osteoblasts growing under optimal conditions *in vitro* and also in the tibial cortex of the newly-hatched chick. She states: "every cell in the ossifying area . . . showed a distinct Golgi apparatus, usually in the form of a network, very occasionally represented by a less organized aggregation of osmiophile elements . . . marked hypertrophy of the network became apparent in the younger osteoblasts before much ground substance has

been laid down, while older cells contained a more compact Golgi apparatus." She also states that "the appearance of the Golgi material in osteoblasts of the embryonic mandible *in vitro* is exactly similar to that found *in vivo*."

If the conditions were unfavorable for differentiation *in vitro* the apparatus tended to break up and disperse throughout the cytoplasm, and if the medium was very unfavorable the apparatus might disappear altogether. In its dispersed form the Golgi apparatus superficially resembled scattered fat granules, but simultaneous staining for the apparatus and for fat showed them to be quite distinct. Hill concluded that there is a close correspondence between the form of the Golgi apparatus and the state of differentiation and functional activity of the osteoblasts; and that it is probable that there is a relation between the production of the bone matrix and the condition of the Golgi material. A similar conclusion was reached from the study of the apparatus in the osteoblasts of developing membrane bone in the rat, using Aoyama's silver method (Pritchard, 1952) (Figs. 9 and 12). In these studies the reticular form was less frequent than the appearance of a collection of discrete but closely packed granules. Both appearances are probably artefacts, however, for it is now well known that the Golgi apparatus is very easily distorted by fixatives and its lipoidal constituents are readily transformed into "myelin forms" which have a spurious resemblance to organized structures. Further studies may well indicate that the Golgi element of the osteoblast is composed of small vesicles with lipoidal envelopes similar to those found in the majority of cells.

6. MITOCHONDRIA

The active osteoblast is characterized by an abundance of mitochondria (Figs. 8 and 11). Dubreuil (1913) and Deineka (1914) describe them as short thick rods lying parallel to the long axis of the cell and present in all parts of the cytoplasm except the juxta-nuclear vacuole. Dubreuil, who studied them in the osteoblasts of sheep bones after Regaud fixation, nitric acid decalcification and iron hematoxylin staining, found granular and long filamentous forms in addition to short rods. Many of the filamentous forms were beaded. They were found in the cytoplasmic processes as well as in the main body of the cell. He stated that their numbers fluctuated with the apparent activity of the cell in bone formation, becoming sparse in the inactive form of the cell and in the osteocyte. He also found an apparent correlation between the numbers of mitochondria and certain neutral-red vacuoles which could be demonstrated by supravital staining and which he regarded as secretory

granules. He inferred that the mitochondria were transformed into the secretory granules during active bone formation.

Fell (1925) studied the mitochondria of the osteoblasts in the developing chick by staining with iron hematoxylin after Flemming fixation with post-chromation. She found large numbers of filamentous mitochondria arranged around the juxta-nuclear vacuole in radiating, fan-like fashion. She states that when osteoblasts fused together to form osteoclasts the mitochondria became swollen and then broke up into granules.

Hill (1936) confirmed Fell's main observations and showed that a concentration of the mitochondria around the juxta-nuclear vacuole was characteristic of osteoblasts apparently engaged in bone formation, *in vitro*. The outwandering less differentiated cells in cultures, however, showed filamentous mitochondria throughout the cytoplasm, generally lying parallel to the long axis of the cell, and no longer concentrated around the vacuole. At the extreme periphery of the culture they were often swollen, vesicular or fragmented to a granular form, probably indicating degeneration. With re-differentiation the mitochondria once again became concentrated around the vacuole. In resting osteoblasts and osteocytes they became fewer, thicker, shorter, and more dispersed. Hill concluded that there was a definite correlation between the number and distribution of mitochondria in the osteoblasts and their bone-forming activity, a perivacuolar concentration being characteristic of the active phase. Fischer (1948) studied the mitochondria of osteoblasts *in vitro* cultivated from the embryonic frontal bone of the chick. In adequate media they were shown by supravital staining with Janus Green B to be long smooth rod-like bodies; but if the medium was inadequate these became beaded and then broke up into separate granules.

In studies on developing bone in the rat (Pritchard, 1952) it has been found, as previous workers have done, that the mitochondria are filamentous or rod-like in form and extremely numerous in active osteoblasts (Fig. 11). No special tendency for them to be grouped around the juxta-nuclear vacuole, was observed. On the contrary they appeared to be fairly evenly distributed throughout the cytoplasm, apart from the vacuolar region, and to be generally arranged parallel to the long axis of the cell, although there were many exceptions to this. Good fixation was essential for the exhibition of smooth filamentous forms: where the fixative had been slow in penetrating, the mitochondria were beaded or fragmented or might even have disappeared. This sensitivity of mitochondria to post-mortem changes was confirmed by supravital examination, using Janus Green B, of osteoblasts scraped from the surface of

the parietal bone of young rats. When fresh the mitochondria exhibited the characteristic filamentous form but as the cells died they rapidly broke up into fine particles. Like Hill, the present author found that undifferentiated pre-osteoblasts, and mature inactive osteoblasts and osteocytes possessed relatively few mitochondria, and these were thicker and shorter than those of the active osteoblasts.

7. OTHER CYTOPLASMIC PARTICULATES

From time to time, after various methods of fixation and staining, fine granules and vesicles have been described in the cytoplasm of the osteoblast (Weidenreich, 1928).

Dubreuil (1913) reported a collection of neutral-red vacuoles in the position of the juxta-nuclear vacuole after supravital staining. Their relation with the Golgi apparatus was not investigated. He also found rounded and irregularly-shaped bodies, stainable with iron hematoxylin after Tellyesnicky's or von Lenhossek's fixatives. Dubreuil regarded these as pre-secretion granules, but it is possible that in reality they were badly-preserved mitochondria. Weidenreich (1928), however, maintains that in well-fixed preparations fine vesicular bodies are always to be found in some part or other of the cytoplasm. He thinks they may be secretory antecedents, and not, as Leriche and Policard (1926) believe, a sign of cell degeneration.

Recently Heller-Steinberg (1951) has described granules $0.3\text{--}0.6\mu$ in diameter, stainable by the periodic-acid-Schiff method of Hotchkiss after freeze-drying or alcohol fixation. Their number waxed and waned with the apparent activity of the osteoblast. Thus after the injection of parathyroid extract they disappeared during the phase of bone resorption, only to reappear as bone formation began once again. The author suspects that the granules are composed of glycoprotein, and are the forerunners of the mucopolysaccharide cement substance of the bone matrix.

III. Cytochemistry

1. BASOPHILIA AND NUCLEIC ACIDS

One of the most characteristic features of the osteoblast cytoplasm is its strong basophilia, very few cells, either embryonic or adult, approaching it in this respect. With methylene blue or other basic dyes, the entire cytoplasm, except for the juxta-nuclear vacuole which remains unstained, is intensely colored. A fine granularity of the stained material is often apparent. In order to demonstrate the basophilia, however, acid fixatives and decalcifiers must be avoided as these remove the basophilic material. The present author has had the best results after fixation with

absolute alcohol, sections being cut without decalcification. Metachromatic reactions are not given (Pritchard, 1952).

The intensity of the basophilia varies markedly with the apparent activity of the cell. Heller *et al.* (1950) pointed out that large columnar or cuboidal osteoblasts are more intensely stained than spindle-shaped and flattened ones. In experimental scurvy Follis (1951) showed that the medullary osteoblasts of the long bones became spindle-shaped and lost their basophilia. When vitamin C was now given the cells regained their basophilia and their characteristic form. In developing membrane bones in the rat embryo it has been shown (Pritchard, 1952) that basophilia increases as osteoblasts differentiate from the pre-osteoblasts of the periosteum, reaching its maximum as bone matrix begins to be deposited, and then declining in intensity as the osteoblasts become quiescent in the interior of the bone, or as they become surrounded by the matrix to form osteocytes.

Cappellin (1948) described the genesis of the basophilic material in differentiating osteoblasts from a 17-day chick embryo. He stated that it first appears in the nucleolus, then just outside the nucleus close to the nuclear membrane and finally, as bone matrix begins to be deposited, throughout the cytoplasm. As the cell becomes surrounded by matrix and is transformed into an osteocyte, the peripheral basophilia disappears first, then the perinuclear staining goes and finally the nucleolus disappears completely.

For some time it has been suspected that the basophilia of the osteoblast is due to the presence of large quantities of ribonucleic acid, as in other basophilic cells. The great affinity of the cytoplasm for pyronin in the pyronin-methyl green staining mixture and the ease with which this pyroninophilia could be removed by previous treatment with acids and alkalis pointed strongly to this conclusion. The matter was settled when Follis (1951), and the author (Pritchard, 1952) showed that ribonuclease prepared from the pancreas removes the cytoplasmic basophilia of the osteoblast as it does the basophilia of other ribonucleic acid-containing cells. It is to be noted that the pyroninophilia of the central portion of the nucleoli is also removed by treatment with this enzyme.

In contrast to the marked basophilia of the cytoplasm of the active osteoblast, the nucleus shows little affinity for ordinary basic dyes. The Feulgen reaction is in agreement with this, for only scattered chromatin granules, and the nuclear and nucleolar membranes, give a positive reaction. This is in marked contrast to the pre-osteoblasts and general connective tissue cells which give a relatively strong nucleal reaction. In my studies the Feulgen reaction failed to demonstrate mitotic figures in active osteoblasts, although they were abundant in the pre-osteoblastic

layer of the young periosteum (Pritchard, 1952). The Feulgen reaction is generally regarded as specific for desoxyribonucleic acid. Follis (1948) confirmed this in the case of the osteoblast by showing that the reaction could not be obtained after previous treatment with a preparation of desoxyribonuclease. Whether there is an absolute loss of desoxyribonucleic acid as the osteoblast differentiates, or whether the loss is only an apparent one due to dilution associated with nuclear hypertrophy, has not been determined. With the pyronin-methyl green mixture, the staining of the nucleus with methyl green is in every way comparable with that given by the Feulgen reaction. In particular, only the nucleolar membrane is stained green, the interior being pyroninophil. Likewise, after treatment of sections with normal hydrochloric acid at 60° C for 5 minutes, methylene blue and toluidine blue simulate the Feulgen picture almost exactly (except for the difference in color). Unlike the ribonucleic acid of the cytoplasm and center of the nucleolus, the desoxyribonucleic acid of the nucleus is moderately metachromatic, indicative probably of a higher degree of polymerization (Pritchard, 1952).

2. GLYCOGEN

The presence of glycogen in cartilage and developing bone has long been known but it was not until Harris in 1932 suggested that it might cooperate with phosphatase in the processes leading to the deposition of calcium salts in the matrix that much attention was given to it. Harris found glycogen to be present abundantly in hypertrophic cartilage cells and pre-osseous mesenchymal cells but he stated that it was absent from the active osteoblasts on the surfaces of bone matrix. Hill (1936) likewise could not find any glycogen in active osteoblasts either *in vivo* or *in vitro*. Gendre (1938), Parvisi (1938), Glock (1940), and Horowitz (1942) confirmed the presence of glycogen in large quantities in the pre-osseous mesenchymal cells of future centers of ossification well before osteoblasts had differentiated or bone matrix deposited. Horowitz also stated that there is much less glycogen in fully differentiated osteoblasts than in the differentiating pre-osteoblasts.

Bevelander and Johnson (1950, 1951a, b) studied glycogen distribution in the developing membrane bones of the pig. They showed that it was present in large quantities in the osteogenic mesenchyme which precedes the appearance of a center of ossification and in the differentiating pre-osteoblasts of the periosteum. As active osteoblasts appeared and the first spicules of bone matrix were formed, however, glycogen momentarily disappeared, but when mineralization began they stated that glycogen reappeared in maximal amount in the osteoblasts and per-

sisted in the osteocytes derived from them. The present author has made similar observations on the developing membrane bones of the rat (Pritchard, 1952) and can confirm the loss of glycogen in active osteoblasts (cf. Figs. 16 and 17). However, only trivial amounts of glycogen could be demonstrated in the less active osteoblasts and osteocytes of the more mature bone. Abundance of glycogen in the differentiating osteoblasts, but complete absence in active osteoblasts, was also observed in the periosteum of developing long bones. Follis (1948), and Follis and Berthrong (1949), however, reported the presence of glycogen both in active osteoblasts and in osteocytes. In experimental scurvy the glycogen disappeared, but rapidly reappeared when vitamin C was administered (Follis, 1951).

In view of the importance attached to the presence of glycogen in osteogenic cells in Gutman's theory of calcification (Gutman and Yü, 1950) it is of interest that in fracture callus, unlike embryonic bone, the present author could find no glycogen in either differentiating or mature osteoblasts and only minimal quantities were present in resting osteoblasts and osteocytes (Pritchard, unpublished observations).

3. PHOSPHATASE

Between 1923, when Robison discovered a phosphatase in calcifying tissues and put forward the hypothesis that it was responsible for the preliminary accumulation of phosphate ions in calcification, and 1939, when Gomori and Takamatsu almost simultaneously published methods for the detection of phosphatase activity in thin histological sections, a great deal of ingenious work was carried out on the problem of the relationship of the enzyme to the calcification process in bone and cartilage (see Chapter IX). From such work the universal presence of an alkaline phosphatase in calcifying and calcifiable tissues was established, although the precise role of the enzyme in the calcification process remained, and still remains, obscure.

It was generally assumed that the enzyme was manufactured and secreted by osteoblasts and hypertrophic cartilage cells (Martland and Robison, 1924; Fell and Robison, 1930, 1933), but histological localization of enzyme activity using the tissue slice method was too crude to establish this with certainty. The advent of Gomori's and Takamatsu's techniques made possible a much more precise localization of the enzyme and the presence of relatively heavy concentrations of phosphatase in osteoblasts and hypertrophic cartilage cells was soon confirmed (Gomori, 1939; Freeman and McLean, 1941; Kabat and Furth, 1941; Horowitz, 1942; Gomori, 1943; Bourne, 1943; Rodová, 1948; Follis and Berthrong, 1949; Bevelander and Johnson, 1950, and many others).

The first-mentioned workers were content to demonstrate the enzyme in a particular type of cell, but lately the relative amounts in nucleus, cytoplasm, and extracellularly, and at different stages in differentiation and functional activity, have received attention. It has been questioned (Martin and Jacoby, 1949) whether the histochemical methods usually employed are competent to localize the enzyme so precisely in view of the possibility of diffusion of the enzyme and its reaction products. Doubt has also been thrown (Gomori, 1950) on the validity of quantitative assessments in view of the large and variable destruction of the enzyme in the course of preparation of histological sections. However, precautions can be taken to minimize diffusion artefacts; and relative activities as between one group of cells and another in the same section may be inferred even though absolute activities cannot be determined.

Bourne (1943) showed that in the active osteoblasts appearing in the periosteum after holes had been drilled in the femora of guinea pigs, both nucleus and cytoplasm gave a diffuse reaction for the enzyme as did the newly-formed osteogenic fibers around the cells. Rodová (1948) likewise showed intense phosphatase activity in both nucleus and cytoplasm in osteoblasts cultivated *in vitro* from the frontal bone periosteum of the chick embryo. He found that ordinary fibroblasts, on the other hand, showed nuclear activity only. As in Bourne's studies, the osteogenic fibers were strongly positive.

Lorch (1949), in the skull of the embryo trout, found only nuclear activity in cartilage cells and osteogenetic mesenchyme before cartilage calcification and bone matrix formation, respectively, had begun. With the onset of calcification in cartilage and matrix formation in bone strong extracellular activity made its appearance while nuclear activity tended to diminish. In cartilage extracellular phosphatase was associated with impending calcification of the matrix. In developing bone on the other hand extracellular phosphatase was associated with osteogenic fiber production, as well as with calcification. Bevelander and Johnson (1950) and the present author (Pritchard, 1952) actually found a *diminution* of phosphatase activity in the osteoblast as calcification began in the bone matrix (cf. Figs. 15 and 18).

Horowitz (1942) and Bevelander and Johnson (1950), from the study of developing bones in the heads of rat and pig embryos respectively, stress that considerable phosphatase activity appears in the mesenchymal cells in presumptive areas of ossification before osteoblasts have differentiated and well before bone matrix has appeared. In a study of phosphatase activity in rat fetuses (Pritchard, 1952), by keeping the incubation time short to minimize diffusion artefacts and to show up sites of considerable enzyme activity preferentially, the present author was

able to confirm that areas of mesenchyme in which centers of ossification later appeared showed phosphatase activity before osteoblasts had differentiated (Fig. 15); and also that the definitive osteoblasts showed less activity than incompletely differentiated ones (Figs. 18 and 19). The enzyme first appeared in the nuclei of mesenchymal cells which were no different in outward form from the general mesenchymal cells of nonossifying areas. As the cells enlarged and began to undergo differentiation into osteoblasts, enzyme activity became much more intense and appeared in both nucleus and cytoplasm and also extracellularly on the collagenous osteogenic fibers. With the appearance of definitive osteoblasts, however, there was an abrupt fall in enzyme activity, particularly in the nucleus, less so in the cytoplasm, and least of all extracellularly. In the cytoplasm activity was often concentrated in the Golgi area at this stage. The osteoid matrix being deposited between and around the osteoblasts gave no reaction for the enzyme. Less active osteoblasts of the primitive marrow spaces showed even less phosphatase activity in nucleus and cytoplasm, although extracellular activity persisted in a thin zone immediately adjacent to the bone matrix. Resting osteoblasts and osteocytes showed little or no activity. These changes in enzyme activity during differentiation, and the various phases of functional activity, of the osteoblast, were essentially similar in developing membrane bones like the mandible and maxilla and in the periosteum of developing long bones. Analogous changes were observed in cartilage preceding its replacement by bone in the course of endochondral ossification. As the cartilage cells began to hypertrophy enzyme activity appeared in the nucleus first of all, then in the cytoplasm and then extracellularly. Soon after the appearance of the enzyme in the matrix calcification began. As the cells became grossly hypertrophic and began to degenerate enzyme activity disappeared.

In bone repair in the adult, changes in phosphatase activity were found to be essentially similar except that nuclear activity was everywhere much less in evidence. Highest activity was found in the cytoplasm of *differentiating* osteoblasts, and in the osteogenic fibers immediately before their incorporation into the bone matrix (Pritchard and Ruzicka, 1950).

The above conclusions were drawn from studies using the Gomori technique in which sodium glycerophosphate is employed as substrate for the enzyme, and the precipitate of calcium phosphate at sites of enzyme activity is converted into cobalt sulfide. Recently the present author has repeated some of these observations using a dye-coupling technique in which α -naphthol phosphate is the substrate and the free α -naphthol liberated by the enzyme is converted into an insoluble brown

dye at sites of activity by interaction with 5-chloro-o-toluidine. The earlier results were confirmed by this method in the main, although nuclear phosphatase was difficult to demonstrate. This raises the question whether apparent nuclear activity is really an artefact, as has recently been suggested for other tissues. From a critical appraisal of these results it appears that most nuclear activity in ossifying tissues can be discounted except that of the mesenchymal cells about to differentiate into osteoblasts and the cartilage cells about to hypertrophy. In such situations nuclear activity was consistently found after very short periods of incubation, by both the Gomori and the dye-coupling methods, and when every precaution was taken to minimize diffusion. In the cells mentioned one can believe with Moog (1944, 1946) that nuclear activity is genuine and is an expression of nuclear metabolic activity associated with the processes of cell differentiation, and that it has an entirely different significance from that of the cytoplasmic and extracellular phosphatase which appears later at the stage of matrix deposition.

4. LIPOIDS

The lipoid content of osteoblasts has received little attention. Dubreuil (1910) believed certain fine vacuoles which he observed in the cytoplasm after staining with iron hematoxylin were of a fatty nature but the presence of fat was not confirmed until Hill (1936) found that functional osteoblasts both *in vitro* and *in vivo* exhibited fine sudanophil granules which increased in number but not in size as the matrix adjacent to the cells became calcified. She also showed that these granules were independent of the Golgi apparatus. Large granules and globules of fat were only found in badly fixed specimens and in cells undergoing degeneration. By the Smith-Dietrich technique she showed further that the granules apparently contained phospholipins.

Using Sudan black on formol-calcium fixed frozen sections of fetal rat heads I have observed small sudanophil granules in the region of the Golgi apparatus, but elsewhere the cytoplasm showed only a very fine and faint sudanophil dust. Young cartilage cells, however, regularly showed comparatively coarse, discrete, heavily-stained granules. It was not possible to confirm the presence of phospholipins in osteoblasts with Baker's acid hematein.

5. ENZYMES OTHER THAN PHOSPHATASE

Follis (1948) and Follis and Berthrong (1949) tested bone and cartilage histochemically for the presence of cytochrome oxidase, and succinic and citric dehydrogenases, using thin freehand slices of fresh,

unfixed tissue. They found abundant cytochrome oxidase in active osteoblasts, using the Nadi reagent. None, however, was found in cartilage. The other enzymes mentioned were present in cartilage cells but were not unequivocally demonstrated in osteoblasts. In experimental scurvy cytochrome oxidase was found to disappear from the osteoblasts as they reverted to the peculiar spindle cells of the "gerüstmark," but it rapidly appeared again when vitamin C was administered.

6. MISCELLANEOUS HISTOCHEMICAL STUDIES

Follis (1948) demonstrated iron and vitamin C in osteoblasts and he also studied the redox potentials in fresh tissues. His report was of a preliminary nature and his results have yet to be confirmed.

IV. Integration of Morphological and Histochemical Studies

In a recent paper (Pritchard, 1952) an attempt was made to summarize and integrate the morphological and histochemical changes taking place in the osteoblast during its maturation in early centers of ossification. The conclusion was that the onset of osteoblastic differentiation is heralded by the appearance of alkaline phosphatase in the nucleus and considerable quantities of glycogen in the cytoplasm. The Golgi element also begins to enlarge, the number of mitochondria increases and ribonucleoprotein begins to accumulate in the cytoplasm. The nucleus is hyperchromatic at this early pre-osteoblastic stage, giving a strong Feulgen reaction, and many dividing cells are seen. Between the cells the matrix consists chiefly of an irregular fine network of argentophil, reticular (pre-collagenous) fibers.

With further differentiation cytoplasmic phosphatase activity appears and rises to a maximum, as does glycogen storage. The Golgi element hypertrophies further and the mitochondria continue to increase in number. Cytoplasmic basophilia becomes still more marked. The Feulgen reaction, however, becomes less intense and fewer mitotic figures are seen. The intercellular matrix now contains numerous bundles of osteogenic collagen fibers which give a strong reaction for alkaline phosphatase and are strongly metachromatic to toluidine blue. This last fact, together with a strong positive periodic-acid-Schiff reaction, indicates the presence of considerable amounts of mucopolysaccharides in the fibrillar matrix.

With the appearance of definitive osteoblasts phosphatase activity abruptly declines from its former level in both nucleus and cytoplasm, and glycogen disappears. The Golgi element, however, reaches its greatest size and apparently pushes the large and now hypochromatic nucleus

to one end of the cell. The juxta-nuclear vacuole, or negative Golgi image, is consequently very conspicuous at this stage. The nucleoli are very large and are mainly composed of ribonucleic acid. Long filamentous mitochondria are very numerous in all parts of the cytoplasm except the vacuole. Cytoplasmic basophilia, indicative of ribonucleoproteins, is at a maximum. Mitotic figures are no longer seen.

Uncalcified bone matrix is deposited adjacent to the nonnuclear end of the polarized cell. This matrix is highly metachromatic and gives a strong periodic-acid-Schiff reaction. It shows no phosphatase activity, however; what extracellular phosphatase there is lies on the surface of the matrix close to the osteoblasts.

In the older parts of the bone where the deposition of matrix is less active the osteoblasts become smaller, and the characteristics just described become less pronounced. Alkaline phosphatase activity further declines, the Golgi element gets smaller, mitochondria fewer, cytoplasmic basophilia less intense. Glycogen, however, once more appears, though in small quantities. Finally, the flattened resting osteoblasts lose almost all the characteristics of the active cells and are virtually indistinguishable, except for their position, from fibroblasts and marrow reticulum cells.

A similar series of observations on the morphology and histochemistry of fracture callus cells has recently been made by the present author. The basic cell type from which osteoblasts are differentiated is a spindle-shaped cell whose characteristics are almost identical with those of the general connective tissue fibroblast. When bone is about to form, these cells undergo transformations similar to those which take place in the mesenchymal cells of early centers of ossification, with two principal exceptions. Firstly, alkaline phosphatase activity is less evident in the nucleus; almost from the inception of activity it is found extracellularly associated with collagenous osteogenetic fibers. Secondly, glycogen cannot be demonstrated at all during the stages of differentiation and up to the definitive osteoblastic condition. Small amounts, however, do appear in less active and resting osteoblasts at a later stage. Changes in the Golgi element, mitochondria, basophilia, nucleus, and juxta-nuclear vacuole are identical in fracture repair and normal development.

V. Functional Significance

The fully differentiated osteoblast possesses a combination of morphological and cytochemical characteristics unique among connective tissue cells. Moreover, such cells are only found in topographical association with sites of active bone formation. The definitive osteoblastic condition,

further, is a temporary one, for as the rate of bone formation diminishes the osteoblasts become more and more like ordinary connective tissue cells. The conclusion is inescapable that the definitive osteoblastic condition is either the cause or the consequence of matrix deposition. Leriche and Pollicard (1926) believe the latter, namely that the condition is one of degeneration in the face of advancing matrix formation for which the cell is in no wise responsible. Most workers, however, interpret the condition as indicative of an active role in matrix formation. My reasons for accepting the latter alternative are that the osteoblast is quite unlike a degenerate cell, but on the contrary gives much evidence of being an actively secreting cell, and, moreover, a cell which is secreting substances known or believed on other grounds to be essential for bone matrix formation. Firstly, the hypertrophic Golgi apparatus, the very numerous mitochondria and the nucleus pushed to one end of the cell by the accumulation of cytoplasmic products, as well as the polarity of the cell with respect to the site of matrix formation, are characteristics of actively secreting cells almost everywhere. By no valid stretch of imagination could these cytoplasmic characteristics be regarded as indicative of cell degeneration.

The very large nucleoli filled with ribonucleic acid and the great ribonucleic acid content of the cytoplasm are, in other cells, invariably linked with a high rate of new protein formation (Caspersson, 1947) and it would be surprising if these features had a different significance in the osteoblast. The combination of high alkaline phosphatase activity and cytoplasmic ribonucleic acid shown by the osteoblast strongly suggests that the protein manufactured, and presumably secreted, is of the fibrous variety, for this combination is widely found outside bone where fibrous proteins are being formed (Bradfield, 1949, 1950). Extracellular phosphatase is characteristic of sites of bone formation, and the close association of the enzyme with the osteoblasts topographically, indicates that the phosphatase found extracellularly has almost certainly been secreted by them.

There is thus strong presumptive evidence that the osteoblast is at least responsible for the secretion of a fibrous protein, and alkaline phosphatase. The fibrous protein is presumably collagen or a collagen precursor. Current theory also demands a substrate for the phosphatase in the preliminaries to matrix calcification. Gutman and Yu (1950) have suggested that intracellular glycogen might break down to provide such a substrate in the form of hexose phosphates. The high content of glycogen in differentiating osteoblasts in the embryo and its disappearance in the definitive osteoblast, seems to provide good circumstantial evidence for Gutman's views. However, the absence of glycogen from

differentiating osteoblasts in fracture repair is a grave difficulty in the way of accepting the hypothesis unless one postulates that in fracture repair the carbohydrate cycle is so rapid that glycogen has no chance to accumulate. Glycogen in embryonic pre-osteoblasts may therefore not have the significance attributed to it by Gutman. Glycogen is abundant in many embryonic tissues which show none in their adult condition. The presence of glycogen in embryonic osteoblasts may therefore merely indicate a general biochemical adaptation to the conditions of embryonic life and have no specific significance in bone formation.

The mucopolysaccharides which are found in the organic bone matrix may also have been manufactured by the osteoblasts. It is difficult to see where else they could have come from. So far, however, there is no very definite evidence from histochemical studies, apart from Heller-Steinberg's (1951) discovery of periodic-acid-Schiff positive granules in the cytoplasm, which may be glycoprotein. Breakdown of glycogen could provide raw materials for the manufacture of mucopolysaccharides, but the absence of glycogen in fracture callus pre-osteoblasts makes this hypothesis very doubtful.

To sum up, the morphology and cytochemistry of the osteoblast strongly indicate that the cell is an actively secreting one and that the secretion contains collagen and alkaline phosphatase. It may also contain hexose phosphates and mucopolysaccharides, but the evidence is much less strong. At any rate, the osteoblast produces some at least of the materials essential for matrix formation. The evidence is completely against Leriche and Pollicard's (1926) view that the osteoblast has no essential role to play in bone formation.

VI. Morphological Status and Transformations

We have just seen that the *functional* status of the osteoblast as a cell playing an essential role in bone matrix formation is vindicated by the morphological and histochemical evidence. The *morphological* status of the osteoblast as a unique cell type, in the genetic sense, rests on much less secure evidence, in spite of the unique combination of morphological and cytochemical features which characterize typical active forms of the cell. Difficulty arises because the typical active form of the cell is incapable of reproducing itself by cell division. It must therefore represent a more or less transitory stage in the transformation of some precursor cell into an osteocyte or other derivative, and the precursor cells are not always easy to identify or to distinguish from ordinary connective tissue cells, while the derivative cells other than the osteocyte are just as elusive. Both the origin and the fate of the osteoblast are in fact

difficult to determine and are the subject of much controversy. Two main hypotheses have been advanced. The first states that there is a special race of connective tissue cells resident in and around bone which alone has the capacity to differentiate into osteoblasts when the need arises. The second states that cells which can differentiate into osteoblasts are present everywhere in the connective tissues, and not only in and around bone.

The first hypothesis receives its strongest support from *in vitro* studies, for so far osteoblasts have only been cultured from explants of bone and periosteum and the mesenchyme of future centers of ossification. Likewise bony tissue has only developed *in vitro* in explants of bone or osteogenetic mesenchyme. The second hypothesis rests principally on the spontaneous occurrence and experimental induction of bony tissue outside the normal skeletal territories. Here the osteoblasts can only have differentiated from local connective tissue cells, which normally never show bone-forming powers, but which are in some way stimulated to do so by unusual environmental conditions. Whether at such sites of heterotopic bone formation osteoblasts may be derived from ordinary fibroblasts, or whether osteogenetic potency is restricted to a hypothetical race of undifferentiated mesenchymal cells supposed to be present in small numbers throughout the connective tissues of the organism, has not been determined. This distinction, however, is immaterial for the main argument, for the very existence of heterotopic bone proves that osteogenetic potency is not absolutely restricted to the skeletal territories. The tissue culture evidence to the contrary is not conclusive in this respect, for it is possible that factors necessary to convert fibroblastic cultures into osteoblastic ones remain to be discovered.

Nevertheless it must be emphasized that although the general connective tissues *can* form bone, they in fact very rarely do so; while the bone-forming powers of the cells within the osseous territories are very readily and consistently elicitable. To explain this it is necessary to postulate *either* that the skeletal cells form a race apart, on the grounds of the *ease* with which osteoblastic activity can be induced in them, *or else* that something is present in growing and damaged bone which causes nearby non-specific connective tissue cells to differentiate into osteoblasts. Several suggestions have been made as to the factor in bone which might determine the differentiation of local cells into osteoblasts. Leriche and Pollicard (1926) and many followers believe that the presence of calcium salts is the essential factor. Levander (1945), Lacroix (1946, 1947) and others believe that a chemical substance akin to an embryonic inductor, which they term "osteogenin," is present in bone. Others again (e.g. Altmann 1950), suppose that mechanical stimuli are

the essential factors and that the need for mechanical stability in some way induces osteoblastic differentiation from indifferent cells.

Attempts have been made to support these contentions experimentally, but without marked success. Implants of calcium salts in connective tissues have rarely evoked bone formation in their neighborhood, although spontaneous heterotopic bone formation is apparently favored by pathological deposits rich in calcium. Alcoholic extracts of bone injected into the muscles of rabbits result in ectopic bone formation in a fairly high proportion of cases (Bertelsen, 1944; Levander, 1945; Lacroix, 1946, 1947); but the discovery that alcohol alone will do as well vitiates the notion that the bone extract contains any specific "osteogenin" (Heinen, Dabbs and Mason, 1949). The production of bone in nonskeletal areas by purely mechanical means does not appear to have been accomplished.

At the moment, therefore, there is little to gainsay the view that the cells in and around bone have an inherent capacity to differentiate into osteoblasts and manufacture bone matrix; and that while this capacity is not absolutely restricted to these cells, it is elicitable so much more easily in them than in the general connective tissues that the postulation of a specific race of cells is justifiable. We may conclude then that the typical osteoblast is the active form of a special race of potentially osteogenic cells normally resident in and around the skeleton, and that its morphological peculiarities in this active form are directly related to its bone-forming activities. We can probably go further and state that this race of cells was determined at an early stage of embryonic development at special sites which give rise to centers of ossification, and that the fibrous periosteum which later appears and surrounds the osseous territories serves to isolate these osteogenic cells from the general mesenchyme and connective tissues derived from it. There are few observations on normal growth, bone repair, bone grafting, and bone *in vitro*, which cannot be most readily explained on this view that osteogenic potency is normally restricted to the cells inside the fibrous periosteum delimiting the skeletal territories.

The question as to which of the cells normally resident within the confines of the fibrous periosteum belong to the corps of osteogenic cells, that is, which cells are capable of being converted into osteoblasts, has been discussed in Chapter XVI particularly from the point of view of fracture repair. Some insight into this problem can also be gained by the study of the cellular transformations which appear to be taking place in normal development, various pathological conditions, and in the reaction of bone to a variety of endocrine, nutritional, vascular, and mechanical disturbances as well as by investigation of the bone-forming

potentiaality of transplants and explants of periosteum, bone, and bone marrow.

In normal development of a membrane bone the mode of origin of, and some of the transformations undergone by, osteogenetic cells are readily observed. In the regions of condensed mesenchyme which adumbrate bone formation the osteogenetic cells are readily differentiable by their marked glycogen content and alkaline phosphatase activity (Figs. 15 and 16). The more centrally placed cells of these condensations then become larger and rounder, the Golgi apparatus hypertrophies, mitochondria increase in number, cytoplasmic basophilia becomes conspicuous, and finally the nucleus becomes eccentrically placed. Bone matrix becomes deposited around these cells which are now typical active osteoblasts. Those trapped in the matrix become osteocytes—others are carried into the interior marrow spaces as the bone expands, where they form a pseudo-epithelial layer applied to the surface of the matrix (Figs. 1 and 2). Some of the latter remain plump and active, but many become flattened, inconspicuous, squamous cells, the so-called resting osteoblasts (Fig. 10). At some sites osteoblasts disappear altogether from the surface of the matrix and are replaced by multinucleate osteoclasts. Meanwhile at the boundary of the bony territory a fibrous periosteum has been differentiated beneath which a zone of proliferating and differentiating cells continues to supply new osteoblasts to meet the needs of the expanding bone and to offset the losses incurred in the formation of osteocytes, for active osteoblasts themselves appear incapable of cell division.

In the development of a cartilage bone regions of condensed mesenchyme also appear, but instead of a direct formation of bone, cartilage is first differentiated and then a perichondrium with an outer fibrous layer and an inner layer of proliferating and differentiating chondroblasts makes its appearance. These erstwhile chondroblasts are eventually converted into osteogenetic cells; then osteoblasts are differentiated and bone is formed adjacent to the cartilage. The perichondrium has, in this manner, become converted into a typical periosteum.

In both membrane and cartilage bone development, the reticulum cells of the marrow spaces appear to be derived from cells identical with those which give rise to osteoblasts and osteoclasts.

During the growth period the transformations mentioned continue to take place in much the same way. Eventually, however, growth ceases, the periosteum loses its layer of proliferating, differentiating, osteogenetic cells and becomes a simple fibrous membrane closely applied to the bone, while the active osteoblasts formerly present on the external and internal surfaces of the bone matrix are replaced by a uniform layer of flattened

squamous cells which are not conspicuously different in appearance from either the fibroblasts of the fibrous periosteum, or the reticulum cells of the marrow spaces.

The study of the cellular events in normal development therefore leads to the view that the undifferentiated cells of the fetal periosteum and perichondrium, the large active osteoblasts, the flattened resting osteoblasts, the osteocytes, and the marrow reticulum cells all represent stages in the life history of one and the same race of specialized mesenchymal cells. The osteoclasts may also belong to the series, for it is difficult to see where they could have come from other than from the fusion of a number of osteoblasts or reticulum cells (cf. Fig. 14 and also Chapter VIII). But while the study of normal development makes it clear that active osteoblasts are constantly recruited from the zone of undifferentiated pre-osteoblasts in the deeper layer of the periosteum during the growing period, it does not indicate whether or not the flattened resting osteoblasts and reticulum cells retain their osteogenetic potency and are capable of conversion or reconversion into active osteoblasts. Nor does it clearly demonstrate the origin of the osteoclasts, nor their fate, nor whether osteocytes can be liberated from their imprisoning matrix and once more assume an active osteoblastic role.

Studies on bone repair indicate, however, that the flattened "resting" osteoblasts retain their osteogenetic capacity and indeed are able also to become chondrogenic (Fig. 13, and see the work of Fell, 1933, who showed that cultures of periosteum *in vitro* give rise to both bone and cartilage). This is discussed by Ham and Harris in a later chapter. See also Ham (1930).

It might be supposed that clear evidence of the osteogenetic potency or otherwise of the various cell types in and around bone should be obtained from the study of bone grafts and free transplants of bony tissue to non-skeletal sites, *in vivo*. However, after a hundred years of bone grafting in clinical practice and experimentally in animals it is still not decided whether the new bone and cartilage which develop around grafts and free transplants is formed by the cells of the graft or by the host cells surrounding the graft. For one thing, it is not even agreed whether the grafted cells can survive or not (but see the definite statements on this point by Ham and Harris in the present volume). The majority of observers believe that some at least do survive, provided they are superficially placed and reasonably near blood vessels. The fact that dead homogenous bone is almost as good a grafting material as living autogenous bone adds to the confusion. It might be thought that the behavior of free transplants would be decisive, but the possibility of induction cannot be ruled out and there are many reports of bone having

been formed around free transplants of dead bone—or at least bone thought to be dead, for the viability of the bone cells is very great and osteogenetic cells may well have survived in some of the cases thought to prove that dead bone is osteogenetic. Haas (1923), for instance, showed that osteoblasts could survive removal from the body for as long as 19 hours without special precautions.

In an unpublished experiment the present author transplanted pieces of adult rat parietal bone with intact pericranium to the subcutaneous tissues of another rat. Within a few days osteoblasts and new bone had appeared in the deeper layer of the periosteum of the grafted bone. There was no new bone outside the periosteum. The appearances in fact were identical with those seen in normal parietal growth and in the early stages of repair of a fractured parietal. I can only attribute the result to the re-activation of the potential bone-forming powers of the deeper lying cells of the periosteum of the transplant. Induction seems to be ruled out in this case.

Nevertheless, induction can and does occur and is most clearly demonstrable around pieces of growth cartilage transplanted under the capsule of the kidney (Lacroix, 1946, 1947). The bone which forms around the cartilage is almost certainly derived from the host tissue. Urist and McLean (1952) have also demonstrated induction in transplants of a variety of skeletal tissues to the anterior chamber of the eye. Some tissues produced new bone in this situation from their own osteogenetic cells, some by induction of the host cells, and some, notably fracture callus, by both methods simultaneously.

Free transplants of periosteum have given very different results in different hands—some find no bone formation, others find it regularly. The difference is almost certainly due to whether or not osteogenetic cells in the deeper layer of the periosteum are removed when the periosteum is stripped from the bone, and whether or not they are damaged in the process. The periosteum of young bone, because of its thick layer of osteogenetic cells, is more likely therefore to give rise to bone than adult periosteum, where the single layer of osteogenetic cells is easily destroyed or left adhering to the bone.

Free transplants of bone marrow on the other hand regularly give rise to new bone, the reticulum cells readily undergoing transformation into osteoblasts (Pfeiffer, 1948).

Cortical bone from which both periosteum and marrow have been removed will also form new bone when transplanted to soft tissues. Here the source of the new bone appears to be the cells lining the Haversian canals, but the possibility of induction, though unlikely, is difficult to rule out.

Several references have already been made to the form and function of osteoblasts in tissue culture. The chief points to be made here are:

(i) That they usually revert or dedifferentiate to the generalized fibroblastic or "mechanocyte" form characteristic of all connective tissue cells *in vitro*, that is to say, spindle-shaped cells with branching processes (Willmer, 1954).

(ii) The dedifferentiated osteoblast is nevertheless distinguishable from the fibroblast by a somewhat plumper form, more irregular outline and by the possession of cytoplasmic as well as nuclear phosphatase activity (Rodová, 1948). It is also distinguishable from the fibroblast by its vigorous growth in media containing relatively small amounts of embryo extract (Willmer, 1954).

(iii) Redifferentiation to the active osteoblastic form with resumption of bone matrix deposition is possible even after long periods of growth and repeated subculturings in the dedifferentiated condition (Fischer, 1930).

(iv) Osteoblasts may be cultivated from the fetal periosteum, and from bone itself freed of all periosteal and marrow tissue (Fell, 1931, 1933). Certain regions of the mesenchyme where centers of ossification are expected to develop will also give rise to osteoblasts *in vitro* (Fell and Robison, 1930); but no other areas of the mesenchyme, or connective tissue, will do so.

(v) Osteoblastic cultures are as likely to form cartilage as bone (Fell, 1933).

(vi) Osteoclasts *in vitro* behave as independent, freely amoeboid cells, and do not appear to be formed from osteoblasts or to be transformed into them (Hancox, 1946, 1949).

The *in vitro* evidence thus affords good grounds for belief in the existence of a special race of osteogenetic (and chondrogenetic) cells which can exist in either an active differentiated or an inactive dedifferentiated form. In the latter condition the osteogenetic cells may be mistaken for ordinary connective tissue fibroblasts owing to their general similarity of form.

Cellular transformations, new bone formation, and bone destruction, are readily invoked in bone by pathological stimuli of many kinds. Almost any interference with bone, if of sufficient intensity, will result in the replacement of the carpet of flattened resting osteoblasts lying against the surface of the bone matrix by a layer of active osteoblasts or osteoclasts. Such a transformation was clearly shown in experiments on the adult rat parietal bone (Pritchard, 1946) in which inflammatory changes in the periosteum were provoked by placing various substances, including turpentine, scalding water, pieces of spleen, placenta, testis, etc.

under the scalp. Within a day or two the flattened cells in the depths of the periosteum had swollen and begun to divide. Soon the periosteum had the two-layered appearance typical of a growing bone and osteoblasts had differentiated. Spicules of fibrous membrane bone then appeared among the osteoblasts, then a network of primary cancellous bone, and finally lamellar bone was deposited on the surfaces of the cancellous bone, giving rise to simple Haversian systems. During all this, the fibrous outer layer of the periosteum remained relatively inactive. Where the inflammatory response was very great, however, the opposite picture was seen: the cells of the deepest layer of the periosteum were converted into osteoclasts and extensive erosion of the old bone took place.

Endocrine secretions have a profound effect on the cells of bone. Parathyroid hormone appears to result in the production of numerous osteoclasts and the reversion of normal osteoblasts to the spindle-shaped resting form (Heller *et al.*, 1950. See also Chapter XXII by McLean). Estrogenic hormone on the other hand stimulates osteoblastic activity. Bloom, Bloom, and McLean (1941) from the study of such cell transformations in the pigeon concluded that the marrow reticulum cells, the osteoblasts, osteoclasts, and osteocytes are reversible modulations of one and the same cell type. A similar conclusion was reached by Heller *et al.* (1950).

The results of Bloom *et al.* (1941) on the pigeon were supported by Clavert (1948) and similar results were obtained in the mouse by Gardner and Clouet (1944) and Urist, Budy, and McLean (1950).

It is of interest too that vitamin C deficiency results in regression of osteoblasts to fibroblastic forms with a cessation of their bone building role. Such fibroblast-like cells show no cytoplasmic basophilia, glycogen, alkaline phosphatase, or cytochrome oxidase. The administration of vitamin C, however, causes a reversion to the typical osteoblastic form, with a return of normal histochemical characteristics as well as normal function. Vitamin A also appears to affect osteoblastic activity, deficiency leading to the differentiation of great numbers of active osteoblasts while excess does just the opposite and osteoclasts dominate the scene (Mellanby, 1946; Irving, 1949; Strauss, 1934; Wolbach, 1947; Barnicot, 1950).

VII. Conclusion

Many other observations could be cited in support of the conclusion that the osteoblasts, osteoclasts, marrow reticulum cells and spindle-cells, osteocytes, and chondroblasts form a labile, mutually convertible, series of cells; but enough has been said to give some idea of the

catholicity of the evidence on which this conclusion is based. Bone is a very plastic tissue, responsive to a great variety of stimuli in two basic ways—by new formation with osteoblastic activity, and by destruction with osteoclastic activity. If we agree, as I think we must, that cellular activity is essential for these changes in the matrix to take place, then we are led to the conclusion that osteoblast-osteoclast-reticulum cell transformations lie at the heart of every problem of bone physiology or pathology, for this cell system must act as the final common path through which the great variety of stimuli affecting bone structure must pass. We envisage formative stimuli disturbing the cellular equilibrium in the direction of osteoblastic differentiation, destructive stimuli leading to excess of osteoclastic activity. Genetic, hormonal, nutritional, mechanical, vascular, biochemical, and pathological stimuli to bone formation and destruction—all in the final analysis must act by disturbing the cellular balance. But how they do this, and even more important, how they manage to affect some cells in some places in one way, and other cells in another place in another way, while throughout all the changes the bones maintain their general form and functional integrity, are matters about which we are almost entirely ignorant.

One plausible suggestion is that some parts of a bone are inherently more labile than others and are therefore the sites of most of the transformations, while other, more stable, parts maintain the general form on a long-term basis, acting as a sort of "skeleton within the skeleton." Another suggestion, which has much to commend it, is that stability and lability are not inherent properties, but simply depend on proximity to blood vessels. Studies using radioactive tracers, for example, indicate that ionic exchanges between bone matrix and tissue fluid take place primarily around the blood vessels. However, the precise manner in which the circulation influences cellular activity, and hence bone formation and destruction, remains to be worked out, despite the confident assertion of Leriche and Policard (1928) that increased vascularity promotes bone resorption and decreased vascularity, bone formation.

From the evidence presented in this chapter the following conclusions may be drawn with some confidence:

(i) The typical osteoblast, despite its unique combination of cytological and cytochemical features, is but a temporary modification, or modulation, of a series of mutually transformable connective tissue cells resident in and around a bone within the confines of the fibrous periosteum, and including the osteocyte, osteoclast, marrow reticulum cell, flattened "resting" osteoblast, spindle-cell and chondroblast as alternative forms.

(ii) The cell types mentioned belong to a race of connective tissue

cells differentiable from other connective tissue cells resident outside the skeletal territories by the ease with which they can undergo transformation into osteoblasts. In other words they constitute a specific race of potentially osteogenetic cells.

(iii) The situation of osteoblasts relative to sites of active bone formation, and their highly specialized morphology and cyto-chemistry, indicate that they have an essential role to play in bone formation, including the production of collagen and alkaline phosphatase, and possibly of organic phosphate substrates and mucopolysaccharides as well.

(iv) The cellular transformations mentioned, especially the osteoblast-reticulum cell-osteoclast transformations, mediate the reactions of bone to stimuli of all kinds, as they are the proximate cause of the changing patterns of bone formation and destruction into which all bony reactions can be analyzed.

PLATE I

(Magnifications are only approximate)

FIG. 1. Osteoblasts lining bony trabecula of embryonic sheep mandible. Mallory. $\times 725$.

FIG. 2. Osteoblasts carpeting surface of woven, fine cancellous bone. Rat embryo. H. & E. $\times 135$.

FIG. 3. Osteoblasts lining surface of new bone. Note juxta-nuclear vacuoles. Rat fracture callus. Methylene blue. $\times 560$.

FIG. 4. Osteoblasts showing anastomosing processes. Sheep embryo. Mallory. $\times 510$.

FIG. 5. Differentiating osteoblasts from mandibular ossification center. Rat. H. & E. $\times 355$.

FIG. 6. Pre-osteoblasts (above) and highly-polarized osteoblasts (below). Mandibular center of ossification. Rat. H. & E. $\times 1275$.

PLATE II

(Magnifications are only approximate)

FIG. 7. Osteoblast showing eccentric nucleus, large nucleoli, juxta-nuclear vacuole and basophilic cytoplasm. Rat embryo. Toluidine blue. $\times 2125$.

FIG. 8. Osteoblasts showing mitochondria. Rat embryo. Author's silver method. $\times 1700$.

FIG. 9. Osteoblast showing eccentric nucleus, large nucleoli and large reticular Golgi apparatus. Rat embryo. Aoyama. $\times 2125$.

FIG. 10. Osteoblasts on trabecula of embryonic bone. Note flattened resting osteoblasts above and typical pyriform active osteoblasts below. Sheep. Mallory. $\times 215$.

FIG. 11. Osteoblasts showing filamentous and rod-shaped mitochondria. Rat embryo. Author's silver method. $\times 680$.

FIG. 12. Osteoblasts with conspicuous Golgi apparatus. Rat embryo. Aoyama. $\times 220$.

FIG. 13. Osteogenic cells becoming chondroblasts above and osteoblasts below. Lizard fracture callus. Weigert & Van Gieson. $\times 425$.

PLATE III

(Magnifications are only approximate)

FIG. 14. Osteoblasts apparently fusing together to become osteoclasts. Rat embryo. Weigert & Van Gieson. $\times 935$.

FIG. 15. Phosphatase activity in mesenchyme of maxillary ossification center. Rat. Gomori. $\times 135$.

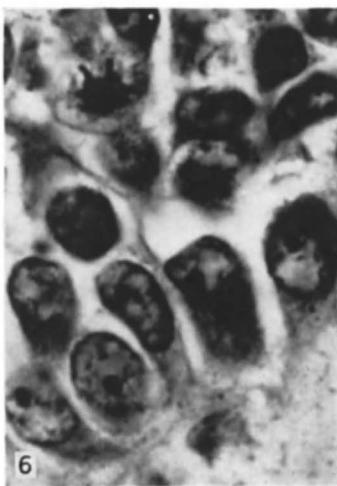
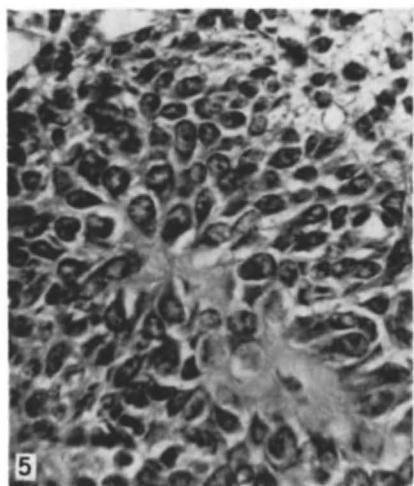
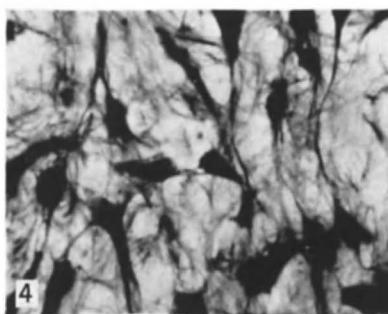
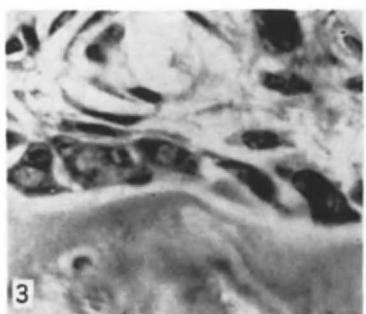
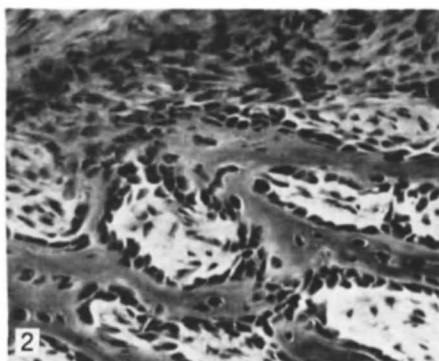
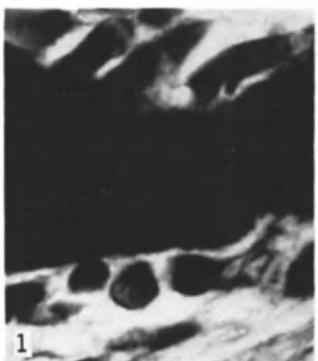
FIG. 16. Glycogen in mesenchyme of mandibular center of ossification and Meckel's cartilage. Rat. Periodic-acid-Schiff method. $\times 135$.

FIG. 17. Glycogen in part of embryonic mandible and Meckel's cartilage. Note absence of glycogen from centrally-placed osteoblasts compared with abundance in outer pre-osteoblasts. Rat. Periodic-acid-Schiff method. $\times 135$.

FIG. 18. Phosphatase activity in embryonic mandible. Note relatively slight activity in centrally placed osteoblasts compared with great activity in outer pre-osteoblasts. Rat. Gomori. $\times 135$.

FIG. 19. Phosphatase activity in osteogenetic layer of periosteum. Note decrease in activity as cells mature below. Rat parietal. Gomori. $\times 465$.

PLATE I

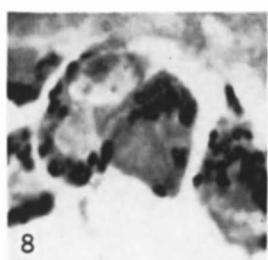


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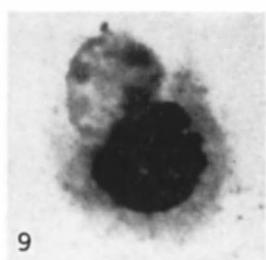
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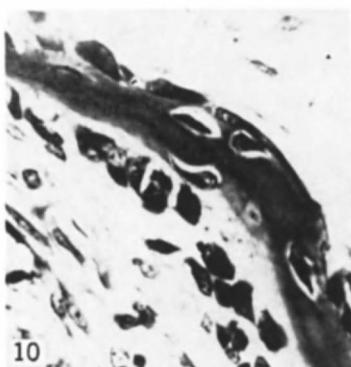
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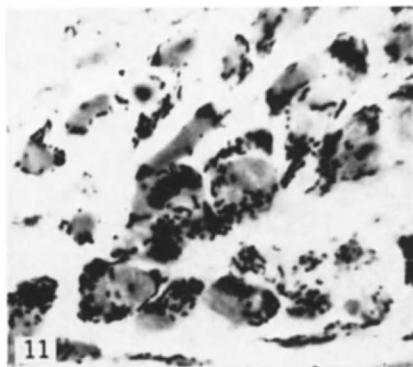
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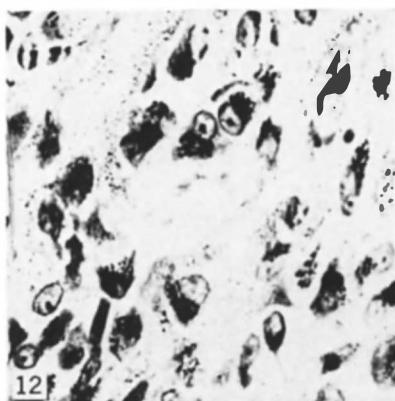
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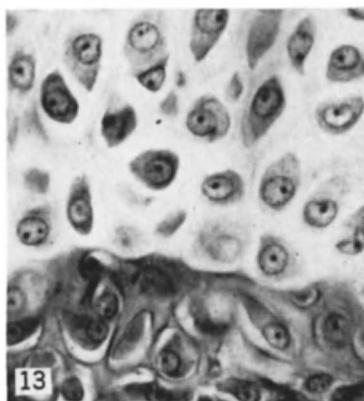
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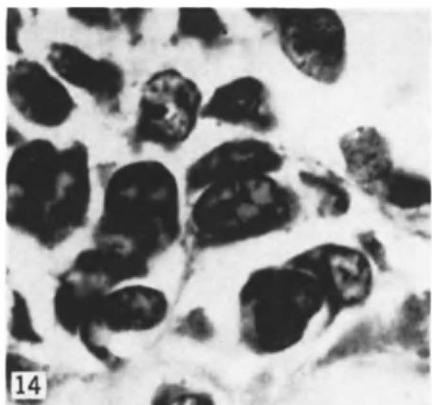
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For explanation, see p. 207.

PLATE III



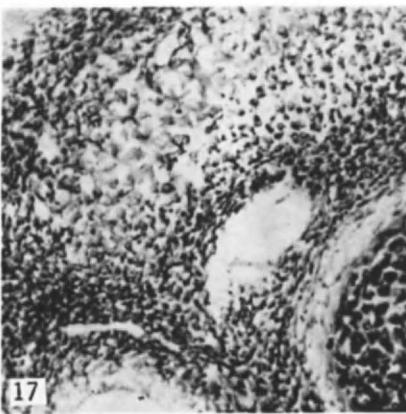
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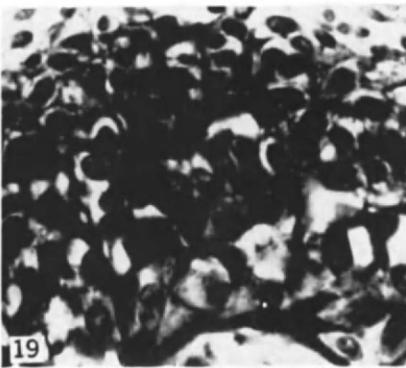
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For explanation, see p. 207.

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CHAPTER VIII

THE OSTEOCLAST

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I. Introduction

The very large size of the osteoclast, its multinuclearity, and the striated border in contact with absorbing bone form a striking histological picture which suggests that the cells are highly active and expend energy on a large scale. However, even up to the present time comparatively little is known about their source, function, lifespan or ultimate fate, although it is now over a century since their histological features were first described and illustrated.

Robin (1849, 1864) was able to distinguish what we now call osteoclasts from megakaryocytes and to describe most of the essential histo-

logical attributes of the cells. He was not able to make any suggestions as to their functions, but Rollet (1870) observed them in sites of endochondral ossification. Kolliker (1873) first suggested that the cells are the universal agents of bone absorption and he christened them with the descriptive title of "ostoklast" but in the literature the French term "osteoclast" seems to have been preferred.

The subject has been reviewed several times in recent years, (Arey, 1919; Weidenreich, 1930; Jaffe, 1930, 1933; Giovanni, 1937; Hancox, 1949a) and there seems little point in considering the historical aspects of the subject any further here; there are excellent accounts of the earlier work to be found in these reviews. In any case it would be chiefly a narrative of the various ways in which a similar histological picture has been interpreted by different microscopists, for, until quite recently, study of the osteoclast has been mainly subjective and descriptive.

Inquiry into the biology of the osteoclast has passed through two main epochs and has now, perhaps, entered a third. In the beginning, the microscopical techniques that were available would nowadays be judged as crude in the extreme. Freehand sections, uncorrected lenses, and the coloration rather than the staining of preparations naturally restricted microscopical investigation. Nevertheless, the fundamental facts were established; osteoclasts were observed to be constantly associated with bone absorption, whether it be physiological (as in the embryo), experimental (as in the case of ivory pegs driven into bone) or pathological (first described by Rindfleisch in 1873).

The latter part of the nineteenth century saw the introduction of such technical advances as microtome sections, biological staining more or less as we know it today, and improved optical systems. This period, out of which we are now beginning to move, was associated with what may be called the cytology of the osteoclast. Microscopists studied the appearances of their colored fixed tissue sections and attempted to decide such questions as the origin, fate, and function of the cells. The validity of conclusions based upon this method of investigation is liable to be affected first, of course, by morphological artefacts, which are well known, and second by what may be called temporal artefacts, the significance of which does not seem to have been fully appreciated. The histological preparations of the older microscopists can be likened to a disconnected series of still pictures; these they endeavored to arrange in order so as to build up a moving picture of the stream of events which take place in the living animal. As will be seen later the snapshots were not always placed in the right order.

The phase upon which we are now entering seems to be full of opportunity. We can produce osteoclastic absorption of bone experi-

mentally in the intact animal by the injection of parathyroid extract and by altering the dosage the intensity of absorption can be varied at will. There are the modern methods applicable to sections such as freeze-drying, histochemical technique and the phase contrast and electron microscopes. A beginning has been made in the study of osteoclasts in the living state and it is very much to be hoped that this line of inquiry can be extended to answer the still unsolved questions of how the cells arise, what they do when in contact with bone, and what happens to them afterwards.

II. Microscopical Appearances

1. FIXED TISSUE SECTIONS

The typical osteoclast is, of course, a large, multinucleated cell, characteristically found on or near bone in process of absorption. This may be physiological, as for instance in the normal modelling of bone, or it may be the result of some pathological cause. The bone around the tooth germs in the embryonic jaw provides an excellent example of physiological absorption. As the developing teeth expand, the subjacent bone is absorbed and osteoclasts are seen in large numbers. This was the site chosen by Arey (1919) and many of the present text figures are from preparations of this region. It is proposed first to describe the principal features of the appearance of the cells as seen in ordinary histological preparations, second to discuss such investigations as have been carried out with histochemical technique, and third to review the observations which have been made on the living cells.

An account will be given in this section of the main features of the cells as seen in ordinary microscopic preparations.

a. Size

Osteoclasts vary greatly in dimensions. At one end of the scale there are the enormous plasmodial masses encountered from time to time. Arey (1919) described and illustrated such a mass; its long axes measured $85 \times 105\mu$ and while its thickness is not stated it could scarcely have been less than 20μ and in all probability was very much more. Assuming 20μ , and allowing 10% loss of volume for shrinkage due to fixation and dehydration (Arey used celloidin sections) the volume of the mass works out at around $200,000\mu^3$. Large osteoclasts are therefore very large cells indeed, certainly the largest in the mammalian body. These very large osteoclasts have a hundred or more nuclei.

At the other end of the scale are individuals with 2 or 3 nuclei and long axes measuring perhaps $20 \times 10\mu$. However, it is much more difficult to measure the volume of osteoclasts than, for instance, of osteo-

blasts. First, on account of their large size, the factor of thickness is important. The cytoplasm of the osteoclast may extend through many serial sections of the thickness usually employed for high power observations. Second, the shape of the cells is very often far from regularly spherical or cuboidal. As will be described more fully below, the cells are often lobed and sometimes they form extremely thin sheets.

In tissue sections, the largest cells are generally seen at a little distance away from bone, while those in actual contact are of medium or small size. Arey (1919) believed that the very large plasmodial masses were left behind in areas previously the site of massive absorption.

b. *Shape*

To appreciate the assortment of shapes adopted by the osteoclast it is necessary either to study serial sections or to examine the living cells as will be described below. As seen in the usual microscopic section ($7.5\text{-}10\mu$ thick) there are two extremes of form between which all sorts of transition may be traced. There are, first, round or oval masses which generally possess 10–20 nuclei. These individuals often seem not to be in close contact with bone tissue but to lie at a little distance from it perhaps separated by a thin strand of connective tissue; the nuclei are generally round and vesicular (Fig. 1).^{*} Secondly, thin sheets occur. These are flattened against bone tissue (Figs. 2 and 3); their nuclei are also often flattened. The apparent length of such cells, as well as the number of nuclei, obviously depends upon whether section plane chances to pass through their longest axis or not.

When occupying absorption lacunae (Howship's lacunae) (Figs. 4 and 5), the cells usually have a roughly semi-circular outline which fits the border of the lacuna while the external surface is smooth. The cell mass is quite commonly divided into two or more lobes each with its own quota of nuclei; the lobes are often separated quite widely and are connected by a thin filament of cytoplasm. Each lobe may occupy its own lacuna (Fig. 6). It is interesting that Barnicot (1947), in his supravital studies on mouse osteoclasts, observed adjacent small individuals to be pear-shaped with the stalks pointing towards each other as if they had originally been joined together. In tissue culture, too, the osteoclast form lobes which move apart from each other (Fig. 23) and then flow together again. (Hancox, 1949b). Cells with three lobes are not uncommon (Figs. 7 and 13), and finally some individuals possess long filiform or short branching processes. This diversity of shape becomes more intelligible when the motility of the cell is borne in mind.

* Figs. 1–15 appear in Plate I, Figs. 16–25 in Plate II, Figs. 21–35 in Plate III.

c. Nuclei

The large masses contain upwards of 50–100 nuclei, or even more, while the smaller individuals possess 2 or 3. An average figure would be between 10 and 30.

The nuclei are rounded or oval in shape with one or two nucleoli; they are generally rather vesicular. There is, however, quite considerable variation in their appearance. Sometimes they are regular in outline; sometimes they appear crenated, shrunken, or box-like, and in a third category are individuals which shew frank pyknosis or karyolysis. Many authorities have maintained that these appearances represent serial changes which indicate different stages in the life span or physiological activity of the cells. For instance, in birds, according to Benoit and Clavert (1952), young osteoclasts possess crenated nuclei, whereas in osteoclasts in full activity they are rounded and vesicular. In their opinion the more condensed nuclei with irregular contours are associated with diminution of physiological activity. However, nuclei of rather irregular shape are often seen in osteoclasts whose appearance in other respects suggests that they are in their physiological prime. Conversely, rounded nuclei are almost characteristic of the large spherical plasmoidal masses at a distance from bone.

It may very well be that movement and temporary shifting of internal pressure in the cells, when physiologically active, may account for some of the variations in shape. There is no proof that the changes are irreversible and of course the shape of the nuclei of most motile cells alters from time to time. On the other hand, frankly, pyknotic nuclei are usually to be seen in cells which, on other grounds, such as irregularity of form and gross cytoplasmic vacuolization, should probably be considered degenerate and irreversibly damaged (Figs. 10 and 11).

d. Cytoplasm

The staining reaction is somewhat variable. According to some authorities it is basophil (Prenant, 1911; Askanazy, 1902); others have described it as oxyphil (Weidenreich, 1930; Jaffe, 1930). There seems to be some support for the view that it is basophil in the young cells and becomes oxyphil as the cell enters upon physiological activity (Arey, 1919; Benoit and Clavert, 1952). The cytoplasm, however, does not usually seem to possess any very marked affinity for either category of dye except when the cells appear obviously degenerate; under these circumstances it is undoubtedly strongly oxyphil. Various types of cytoplasmic granule have been described by many authors. In recent times Barnicot (1947) speaks of "numerous granules of regular size scattered throughout the cytoplasm." Arey's (1919) illustrations of osteoclasts show

the cells as coarsely stippled with granules and the same sort of appearance is to be seen in the drawings of Klein and Noble Smith (1880). Curiously enough, such granules are not always very obvious. Their function does not seem to be known although Barnicot (1947) considers that they may possibly be secretory.

Weidenreich (1930) demonstrated rounded cytoplasmic bodies by means of gram-Weigert staining. What seem to be analogous structures may be brought to light by silver impregnation techniques (Jaffe, 1930) and the use of the Schmorl picrothionine method (Figs. 8 and 12). The nature of these bodies is unknown; it seems that they are solid, and provide surfaces against which silver may be precipitated and insoluble dyes flocculated. They might possibly be the remains of mitochondria which take the form of short rods (Fell, 1925; Hyslop, 1952).

The cytoplasmic vacuoles which are present can be tentatively classified as those occurring in cells at their physiological prime and those seen in degenerating individuals. In active cells, if due attention is paid to matters of technique, a series of cytoplasmic vacuoles can be seen arranged in an orderly manner just beneath the striated border where the cells are in contact with bone. These, which suggest very strongly some sort of secretory mechanism, will be discussed again more fully when considering the striated border itself.

In the cells which appear degenerate, that is, possess pyknotic or karyolytic nuclei and an uneven contour, the cytoplasm is riddled with coarse vacuoles. These may be so numerous as to fill up the whole cell and reduce the cytoplasm to a mere intervacular tracery (Figs. 10 and 11). Barnicot (1947) has shown that when living osteoclasts are immersed in dilute solutions of neutral red they segregate the dye in the form of cytoplasmic droplets of varying size and intensity. It would be interesting to know whether all osteoclasts behave in this way or whether it is especially common among the older cells which already possess large vacuoles in their cytoplasm.

e. Anatomical Relationships with Bone

The osteoclast apposes itself against bone in two ways. First, its cytoplasm may be wrapped around the end of a bone spicule (Figs. 9 and 14) and second, it may be flattened against the bone surface (Figs. 2 and 3) or occupy erosion pits (Figs. 4, 5, and 6). Such lacunae or punched-out cavities were originally described by Howship (1817). Erosion pits lacking osteoclasts are quite often seen. This, as we shall see below, has been interpreted by many observers as meaning that pits can develop without osteoclasts but this view is almost certainly erroneous.

f. Inclusions

The majority opinion is that osteoclasts do not contain phagocytosed bone matrix. Jordan (1920), however, thinks otherwise and Jaffe (1930) has an illustration which is said to show phagocytosed matrix within the osteoclast. This, however, is probably a section plane illusion, the bone being the end of a spicule surrounded by osteoclast cytoplasm.

In embryonic tissues, at least, cells resembling osteoblasts are quite commonly seen within osteoclasts, where they are generally surrounded by a clear space (Fig. 15). It may be that this appearance of phagocytosis is also a section plane illusion. The case of the osteocytes, however, is slightly different. The histological picture suggests that as the osteoclast moves nearer and nearer to an osteocyte, a protrusion of the former's cytoplasm flows along a canalculus into the osteocyte lacuna (Fig. 16). This pseudopod then seems to fill up the lacuna (Fig. 2), the osteocyte is drawn into the osteoclast body but is not apparently assimilated, being contained in a vacuole in the cytoplasm. After a period it begins to degenerate, and finally all that can be seen is an empty sphere bordered by a pellicle of darkly staining material (Fig. 17). Arey (1919) describes other inclusions such as erythrocytes and cell debris.

g. Relations with Blood Vessels

The absorption of bone is often accompanied by an intense hyperemia. As we shall see later, there are those who believe that the hyperemia is the cause of the absorption; terms such as vascular resorption and superficial osteolytic resorption are employed by them in this connection. A typical example of this sort of mechanism is to be found in fracture callus. Here, the anastomosing trabeculae of woven bone enclose large, thin-walled venous sinuses, whose endothelial lining is very close to the edge of the bone and may even touch it. Osteoclasts, where they are to be seen, fit in between bone and the endothelium which may actually invest them. Under these circumstances the osteoclasts are generally arranged in the form of rather thin sheets which surround perhaps half of the circumference of the vessel and of course are much flattened against the bone. In poorly fixed material, they may be difficult or even impossible to find (Fig. 18).

There is abundant evidence that, in the intact animal (Maximov, 1910; Danchakoff, 1909; Arey, 1919) as in tissue culture (Hancox, 1946; Hyslop, 1952) osteoclasts are freely mobile. Sometimes the histological picture suggests that osteoclasts move towards, and possibly out of, blood vessels (Fig. 19). Much more rarely they can be identified actually within the lumina of vessels (Fig. 20) as illustrated by Arey (1919) and

Jaffe (1930). It is not often that one chances to encounter an osteoclast in this position for obviously, in life, the flow of the bloodstream would very soon hurry the cell away from the site. It is interesting to speculate upon the fate of such cells. They may be held in the spleen or lung, for instance.

Irrespective of events in fracture callus with its profusion of thin walled sinus-like vessels, in the embryo the relationship between osteoclasts and blood vessels is rather less clear. Sometimes the cells are seen to be in a highly vascular environment, their cytoplasm again being virtually in contact with vascular endothelium. At other times vessels cannot be seen in their vicinity. This seems to indicate that the relationship of endothelium to osteoclast is of secondary importance.

h. The Striated Border

This is perhaps the most outstanding microscopical feature of the osteoclast. It is found, under conditions to be described below, as a striated or fibrillated zone between the cell and the subjacent bone. Clearly, this zone is the most important structural feature in bone absorption and will therefore be discussed in detail.

Since there has been much disagreement about its structure and function, it seems best to begin by emphasizing that the striated border is not an easy subject for study, nor, unfortunately, for photography. On the one hand, the structures involved are not so very much above the limits of ordinary microscopic visibility, so that the possibility of being misled by technical artefacts looms very large. On the other, the osteoclast is a particularly difficult cell to preserve in a life-like manner for it is surrounded by a barrier of bone, one of the most impermeable of all tissues. The penetration of fixatives must therefore be hindered and their action cannot be expected to be almost instantaneous as is required for cytological purposes. Similarly, prolonged treatment with dehydrating agents is unavoidable, and, if the tissue is to be embedded in paraffin, lengthy periods in clearing agents and hot molten wax are needed. Again, for most purposes the tissue must be decalcified, another possible cause of distortion. It is true that sections can be cut "undecalcified" by celloidin embedding (Bloom and Bloom, 1940; McLean and Bloom, 1940) but for most purposes, and especially where thin sections (less than 7.5μ) are required, decalcification is virtually unavoidable. Even if the osteoclasts are perfectly preserved in the embedded, decalcified tissue, there is a strong probability that they will finally be dislodged mechanically from the bone by the microtome knife when sections are cut; this can be verified by glancing at almost any illustrations of the cells in the literature. Many of the shortcomings of the older methods

can be avoided by the use of polyethylene glycols (water-soluble "waxes") for embedding; most of the text illustrations are from material embedded in Nonex (Miles and Linder, 1953). With this substance, alcohols, benzene, etc. are unnecessary and the tissue is not subjected to a temperature higher than 39° C. Shrinkage and distortion are minimal and the cells seem to retain their physiological attachment to bone in the final sections.

The cells and their borders may be made visible microscopically in sections by two main techniques. There is, of course, first, the traditional staining. Many of the standard techniques give excellent results, particularly Heidenhein's iron hematoxylin. Secondly, there is the more recent method of studying unstained sections with the phase-contrast microscope. Ham (1952), for instance, provides an illustration of an osteoclast photographed in the phase microscope. One of the advantages of this method is that the bone fibrillae are more easily seen and that by mounting sections in media of differing refractive indices the contrasts can be varied. One inherent disadvantage of the method is, of course, that the proper phase conditions can be obtained only in rather thin sections which are not usually the most instructive.

The striated border is seen only in a proportion of the cells; and in these it is confined quite sharply to the zone where contact between cell and bone is closest. Some cells although in contact with bone lack border striation even when followed through serial sections; this might be a reflection of functional variation. Cells which are near but not in contact with bone do not possess striated borders.

The striations are directed roughly perpendicular to the surface of the bone. Thus, in cells occupying absorption lacunae, the striations seem in general to radiate outwards from the cell surface (Figs. 21, 26); in cells flattened against bone (Figs. 27, 29), the striations form a more or less regular row; when the cells envelop a short spicule, the border often seems to radiate inwards towards the bone (Fig. 14). The appearance of the striae is variable. Sometimes, they are long and fine (Fig. 21), sometimes short and relatively blunt (Fig. 35). This point will be amplified below.

Since the earliest days there has been lively debate as to the origin of the brush border. Kolliker (1873) believed that it belongs to the cell. Pommer (1883), however, considered it as part of the bone; he described brush-like borders on the walls of some lacunae produced by bone fibrils or fibril bundles which remained after dissolution of the ground substance and which could be traced to the fibrils of the surrounding bone. In addition, it seems, he believed that the cytoplasm of the cell also formed fringed borders which matted with the lacunar wall border

of bone fibrillae. Jaffe (1930) also speaks of border "fibrils, the free edges of which extend from the cell and are in contact with the surrounding tissues." Ham (1952) has recently restated the view that the brush border consists of exposed bone fibrillae, basing his conclusions upon observations of four kinds. First, that the exposed bone fibrillae and the striated border can be seen to be the same thing; second, that striated borders are only to be seen "where the collagenic fibres of the bone are disposed roughly at right angles to its surface" i.e., approach the surface; thirdly, that at the edges of bone trabeculae the fibrils can sometimes be seen protruding a little to form a pattern resembling a striated border, even though no osteoclast is present. Lastly, he points out that when osteoclasts shrink away from bone in tissue sections the border often adheres to the bone, which he takes to indicate that the border belongs to bone rather than osteoclast.

The evidence however seems more in favor of Pommer's original contention that there are two components to the border. On the one hand, some of the striations are undoubtedly exposed bone fibrillae but there are others which are cytoplasmic. These will next be discussed in turn.

It is often quite easy to confirm that bone fibrillae and striated border are continuous, especially in tissue sections embedded in Nonex, mounted in bergamot oil and examined with the phase contrast microscope. This oil has a refractive index lower than Canada balsam and the bone fibrillae are relatively easy to distinguish; typical results are shown in Figs. 26, 28, 30, 31, 32 and 33.

It is also possible to extend Ham's (1952) observations somewhat. He remarks that striated borders are only to be seen where bone fibrillae run at right angles to the surface on which the osteoclast is located; but this is by no means always the case. Quite often, distinct striated borders are visible where osteoclasts associate with bone tissue whose fibrillae run parallel to the surface. Here, the bone fibrillae often form quite a sharp angle at the edge of the erosion zone, bending radially inwards towards the cytoplasm of the osteoclast. This is extremely difficult to photograph.

The overall appearance of the border is governed by two variables. On the one hand the striae may be exceedingly fine, at the threshold of visibility (Fig. 21), or coarser and block-like in appearance (Fig. 35). This depends upon whether the individual bone fibrillae or their fibrillar bundles respectively are exposed. On the other hand, the striae may be short or long, or even apparently granular, depending upon the chances of section plane. A further factor is the variable distribution of secretion amidst the striae; this will be discussed again below.

In the second form of striation the striae are shorter, thicker, and

fewer. They are arranged quite without relation to the organization of the subjacent fibrillae to which they may often be seen to be at right-angles. Even in different parts of the same cell both varieties of striae can occasionally be distinguished. This appearance is difficult to photograph in a convincing way; for one thing, it is easiest to see by focusing up and down in relatively thick sections ($7.5-10\mu$) which, of course, do not photograph clearly with high magnifications. Figures 32, 33, and 34 show the sort of appearance encountered, but do not convey fully what can be seen in originals. These striae, whose pattern is independent of the bone fibrillae, may well be minute pseudopod-like protrusions of cytoplasm and it seems at least possible that they extend into the bone between, and at right angles to, the long axes of the fibrillae. In the living cell they may be continuously in motion. In time-lapse motion pictures of osteoclasts *in vitro*, rather similar surface protrusions form and withdraw.

Ham (1952) has drawn attention to the pattern of fibrillae often seen protruding from the edge of bone, resembling the fine hair-like striated border, in the absence of osteoclasts. This of course adds further strength to the view that the border consists of bone fibrillae. It does not indicate that absorption occurs without osteoclasts, for an exactly similar pattern of fiber organization is to be seen where bone is in process of deposition. The fibrillae run between osteoblasts. If osteogenesis is temporarily (or permanently) at a standstill, the osteoblasts disappear but the fibrillae remain.

Description of the zone of striation would be incomplete without some mention of the vacuoles found in this area. In well-preserved cells, the vacuoles can be seen in a narrow zone just beneath the cytoplasmic boundary. They vary in size; they appear empty by phase illumination (Figs. 26, 28, 30, 31), and do not stain by ordinary methods, as if their contents had been dissolved out. They can also be seen, lying between the striations, free of the cell border (Figs. 25, 28, 30). They suggest very strongly some form of cellular secretion. It is interesting that they are not generally seen in sections from material embedded in paraffin; they are not shown, for instance, in the illustration given by Ham (1952), Benoit and Clavert (1952), Jaffe (1930), etc. They are preserved after embedding in celloidin (Fig. 25), although Arey (1919), who used this technique, does not seem to have encountered them in his preparations.

The foregoing observations make it possible to draw in a rough sketch of the striated border. There would be a zone between cell cytoplasm and unaltered bone consisting of bone fibrillae, either in bundles or as individuals, more or less denuded of ground substance and bone salt. If the fibrillae were not denuded they would not be seen so clearly. Into this zone, the osteoclast pours forth a secretion of some

sort. Meantime, if the cell surface happens to lie wholly or partly over bone where the fibrillae are parallel to the surface, the cell sends forth exploratory processes which push between the fiber bundles and, it may be, loosen and digest away the ground substance so that the fibrillae fall apart. However, speculation must not be carried any further at this stage; the mode of action of the cell will be discussed more fully below.

2. HISTOCHEMICAL OBSERVATIONS

Histochemical work in this field is sparse, and has generally been concerned with the wider issue of bone absorption rather than with the osteoclast itself.

So far as enzymes are concerned, the distribution of alkaline phosphatase has been studied by several investigators. Their results have been conflicting and rather inconclusive. For instance, Greep, Fisher, and Morse (1948) describe a faint positive reaction for the enzyme; Lorch (1947) obtained a positive reaction in the nuclear membranes and nucleoli. Barnicot (1948c) did not obtain any indications of enzyme in his mouse preparations. In any case, in the light of modern knowledge of diffusion artefacts which occur with prolonged incubation of sections in the substrate bath used for demonstration of sites of enzyme activity (Martin and Jacoby, 1949; Leduc and Dempsey, 1951; Lison, 1953), the validity of the earlier experiments seems doubtful. There is always the possibility of migration of enzyme and its reaction products from bone, itself rich in the enzyme, to osteoclast, resulting in a false positive reaction.

Lack of a high degree of chemical specificity does not detract significantly from the work of McLean and Bloom (1940) upon the localization of bone salt in tissue sections. These investigators were able to prepare quite thin sections of bone in which the bone salt was left intact; the latter was brought to light by "staining" with a modified von Kossa silver nitrate technique. While it is true that other substances besides calcium phosphate or carbonate are capable of reacting with silver (Cameron, 1930; Lison, 1953), they are not present in bone. In both normal bones and in bones absorbing under the influence of injections of parathyroid extract (McLean and Bloom, 1940), the osteoclasts did not contain demonstrable bone salt. Also it seemed that the walls of the absorption lacunae were not less mineralized where actually in contact with the osteoclasts than at a distance. The striated border zone, however, is not mentioned in these reports. It has to be borne in mind of course that the von Kossa technique brings to light only particulate matter; any local increase in diffusible calcium phosphate or its constituent ions would not be detectable.

Heller-Steinberg (1951) working in Bloom's laboratory, has recently carried out some investigations on frozen-dried bone by means of the Hotchkiss periodic acid-Schiff procedure. She described small spherical granules, $0.3\text{--}0.6\mu$ in diameter, in the cytoplasm of osteoclasts. These stained intensely red, and were thought to consist of a polysaccharide-protein complex rather than of lipid, lipo protein or polysaccharide. However, since similar granules were to be seen in osteoblasts and osteocytes, they cannot be considered specific to the osteoclast. Heller-Steinberg (1951) does not mention the region of the striated border. It would have been interesting to know whether the vacuoles occurring in the osteoclast cytoplasm at the edge of the border contain material oxidizable by periodic acid to produce aldehydes or dialdehydes.

Heller-Steinberg (1951) also applied a modified silver nitrate (von Kossa) technique to her material and was able to distinguish varying intensities of staining of the bone salts. She interpreted this as indicating varying reactivity of the salts.

Cretin (1951) has also recently described some histochemical work upon absorbing bone; unfortunately his article is not illustrated. His results fall, roughly, under two headings; information concerning firstly the chemical mechanism of bone absorption which will be discussed in a later section, and secondly, concerning the pH around osteoclasts. This latter work entailed the study by means of polychrome indicator solutions, of freehand sections of fresh (unfixed) bone absorbing in the region of a fracture. A range of pH, in the sections, of from 6.4 to 9.0 was encountered. If osteoclasts were present, the pH was always below 7.0; if they were not, it was always very alkaline. Subjective difficulties were found to be such that caution was needed in interpreting the results unless the observed differences were large. Cretin concludes that the osteoclast has a certain climate, more accurately to be called a "regional chemistry" covering $20\text{--}30\mu$, the adjoining territory being different.

We need much more information on the histochemistry of the region of the striated border. Does the lowering of pH around osteoclasts, described by Cretin (1951), originate from this region? What is the chemical nature of the secretion formed in the vacuoles at the cell edge? Can free Ca^+ or PO_4^- be demonstrated in the border region? Are there changes in the state of polymerization of the ground substance binding the fibrillae, and also the physical carrier of the bone salt?

3. THE LIVING CELL

Apart from Cretin's (1951) work on osteoclasts in freehand sections of fresh bone, mentioned above, there are three main techniques by means of which the cells have been studied in the living state. These are

first, observations on cells in transparent rabbit-ear chambers; second, supravital investigations carried out upon osteoclasts adhering to the skull bones removed from mice, and third, work carried out upon the cells *in vitro*.

a. Ear Chambers

Kirby-Smith (1933) encountered an osteoclast in an ear chamber preparation in which a bone fragment had suffered an accidental fracture 5 days previously. The cell is described as a granular protoplasmic mass, in contact with the bone; it was observed over a 48 hour period, at the end of which time the fragment had disappeared. Camera lucida drawings prepared during this period show the changing shapes of bone and osteoclast as the former was absorbed. Living osteoclasts were encountered also by Sandison (1928); in this instance, bone was transplanted to an ear chamber. In his Fig. 6, a series of camera lucida drawings of the living transplant are given. In the first, a trabecula of bone is shown; in the second, 48 hours later, there is a lacuna in the left hand margin of the bone in which an otseoclast is located. After a further 48 hours the trabecula is bisected and the osteoclast has disappeared.

Some of the observations made by these two workers are of fundamental importance and could not have been obtained in any other way. For instance, Kirby-Smith (1933) was able to satisfy himself that absorption of bone never took place without osteoclasts and, perhaps in some ways even more crucial, he was able to observe the advent of an osteoclast upon the scene before the onset of absorption which was seen to occur around it later. This cell must have been the cause and not the result of the absorption.

Again, although neither worker attempted to do so, it is possible from their camera lucida drawings to make quantitative assessments. The drawings, naturally, indicate only two dimensions so that volume or weight calculations are impossible. Unfortunately even surface area measurements are scarcely practicable, but it is possible to calculate, albeit very roughly, the distance travelled by the osteoclast. In Kirby-Smith's (1933) Fig. 10, this works out at around 2.5μ per hour over a 6 hour period. In his Fig. 9 there is a detached fragment of bone with which an osteoclast is associated. As a first approximation the long axis of the bone can be assessed at 60μ . Next day only a small piece remained; this entails an absorption path of around 60μ in 24 hours, which agrees quite well with a rate of 2.5μ per hour. These figures indicate how astonishingly rapid the absorption must be in terms of volume. The drawings also of course show that the living osteoclast is motile.

The fact that neither author refers to the striated border might be

important. It could mean either that it does not occur or cannot be seen in the living state; there is, of course, the third possibility that although it was there, it caught the eye of neither author. It would not be altogether surprising if it were practically invisible in the experimental conditions employed. First, the inherent thickness of their preparations has to be kept in mind; the border would probably be obscured by the cytoplasm overlying it, or even by bone if the latter were "undercut" by the cell. Second, the difference in refractive index between border and surroundings may be very slight so as to make it invisible by transmitted light anyway. It is unfortunate that neither darkfield nor phase contrast illumination could be applied to transparent chamber preparations unless some method could be developed for a marked reduction of their thickness.

b. *Supravital Studies*

Barnicot (1947) has described in an interesting paper how osteoclasts will avidly segregate supravital neutral red. Mice of varying age from birth to 28 days were killed and the skin stripped from the head; the skull roof was next removed and incubated in dilute solutions of neutral red in Ringer. Staining of the osteoclasts began within 15 minutes and was intense after $\frac{1}{2}$ hour. The parietal bone was used for quantitative studies and comparisons with abnormal animals. The intensity of staining showed individual variation. It took the form of cytoplasmic "granules" of varying size and color; individual differences in staining intensity depended upon the size, number, and coloration of the granules. After brief incubation in dye solution, pale and dark cells could be distinguished. The former contained coarse yellow-red, the latter dark blue-red granules. Barnicot fixed fresh osteoclasts in osmic acid and Champy's fluid and found numerous granules of regular size throughout the cytoplasm; he states "it seems likely that it is these, possibly secretory granules, which stains pale yellow-red supravitally." This is interesting from two points of view. First, as was mentioned in a previous section, cytoplasmic granules of this sort do not seem to be constantly present in osteoclasts in tissue sections. Barnicot's (1947) experimental technique was superior in that fixation of the exposed osteoclasts was probably almost instantaneous, whereas in tissue sections fixation is slower; this fact may explain the apparent conflict. Second, as will shortly be described below, the cytoplasm of living osteoclasts *in vitro* contains many vacuoles and these are the sites at which, in the tissue culture cell, neutral red is segregated. Their size and staining intensity is variable.

Barnicot was able to plot the distribution of the individual cells on outline drawings of the parietal bones of mice of different ages; he contributes interesting facts about the density and distribution of the

osteoclast population which change in a remarkable manner within 14 days. He compared the distribution and number of the osteoclasts upon the parietal bones of normal and grey-lethal mice and also observed differences in the size of the cells, which were often very much larger in the grey-lethal preparations. In the normal animal also, individual osteoclasts were found to vary in their size and shape. In the mouse, as in other species (Figs. 6, 7 and 13), lobed osteoclasts were encountered; sometimes, as in tissue cultures (Fig. 23) (Hancox, 1946, 1949b) the appearances suggested that the connecting filament between two lobes had broken so that two smaller individuals had formed.

It might be possible to utilize Barnicot's method of removing the stripped skull bones in experiments of other sorts; for instance, if suitable oxygenation could be provided, for high power observations on the living osteoclasts *in situ*, warmed to body temperature. It is unfortunate that thickness difficulties would preclude phase contrast microscopy and the application of the technique described by Barer *et al.* (1952) for refractrometry of the living cells.

c. *Tissue Culture*

Tissue culture experiments have provided some interesting information (Hancox, 1946; Hyslop, 1952), but there is always the difficulty of knowing to what extent the behavior of any type of cell *in vitro* mirrors its behavior in the intact animal. However, living osteoclasts *in vitro* constantly exhibit some features which seem in a general way to correspond to what is seen in tissue sections. First, they are highly mobile, wandering away from pieces of explanted bone into the surrounding medium. Second, there is a strong tendency for the cytoplasmic masses (which are often very large indeed with nuclear counts ranging up to 125) to undergo constant changes in shape, particularly to form separate lobes (Fig. 23) connected by fine, even filiform, cytoplasmic threads. The lobes each possess their own endowment of nuclei and, as shown by cine records (Hancox, 1949b), frequently move off away from each other stretching the connecting filament further and further. Barnicot (1947) encountered two small globular cells with pointed ends directed at each other. *In vitro*, the lobes, stretched far apart, suddenly reverse their direction and flow quickly together to form a single mass again. The nuclei are shunted passively, jostling each other in the cytoplasmic stream. Sometimes the cells form flat sheets; sometimes they are globular, sometimes they form two, three, or more lobes. In tissue culture, after 24 hours signs of degeneration became apparent. The cytoplasm becomes loaded with coarse vacuoles and becomes intensely eosinophil; after 48 hours the cells round up and die.

A further property of osteoclasts *in vitro* is that they seem to have unusually vigorous proteolytic abilities; this is demonstrated by a rather striking liquefaction of the nutrient plasma clot as they progress. Suitably stained preparations demonstrate tunnels in the clot along which the cells have moved. (Fig. 24).

Hyslop (1952) has studied osteoclasts *in vitro* further. She has shown that they segregate neutral red from dilute solutions with great avidity. One advantage of tissue culture technique in this particular problem is that phase illumination can be employed. As seen with the phase microscope the cytoplasm of the osteoclast *in vitro* contains many scattered vacuoles. These consist of two components; there is the wall or pellicle and its contents. The latter consist of one or two minute granules which, at body temperature, are jostled around by Brownian movement showing that the remainder of vacuolar contents are in the form of a fluid. Examining individual cells carefully before and during exposure to dilute neutral red, Hyslop was able to show that the dye is segregated in these preformed vacuoles. The granule (or granules) within the vacuole colors first, becoming red within 1 minute. It then abruptly loses its Brownian movement, and suddenly a shower of minute, dust-fine colored particles appear in the vacuolar fluid. These rapidly become deep red and clump or flocculate together. Next, the vacuolar fluid begins gradually to color, first pink, and finally a bright brick red. The walls of the vacuoles remain unstained with neutral red, but were seen to become colored with supravital methylene blue.

Other observations were made upon the mitochondria, which were clearly visible with the phase contrast microscope. They appeared as short rods.

Hyslop also studied the distribution of alkaline phosphatase and polysaccharides in the fixed tissue culture cells by the Gomori and Hotchkiss periodic acid-Schiff techniques, respectively. No traces of phosphatase activity were detected except with prolonged incubation periods; these were thought to be artefacts resulting from migration of enzyme, or its reaction products, from bone or clot to cells. Hyslop obtained some interesting results with the Hotchkiss technique. The walls of the vacuoles showed a positive reaction; in all cells, there were rows and strings of small spherical granules which were usually positioned near the nuclei. Similar granules were to be seen in fibroblasts, arranged at one or other or both poles of their nuclei. These, it seems, are the analogue, in the tissue culture cell, of the granules described by Heller-Steinberg (1951) in the cell in tissue section.

Rumjantzev and Bereskina (1944) have also studied osteoclasts *in vitro*. They believed that increased numbers of osteoclasts resulted

from the addition of parathyroid extract to the culture medium. While they report that osteoclasts are non-motile *in vitro*, they nevertheless describe large osteoclasts migrating out from the original explant. The cells did not survive long *in vitro*, disappearing on subculture.

The experiments that have been carried out with living osteoclasts have not shed very much light upon the part (if any) the cells play in the absorption of bone. On the other hand, they have amplified description of the cell. For instance, they seem to indicate that osteoclasts may undergo rapid changes of shape, which we should bear in mind when scrutinizing the still pictures provided by fixed tissue sections. They also suggest that large osteoclasts may tear asunder to produce smaller individuals; that the lifespan of the cell may be quite brief; and that at any rate *in vitro* they have marked proteolytic ability.

III. Origin and Fate

1. PHYLOGENETIC OCCURRENCE

It is interesting that even sponges have the ability to absorb their calcareous spicules. Minchin (1898, 1910) observed porocytes of *Clathrina coriacea* to contain vacuoles within which fragments of spicule could be identified, and he suggested the name "scleroclast" for them. Theel (1892) remarks "In the Echinoderm too we may with good reason speak of cells homologous to osteoblasts and osteoclasts, the calcareous substance having been formed by the agency of the calciferous cells and absorbed again in case of exigency by others or possibly the same cells." Onoda (1931), speaking of the absorption of arm spicules in developing sea-urchins, remarks that "in the course of absorption the plasm condenses into small spherules at the tip of the arm."

In the vertebrates, osteoclasts have been demonstrated wherever bone tissue occurs in the body of the organism. Stephan (1900) gives beautiful illustrations of osteogenesis in bony fishes; osteoclasts can be recognized. They occur in amphibia (Jordan, 1925), in birds (Fell, 1925; Bloom, Bloom and McLean, 1941; Benoit and Clavert, 1952), and of course in mammals of all sorts.

2. DERIVATION

It is generally agreed that osteoclasts do not constitute a separate fixed rate of body cells but differentiate from precursor cells as occasion demands. In theory there are two ways by which this could be accomplished. First, there could be repeated nuclear divisions, mitotic or amitotic in the precursor cells, without simultaneous cytoplasmic cleavage. Second, uninucleated precursors could amalgamate.

There are a few reports of mitosis in osteoclast nuclei (Jackson,

1904; Jordan, 1925), and of amitosis (Kolliker, 1889) but such events are exceedingly rare and cannot be the general rule. At the present time the majority view is that osteoclasts arise from the fusion of uninucleated cells. At the same time, there is some evidence that large osteoclasts divide, by fission, to produce smaller individuals (Barnicot, 1947); this kind of mechanism might account for the formation of some fresh osteoclasts in zones where absorption is continuous, for instance, around the roots of deciduous teeth, but clearly it could not be involved in the sudden appearance of large numbers of osteoclasts such as occur, for example, after the injection of parathyroid extract. Under these circumstances it seems certain that the cells arise *de novo*. What is uncertain is the nature of the precursor cells.

It is important to bear in mind that most of the conclusions which have been reached on this point are the result of subjective interpretation of fixed material. The sorts of consideration taken into account are the propinquity of various types of cell to one another and to osteoclasts; to direct cytoplasmic connections which can apparently be traced between osteoclasts and other types of cells; to grades of cytoplasmic staining reaction; to transition stages between the appearance of the nuclei of osteoclasts and other types of cell. Once again, the simile of the still picture and the cinematographic record comes to mind, particularly as we know that osteoclasts (and most other cells too) are mobile and may throw out processes. It is easy to imagine that when such processes form, move around, and are withdrawn, they would probably touch upon the cytoplasm of all sorts of cells in their vicinity without effecting cytoplasmic fusion. However, at the present time there seems to be no possibility of investigating the formation of osteoclasts in the living state and we are forced to rely upon the interpretation of fixed tissue sections. The dangers are clear.

The cells which amalgamate to form osteoclasts must be either relatively fixed, sessile individuals on the one hand, or mobile on the other. Possibly both are involved. Regarding the first alternative, embryonic connective tissue or reticulum cells have been selected by Morison (1873), Jackson (1904), Danchakoff (1909), Maximov (1910), and Barnicot (1941). Bloom *et al.* (1941), and Heller, McLean and Bloom (1950) have revised and amplified this view. They base their opinions upon the rapid changes which occur in the medullary bone of laying pigeons and in the bones of animals receiving injections of parathyroid extract. Under both these conditions there is a most intense absorption of bone which is effected with almost unbelievable speed. They came to the conclusion, on the basis of studies of fixed tissue sections, that the formation of osteoclasts is but one example of a series of cellular transformations of which the cells involved in osteogenesis are capable.

They provide (1950, plate 4), a diagrammatic scheme of the transformation stages which they believe they have observed; according to this, the central figure is the "spindle cell," which can transform into osteoblast and vice versa. Osteocytes may become spindle cells; osteoblasts (and osteocytes) may transform into osteoclasts. Under the influence of parathyroid extract, osteoblasts may become phagocytic and then transform into "spindle cells." Osteoclasts themselves may transform into either osteoblasts or spindle cells.

Osteoblasts have been held to be the precursor type by Kolliker (1872), Pommer (1883), Jordan (1921) and by Arey (1919). Ham (1932) thought that osteoblasts and osteoclasts represent the differentiation of osteogenic cells along different pathways.

According to another school of thought, osteoclasts are derived from the fusion of osteocytes freed from the matrix as bone dissolution proceeds. (Loewe, 1879; Lewis, 1913; Retterer, 1917; Haggquist, 1934). This seems unlikely, for various reasons. First, osteoclasts arise around grafts of dead (for instance, frozen-thawed; Ham, 1952) bone. Host cells must have been their source of supply. Second, Kirby-Smith (1933) observed the arrival of an osteoclast before absorption of the bone.

The second alternative is that osteoclasts are formed by the coalescence of wandering cells. The "endothelial cells" and "endothelial leukocytes" which Pommer (1883) and Mallory (1912) respectively considered as the source cell would now be regarded as "histiocytes." Lacoste (1923) observed transition stages between small uninucleate wandering cells and typical osteoclasts which he was certain were formed by coalescence of the former. In several ways, this seems to be the most probable source; osteoclasts resemble wandering cells in the avidity with which they segregate neutral red (Barnicot, 1947; Carrel and Ebeling, 1926). *In vitro*, they resemble wandering rather than connective tissue cells in their mode of progress (Hancox, 1949a, 1949b). Furthermore, they resemble other types of multinucleated cell such as the "foreign body giant cell" in several ways and it is now virtually certain that these arise by the fusion of wandering cells.

To sum up, the origin of the osteoclast is still an open question and until we can develop some technique for watching the process in living tissue the matter cannot be settled with finality. As several authors have said, it may well be that more than one source is involved (Benoit and Clavert, 1952).

3. FATE

The ultimate fate of the cell is as obscure as its origin. There seems to be three chief possibilities. First, there seems little doubt that a proportion of osteoclasts undergo local degeneration, no doubt followed

in time by the phagocytosis of their remains. Such cells can be identified by their intense eosinophilia, grossly vacuolated cytoplasm and the distorted, often pyknotic nuclei.

Second, there are those who believe that osteoclasts may revert to osteoblasts or reticular cells (Jackson, 1904; Maximov, 1910; Heller *et al.*, 1950). Here, again, differences of opinion arise through the inherent difficulties of assessing and analyzing histological minutiae in fixed tissue sections. For instance, Arey (1919) remarks: "Jackson & Maximow, in particular, have upheld the fragmentation of osteoclasts into detached cells which become indistinguishable from the reticulum of the marrow. I have seen several stages which show stellate portions of the osteoclast being cut off by vacuolization. That these moieties, some of which may even lack nuclei, persist as elements indistinguishable from the reticulum is doubtful; on the contrary, the general appearance of such osteoclasts seems rather to point to ultimate degeneration. If my interpretation of the osteoclasts be correct, the entire course from the time of osteoblastic coalescence is one of progressive decline. To return to a healthy, active state, the fragmentation products of such osteoclasts would seemingly have to undergo extensive rejuvenation both as regards nucleus and cytoplasm."

Third, a proportion of osteoclasts find their way through the walls of vessels and enter the circulation. This is illustrated by Arey (1919) and Jaffe (1930), and is shown in Fig. 20. Once in the bloodstream the cells would be hurried away possibly to be trapped in spleen or lung, so that, as might be expected, they are seldom encountered within vessels. This escape route might explain why degenerate osteoclasts are rather rarely encountered.

4. DURATION

It seems probable that osteoclasts are ephemeral structures which rapidly differentiate in response to a stimulus and quickly disappear as Baker (1939) has suggested. In Sandison's (1928) ear chamber experiments an osteoclast is illustrated which appeared, absorbed a mass of bone, and disappeared within 48 hours. Similarly, in Kirby-Smith's (1933) experiments, an osteoclast appeared and vanished within approximately the same period. *In vitro* the cells are generally degenerate after 48 hours (Hancox, 1946). The experiments upon medullary bones of laying birds (Bloom *et al.*, 1941; Clavert, 1952) show how extraordinarily rapidly osteoclasts may form and then vanish. In the pigeon, the process of laying down of a large quantity of medullary bone and its destruction, presumably to provide Ca for the shell as the egg moves down to the uterus is accomplished in the incredibly short span of 48 hours. Further evidence as to the time scales involved is available from the work of

Heller *et al.* (1950). In their preparations, 12 hours after the injection of parathyroid extract into rats, large numbers of osteoclasts appeared; after 48 hours their numbers were greatly reduced.

IV. Function

1. STIMULI

Osteoclastic absorption of bone is seen either as a generalized process, affecting a large part of the skeleton, or confined to circumscribed areas.

Generalized absorption is encountered in a variety of normal and abnormal conditions and, of course, can readily be produced experimentally. One of the most beautiful examples of physiological absorption of bone is to be seen in the medullae of the bones of birds during the period of lay (Bloom *et al.*, 1941; Clavert, 1952), where the Ca required for shell formation is liberated from the bones. The classical example of pathological absorption is, of course, provided by von Reckinghausen's disease of bone, in which a tumor of the parathyroid gland is the causative factor (Jaffe, 1933). From the experimental point of view several procedures lead to a generalized skeletal absorption. Foremost perhaps, is the injection of parathyroid extracts (Jaffe and Bodansky, 1930; Heller *et al.*, 1950), Vitamin A (Mellanby, 1938, 1939, 1944, 1947), glucose and lead salts (Rutishauer, Broccard and Bionchi, 1935) or simply the restriction of alimentary Ca (Jaffe, Bodansky, and Chandler, 1932). The essential change in all these conditions is the appearance of large numbers of osteoclasts and an absorption of bone in all parts of the skeleton though it should be added that certain sites of predilection are affected earlier and to a greater extent.

Localized bone absorption is a common event during the development of bones and a similar process of "modelling" is seen in post-fetal osteogenesis, for instance, in fracture callus and ectopic ossifications. The factors responsible for the appearance and the localization of the osteoclasts are unknown; age changes in the bone or some change in its biological constitution may very well be involved. Localized absorption of bone occurs around embryonic teeth and around the roots of deciduous teeth about to be shed. The usual explanation of this kind of absorption is that local pressure is responsible. The absorption of bone seen beneath aneurysms which press upon bone, and the absorption of alveolar bone which occurs in the jaw around maloccluding teeth are examples of a pathological causation, pressure again being involved. Many experimental procedures are known to lead to localized bone absorption. It is seen as a late result of infection. Prichard (1946) grafted various tissues beneath the scalp in rats and found osteoclastic absorption

of the subjacent bone if infection supervened and Fish (1939) obtained a similar result in his experiments upon the effects of artificial periodontal infections. A sterile inflammation also stimulates the appearance of osteoclasts; Brinche (1935) filled the root canals of teeth with a paste containing formalin which led to absorption of the adjacent dentin by osteoclasts. But perhaps Barnicot's work is the most outstanding in the field of experimental local absorption (1948a, 1948b, 1950, 1951). He prepared grafts consisting of small rectangular-shaped pieces of skull bone against the surfaces of which a series of trial substances were held in place by means of fibrin clots. The prepared grafts were then inserted within the cerebra of littermate mice, recovered after various intervals, and studied either in sections or in total mounts. In the latter case, the spatial distribution of osteoclasts could be followed by the use of his supravital neutral red technique (1947). Barnicot found that Vitamin A, calciferol, and grafts of the parathyroid gland effected a remarkable absorption of the subjacent grafted bone usually confined to the tissue immediately under the trial materials. His work has been confirmed by Chang (1951), working in Bloom's laboratory. A slightly different technique was employed by means of which the substances to be studied were placed in direct contact with the bone of the skull roof of the host animal. Chang observed a constant and intense absorption of this bone beneath grafts of parathyroid tissue. Beneath other tissues which were used for control purposes (such as compact bone, cartilage, urinary bladder and gastric mucosae, anterior lobe of hypophysis, etc.) and beneath inert substances such as silk thread and soft paraffin there was no significant absorption though new bone formation was often considerable.

These facts can be summarized in the following statements. First, injury or damage to bone results in the appearance of osteoclasts; second, mechanical pressure has the same effect; third, injections of the parathyroid gland or even mere propinquity to gland substance lead to intense osteoclastic absorption; and fourth, certain substances of known chemical constitution acting by parenteral injection or by local diffusion can be made to produce a similar effect.

There seem to be two possible ways in which the differentiation and multiplication of the osteoclasts can be explained. First it may be that there is a direct stimulation of the existing cells and their precursors to produce fresh individuals; or second that the primary effect is upon the bone itself, which responds by giving off stimuli which cause osteoclasts to appear.

Several considerations make the first possibility unlikely. Osteoclasts are mobile cells and merely to produce them is hardly sufficient; each

must have its own particular locus of action. Their distribution does not seem at all haphazard, at any rate in experiments such as Barnicot's. Also, it seems unlikely that such a diversity of stimuli could have the same end result save by having at least in part a local action on the bone itself. Again, there is some tissue culture evidence that at least one of the known stimuli do not act in this way. Hancox (1939) added parathyroid extract to the nutrient medium in which osteoblasts were grown *in vitro* and also employed plasma from cockerels injected with massive doses of the extract; osteoclasts did not form in the cultures. However, Rumjantzev and Berezkina (1944) claim to have obtained evidence of a direct effect of parathyroid extract *in vitro*.

It seems more likely that the primary effect is on bone itself, which is altered and liberates some substance (or undergoes physical changes) under the influence of which osteoclasts form. For instance, phthioic acid extracted from the bodies of tubercle bacilli causes the formation of giant cells (Sabin, 1941). A tropic gradient might serve to attract osteoclasts to the affected portions of bone.

In the case of parathyroid extracts, at least, there is clear evidence of change in the constitution of bone tissue preceding its absorption. First, Jaffe (1930, 1933) some years ago described changes in the appearance of bone, especially as seen in ground discs, in the early hours after injection of extracts; and second, there is the more recent work of Heller-Steinberg (1951) who studied the same question by means of histochemical techniques. She found alterations in the distribution and intensity of staining of ground substance when treated with the periodic acid-Schiff technique and of the bone salt when visualized with a modified silver method. It is easy to imagine that, under the less intense conditions of a more physiological and slower absorption, there might be alterations in the biological quality of the bone not discernible by our relatively crude histological methods. The recticulo-endothelial system can distinguish an effete erythrocyte when the histologist cannot.

Assuming that the stimuli act on bone, there are three possible targets; the collagenous fibrillae, the bone salt, and the ground substance. The former do not seem likely; the thin layer of bone tissue which is deposited over a core of calcified cartilage in endochondral trabeculae lacks fibrillae (Renaut and Dubreuil, 1910) but of course is often the site of osteoclastic absorption.

Regarding bone salt and matrix the evidence seems to be as follows. In normal endochondral ossification osteoclasts (or chondroclasts as it would be more correct to call them) are found in association only with the longitudinally disposed bars of calcified matrix, being absent from uncalcified, transverse intercellular septae (Dodds, 1932). Second, in

rickets masses of osteoid accumulate near the growth zone cartilage but there is no attempt at absorption. If healing occurs, the osteoid calcifies; osteoclasts then appear and absorption occurs. Wolbach (1947), in describing these events, remarks that there does not seem to be any bodily mechanism for the removal of osteoid. Weinmann and Schour (1945) have demonstrated a similar principle experimentally. In rachitic rats the interdental bone alveoli consist largely of osteoid, and, following administration of parathyroid extract, while osteoclasts appear on adjacent calcified bone they do not develop on the osteoid. If ducks are injected with estrogen, masses of spongy bone are laid down in the medullae of the long bones and if they are maintained on a diet deficient in calcium beforehand, osteoid is formed instead of bone. After injection of parathyroid extract, osteoclasts are plentiful in association with the normal pre-existing bone but are absent around the osteoid (Benoit and Clavert, 1945a, 1945b, 1947).

The preceding facts may be thought to indicate that osteoclast-provoking stimuli act directly on the bone salt. Something happens to the bone salt as a result of which osteoclasts make their appearance. This would explain why osteoclasts are not found in osteoid. Unfortunately, matters are not so simple. Difficulty arises because we know that osteoid differs from bone in more ways than one. It certainly lacks bone salt, but there is also reason to believe that the organic matrix itself is also different; for one thing, even in decalcified sections, it stains differently from the matrix of normal bone both by routine and histochemical (Heller-Steinberg, 1951) methods. It is therefore possible that the competency (in Needham's sense of the word) of matrix to respond for instance to parathyroid secretion is conditional upon its having been calcified beforehand.

Much more work is needed before the choice between ground substance and bone salt can be made. It seems best to leave the question open and to turn to the osteocyte itself. The role which these cells might play is, unfortunately, largely speculative. They might conceivably be the primary locus of action of blood-borne factors such as parathyroid secretion, calciferol, and Vitamin A; their death might lead to changes in the matrix surrounding them. Similarly during embryonic development they might come to the end of their appointed span of life. In the experiments of Heller *et al.* (1950), the injection of 1,000 units of parathyroid extract into 49-day-old rats was followed in 6 hours by the death of many osteocytes and, of course, later on, by an intense osteoclastic absorption of bone. At 6 hours Heller-Steinberg (1951) found changes in the histochemical reaction of the matrix around the osteocytes. On the other hand in the kitten dead osteocytes were very rarely seen; and

the number of osteoclasts was not significantly different from the normal.

However, the violent changes produced by large doses of parathyroid extract do not necessarily provide the best material for study of this particular point. Certainly, where absorption is more deliberate and "physiological" the osteocytes beneath absorbing bone surfaces are almost always normal in appearance, which suggests that damage to them is not the primary universal precursor of osteoclasia.

In concluding this section it may be of interest to point out how the recent work on the early changes to be found in the bones of animals receiving massive doses of parathyroid extract tend to confirm von Recklinghausen's (1910) views. He maintained that the primary change occurs in the bone cells and their processes ("onkose"), which is followed by changes in the matrix of the surrounding cell territories. These consist of a liquefaction of the ground substance; this would now perhaps be called depolymerization of its structural mucoprotein such as Cobb (1949) and Heller-Steinberg (1951) have described. Structures such as cell capsules and the fibrillar net therefore become easier to see (Kirby-Smith, 1933) in living tissue. This softening and liquefaction ("thrypsis") leads to a leaching-out or halisteresis of bone salt later on; Heller-Steinberg certainly found alterations in the reactivity of the bone salt towards silver solutions in her experiments.

2. SIGNIFICANCE IN BONE ABSORPTION

The part which osteoclasts play in the absorption of bone is still somewhat enigmatical. Kolliker himself believed them to be the primary universal agents and others supported his view (Pommer, 1881). The opposition view was stated by Frey (1874); "I have not the slightest belief in their power of dissolving bone." Others (Cornil and Ranvier, 1882) were doubtful, while Klein and Noble Smith (1880) took the view that osteoclasts sometimes absorb and sometimes deposit. The position today is much the same.

The evidence that osteoclasts erode bone is partly circumstantial. There is the fact that the cells are found only upon bone when there are good reasons for supposing that it is being absorbed; there is the suggestive histological and cytological picture. Also, there is evidence of a more direct nature; in the living condition (Kirby-Smith, 1933) absorption depends upon the presence of osteoclasts which precede absorption, and from the histological point of view bone was seen to be absorbed beneath the cell body.

The view that osteoclasts do not erode bone is founded upon three main contentions. First, there is the undoubted occurrence of Howship's lacunae lacking osteoclasts. This, it is said, indicates that lacunar absorp-

tion can occur without the cells. We know, however, that osteoclasts are mobile and that their life-span is short. It would be surprising if empty lacunae were not found. Second, it has been said that absorption of bone can take place without osteoclasts by what has been called "osteolytic resorption" in which vascular endothelium and granulation tissue play a major part; this subject has been reviewed by Starr (1947). Recently, Fell and Mellanby (1952) have described remarkable lytic changes (without osteoclasts) in explants of cartilage cultivated in the media containing excess of vitamin A. The possibility that bone may be absorbed without osteoclasts does not help us very much in understanding what osteoclasts do. Third, there is the view that the cells are a result and not the cause of absorption, being merely a mass built up from the fused bodies of osteocytes freed by dissolution of the surrounding matrix. Reasons have been given above for considering this mechanism unlikely, although quite recently Heller *et al.* (1950) have considered it a serious possibility.

At the present time the balance of evidence is that osteoclasts bear a causal relationship to the absorption of bone, which they effect by superficial or lacunar erosion. The important question is how they act. Our answer to this question must remain largely speculative until more evidence is forthcoming.

To remove bone is to dispose of its three constituents. There is the ground substance to which the salts are probably bound and there are the fibrillae. The ground substance is believed to be a mucopolysaccharide and according to Gersch and Catchpole (1949) exists in a polymerized state. There is some evidence that it becomes depolymerized during absorption (Heller-Steinberg, 1951). Such a softening would loosen the bonds which bind the bone salt. As an initial speculation, it might be imagined that the osteoclast produces a secretion which digests or depolymerizes the ground substance. Cytologically, this might be in the vacuoles seen just beneath the cell border.

The bone salt would thus be freed. It has been believed since Kollier's time that osteoclasts produce an acid which decalcifies bone and, of course, there are Cretin's (1951) observations that the pH is actually lowered around the cells. There is no cytological evidence as to the part which the cells might play in this process, although their eosinophilia links them with the gastric oxyntic cell and with certain cells in the gills of eels. Both of these are concerned in the lowering of pH and possibly with secretion of chloride; both contain appreciable amounts of carbonic anhydrase (Keys and Willmer, 1932; Davenport, 1940; Leiner, 1940). Cretin's view (1951) is that free phosphoric acid is involved.

The fibrillae, denuded of salts and ground substance, must also be

removed. There seems to be little or no evidence as to how this could be effected. Perhaps the border vacuoles contain a collagenase. *In vitro*, the cells can digest fibrin (Hancox, 1946).

Cretin's (1951) opinion is that osteoclasts initially attack proteins bound with Fe. The latter is liberated and this sets free Ca. The proteins which are attacked are especially rich in phosphorus so that free phosphoric acid is formed.

In conclusion we may draw in our speculative picture of the osteoclast at work. In response to stimuli (physical or chemical) emanating from bone, osteoclasts are formed from the coalescence of their precursors, probably wandering cells, and move along a tropic gradient to their locus of action. They flatten against the bone and secrete something which liquefies the ground substance. The bone salts are freed and dissolved possibly by acid. The fibrillae are exposed and form the classical brush border. This conflicts with Mallory's (1912) idea that the striae are cilia which help to erode bone mechanically. The fibrillae are slowly digested away by the cell which continues to pour out secretion between the fibrillae or fibril bundles so that the process of erosion is continuous. At the end of their short appointed span of life the osteoclast degenerates; it may move away, perhaps into the bloodstream, or perish locally near its site of action.

APPENDUM

Since this chapter was prepared, Kroon (1954) has published an interesting article upon the osteoclasts of pigeons receiving injections of parathyroid hormone. Sections were stained both by microanatomical methods and by a modification of the periodic acid-Schiff technique for polysaccharides. Kroon believes that the striated border consists of cytoplasmic projections from the osteoclast into bone substance. He illustrates and describes vacuoles in the border, similar to those mentioned in the present text, but regards them as bone substance in process of liquefaction rather than a cellular secretion. Some vacuoles gave a positive reaction with periodic acid-Schiff. Others were negative. The staining reaction of bone tissue apparently showed changes in the vicinity of osteoclasts. A series of drawings is given supposedly showing successive changes in the appearance of the border; but it must be remembered that these have, perforce, been arranged in serial order subjectively.

PLATES I-III

PLATE I

All sections are from decalcified tissue and from embryonic jaws unless stated otherwise.

FIG. 1. Low power. Paraffin section. Several rather large osteoclasts are present, surrounded by osteoblasts. Bone spicule above and to left.

FIG. 2. Medium power. Nonex. An osteoclast is apposed against bone, above. The right hand end of the cell is flattened while the other is more spherical; a cytoplasmic pseudopod has entered an osteocyte lacuna.

FIG. 3. Medium power. Nonex. A much-flattened osteoclast is apposed against bone on the right. A brush border is present at its lower end.

FIG. 4. Medium power. Nonex. The edge of a bone trabeculum is eroded by osteoclasts in lacunae.

FIG. 5. High power. Nonex. An osteoclast occupies a lacuna of Howship. Note the cytoplasmic vacuoles near the edge of the striated border. An osteocyte is about to be shed on the left.

FIG. 6. Medium power. Celloidin. A thin stalk, running close to the endothelium of a blood vessel, connects the two lobes of an osteoclast. Each lobe occupies its own erosion lacuna. Note border vacuoles and striae. On the right is another osteoclast in a lacuna.

FIG. 7. Medium power. Celloidin. An osteoclast with two large cytoplasmic processes is visible.

FIG. 8. High power. Paraffin. Bielchowsky silver technique. An osteoclast is apposed against bone; it possesses a long, tapering cell body. The cytoplasm is loaded with coarse silver-reducing granules.

FIG. 9. Low power. Nonex. On the left, above a vessel, an osteoclast is wrapped around the end of a bone trabeculum (embryonic femur). Further along, another osteoclast is visible.

FIG. 10. High power. Celloidin. Human fracture callus. A grossly degenerated osteoclast loaded with cytoplasmic vacuoles.

FIG. 11. Similar to Fig. 10.

FIG. 12. High power. Paraffin. Schmorl's picrothionine technique. An osteoclast containing coarse cytoplasmic granules is wrapped around the end of a bone spicule (embryonic femur) in which osteocyte lacunae and canaliculi can be distinguished.

FIG. 13. Medium power. Celloidin. The osteoclast has three lobes; its cytoplasm possesses some large vacuoles. This may indicate an early state of degeneration.

FIG. 14. High power. Paraffin. An osteoclast is wrapped around the end of a bone spicule and has become somewhat detached artificially. The striae of its brush border radiate towards the bone. Note nearby osteoblasts.

FIG. 15. High power. Paraffin. Much shrinkage is present. Towards the right and lower pole of the osteoclast cytoplasm there is an osteoblast apparently engulfed. At the lower left hand edge of the cell is a small and badly-preserved brush border.

PLATE I

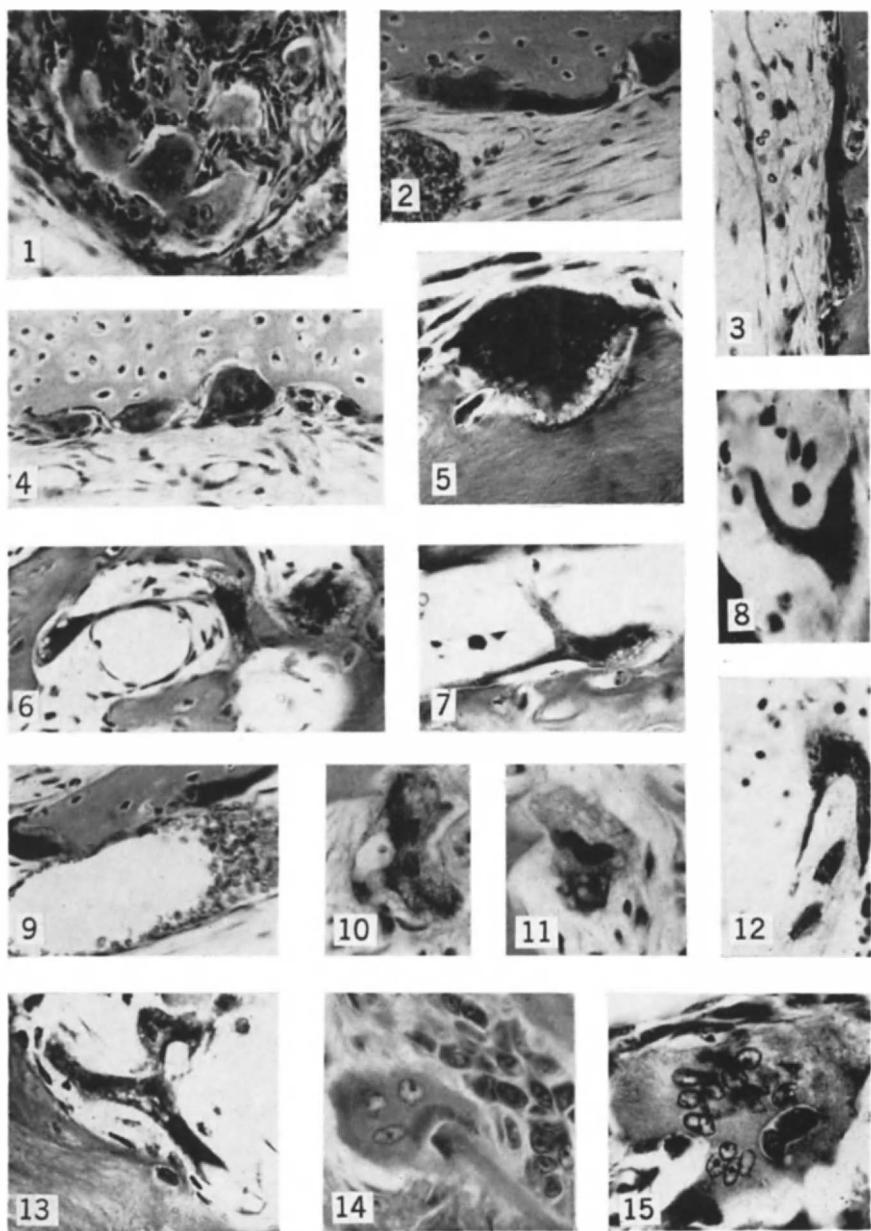


PLATE II

All sections are from decalcified tissue except Figs. 23, 24, and 25, and from embryonic jaws unless stated otherwise.

FIG. 16. High power. Paraffin. Photographic enlargement. Above, a portion of an osteoclast with striated border. Below, bone; an osteocyte may be seen within its lacuna. A process of the osteoclast, resembling a forked tongue, has entered the lacuna along a canalculus.

FIG. 17. High power. Paraffin. The prominent vacuole-like spaces are probably the remains of osteocytes within the osteoclast cytoplasm.

FIG. 18. Medium power. Paraffin. Human fracture callus. An obliquely-cut thin-walled venous sinus is present between two bars of bone. Two osteoclast masses are present, between endothelium and bone.

FIG. 19. Medium power. Celloidin. Human fracture callus. Osteoclasts near blood vessels. They are situated some distance from bone.

FIG. 20. High power and photographic enlargement. The lumen of a vessel is shown and on the left, above, there is a mass of clumped erythrocytes. Surrounded by these is an osteoclast. Human fracture callus.

FIG. 21. High power. Nonex. The striated border of the osteoclast is visible. The individual striae are extremely fine, at the limit of visibility; their arrangement is more or less radial.

FIG. 22. High power. Nonex. The striae of the border of the osteoclast are long and relatively fine.

FIG. 23. High power view of a fixed osteoclast *in vitro*. It has two lobes with connecting filament. Compare Figs. 6 and 18.

FIG. 24. Medium power view of fixed tissue culture. At bottom of picture the edge of the bone explant may be seen and, running upwards, the digested tracks left in the plasma clot by migrating osteoclasts.

FIG. 25. High power. Celloidin. "Undecalcified" human fracture callus. An osteoclast, above and to left, is apposed against bone, below and to right. At the zone of contact between the two, secretion vacuoles formed by the osteoclast may be seen.

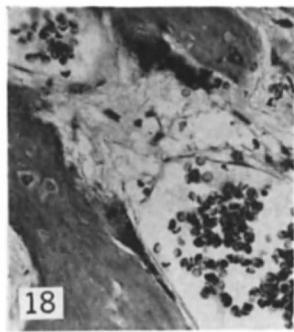
PLATE II



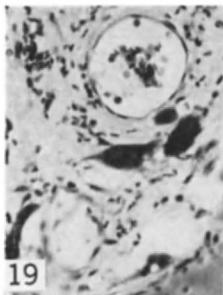
16



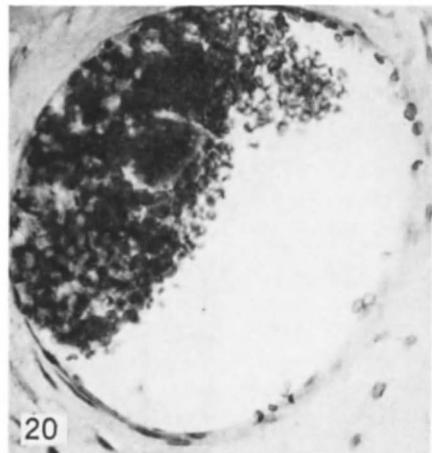
17



18



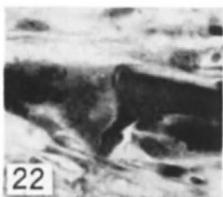
19



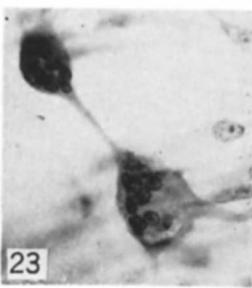
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PLATE III

All sections are from decalcified tissue and from embryonic jaws unless stated otherwise.

FIG. 26. High power. Nonex. Phase contrast; section mounted in Bergamot oil. Bone above; osteoclast below and striated border between. Note cytoplasmic vacuoles beneath border. The striae are exposed bone fibrillae.

FIG. 27. High power. Nonex. Osteoclast above, bone below, striated border between.

FIG. 28. High power. Nonex. Phase contrast; section mounted in Bergamot oil. Bone above (with osteocytes), osteoclast below. It contains an engulfed osteocyte. Note, on left, cytoplasmic vacuoles. The striated border fills in space between bone and cell. On the left it is relatively fine while on the right it is coarser, corresponding to exposure of fibrillae and fibrillar bundles respectively.

FIG. 29. High power. Nonex. Note coarse striae of border between osteoclast, above and to right of the bone (with osteocytes) below.

FIG. 30. High power. Nonex. Phase contrast, section mounted in Bergamot oil. Bone above; note the orientation of its fibrillae and fiber bundles. Osteoclast below; the striated border between comprises the exposed bone fibrillae denuded of ground substance and bone salts. On the right, the fibrillae can be seen to end abruptly at the cell border. The section is fairly thick (10μ); at other focal planes the fibrillae can be seen to extend across the remainder of the border zone. Note secretion vacuoles beneath border on left and, free of the cell between bone fibrillae towards right.

FIG. 31. Photographic enlargement of portion of preceding to show continuity between bone fibrillae, above, and striated border below. At bottom of field is a portion of the body of the osteoclast joined to bone by border.

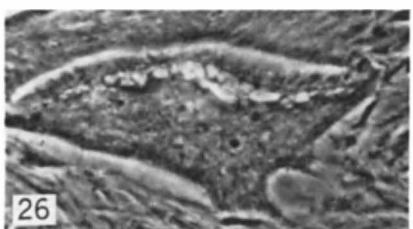
FIG. 32. A photographic enlargement of the cell shown in Fig. 33. Nonex, phase contrast. Bone to right; an osteocyte within its lacuna at bottom of picture. The bone fibrillae can be seen to sweep towards the surface near the osteocyte. They continue as the striated border of the overlying osteoclast. Towards the top of the picture, the fibrillae run parallel to the surface. The overlying cytoplasm of the osteoclast forms irregular, saw-tooth like protrusions at right angles to bone fibrillae.

FIG. 33. High power. Original from which Fig. 32 was obtained by photographic enlargement.

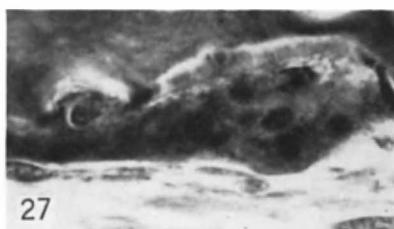
FIG. 34. High power. Nonex. Phase contrast, section in Bergamot oil. Bone above (note two osteocytes). Fibrillae (below them) run parallel at surface of bone. The edge of the osteoclast visible below the bone is serrated; there are probably many minute cytoplasmic processes.

FIG. 35. High power. Nonex. Bone above and to left; osteoclast below and to right with striated border between. The border consists of relatively coarse block-like striae, separated by unstained spaces probably secretion. These coarse striae are probably bundles of fibrillae running down from the bone, denuded of ground substance and bone salt. Note secretion vacuoles beneath border especially on right.

PLATE III



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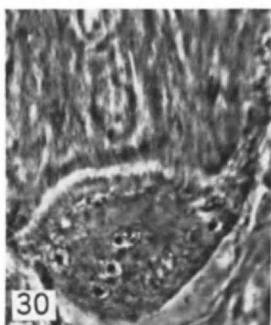
27



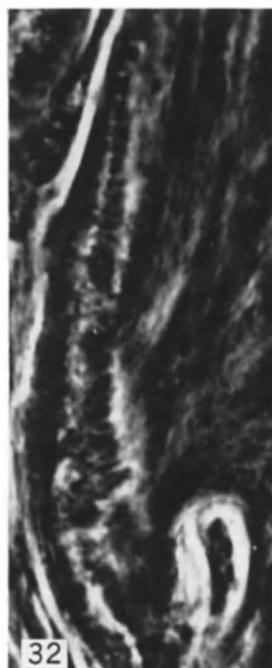
28



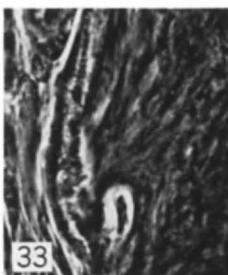
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CHAPTER IX

PHOSPHATASE AND BONE

G. H. BOURNE

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I. Historical

Although phosphatase activity of bone had been discussed as early as 1907 (Suzuki, Yoshima, and Takakishi) not much attention was paid to it until the early 1920s when R. Robison, studying the dephosphorylation of hexose monophosphate by various enzymes, noted that when the soluble calcium and barium salts of this ester were used in his experiments, he obtained a precipitate of calcium and barium phosphate.

This suggested to him that the calcium phosphate of bone might be precipitated *in vivo* in the same way. He therefore placed fresh bones of young rats in solutions of barium hexose monophosphate and in a few hours found a precipitate of barium phosphate on the bone. Further work (Robison, 1923) showed that bone contains a phosphatase, active not only against hexose monophosphate, but against a number of other phosphoric esters as well, e.g., hexose diphosphate, glycerophosphates, nucleotides, etc. (Kay and Robison, 1924). It was also shown that this enzyme was not present in cartilage which was not ossifying but that at the stage of ossification which is represented by hypertrophy of the cells the enzyme appeared. According to Robison, cartilage which never ossifies never shows any phosphatase. The enzyme was found to be present in greatest activity in the ossifying cartilage, bones and teeth of young

animals. Bone, in fact, was found to contain very much more phosphatase than any other organ in the body—twice as much as kidney and twenty times as much as liver. It is of interest that Kay (1926) found that in the embryonic animal there was very little phosphatase activity in the kidney but a good deal in the developing bones. Rossi, Pescetti and Reale (1951a, b & c) have found histochemically, however, a good deal of alkaline phosphatase in the human kidney from the period when it begins to differentiate onwards. Phosphatase has been found to be associated not only with ossifying cartilage but also with regions of membranous ossification. Robison's conclusion (1932) was that "it was considered legitimate to conclude that the production of the enzyme is a part of those cellular activities which result in the formation of bone." It is of interest that Robison recorded the fact that rachitic bones actually contain more phosphatase than normal.

Robison and his coworkers carried out a good deal of *in vitro* calcification experiments, comparing bones from normal animals with those suffering from rickets. They soaked rib junctions or entire heads of the bones of rachitic rats for 8–24 hours in *M/10* solutions of calcium hexose monophosphate or calcium glycerophosphate at 37° C. Some bones were split longitudinally. After incubation the bones were washed and fixed and finally stained by treatment with silver nitrate and exposure to light. Robison found that whereas in the bones of a rachitic animal there was a broad metaphysis of uncalcified hypertrophic cartilage, when this bone was incubated with phosphoric ester calcification occurred in large areas of hypertrophic cartilage in the metaphysis which would be calcified *in vivo* if the animal had been placed on a normal diet. This indicated that phosphatase was present in these rachitic bones and it seemed as though lack of phosphoric ester was a factor in the failure of calcification. It may be of interest in this connection that Zetterström and Ljunggren (1951) have shown that vitamin D₂ activates alkaline phosphatase at pH 9.7. However, the problem of rachitic bones is discussed by L. J. Harris in his chapter (XIX) on "Vitamin D and Bone" in this volume and need not be repeated here.

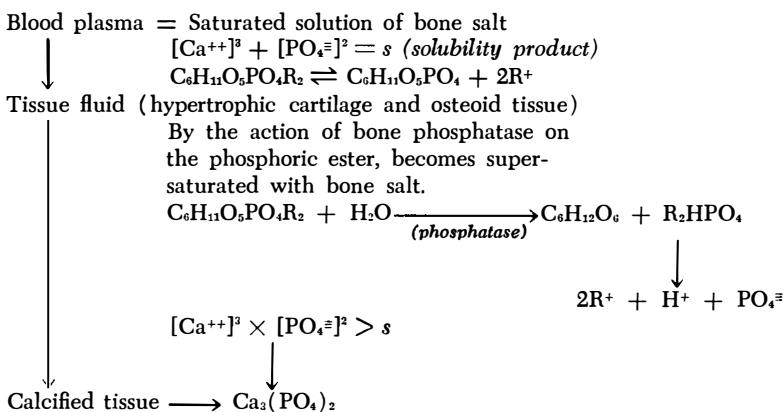
A further series of experiments designed to show further the association between phosphatase and calcification were initiated by Fell and Robison (1929). Fell had previously shown (1925, 1928; Strange-ways and Fell, 1926) that if fragments from the end of the limbs of an 8-day chick embryo were implanted *in vitro*, one of two things was likely to happen: if cartilage was already present in the explanted portion it tended to differentiate into a diaphysial portion with hypertrophied cells and an epiphysial portion with small cells and, in some cases, bone was deposited in the diaphysial portion; if, however, a piece of limb bud

which contained only undifferentiated mesenchyme was planted, eventually cartilage was formed from it. However, this cartilage was of the small-celled type and calcification scarcely ever occurred. Fell and Robison found that if cartilage was present in the transplant, even if it was the small-celled variety and could be shown by chemical estimation of the control limb rudiments of the other side to contain no phosphatase, it developed the enzyme after a period of incubation. In the second type of explant, i.e., containing undifferentiated mesenchyme, the small-celled cartilage which formed but did not calcify did not develop phosphatase. Robison (1932) believed that the phosphatase was synthesized by osteoblasts and by the hypertrophied cartilage cells.

Similarly, explants of Meckel's cartilage which contained a non-ossifying portion which did not calcify *in vitro*, did not develop phosphatase while the neighboring palatoquadrate did both.

These experiments (now nearly a quarter of a century old) of Robison and his colleagues appeared to establish an intimate relation between ossification and phosphatase and Robison adduced the following scheme to explain the role of phosphatase in ossification.

SCHEME OF CALCIFICATION



Robison explained that for simplification he assumed that bone salt was tricalcium phosphate and the ester, hexose monophosphate, but that the scheme would apply equally well if a more complex bone salt such as carbonato-apatite was precipitated or any other phosphoric ester which could be split by the phosphatase were available.

Martland and Robison (1927) subsequently showed that bone phosphatase would synthesize phosphoric esters from glycerol, glycol, mannitol, glucose, and fructose in the presence of inorganic phosphate. Robison's theory of calcification suggests therefore that the bone phosphatase

can synthesize phosphoric esters and then later by dephosphorylating them at specific sites, secure local concentrations of phosphate which in the presence of calcium would precipitate as bone salt. Robison felt, however, that there was probably a second factor in addition to phosphatase involved in the actual precipitation of the bone salt but he did not actually establish the nature of such a factor.

II. Identity of Bone Phosphatase and Comparison with Soft Tissue Phosphatase

An enzyme (or enzymes) which hydrolyzes glycerophosphate and other mono-esters of phosphoric acid at an alkaline pH is widespread throughout the body. Some soft tissues, e.g., the kidney, contain considerable quantities of it. Such alkaline phosphatase is also normally found in serum.

Whether the soft tissue phosphatases are the same as bone phosphatase is not certain. Various substances inhibit the activity of phosphatases derived from different tissues to a different extent. For example, Bodansky (1937) showed that the activity of bone and kidney phosphatase was retarded by bile acids and that intestinal phosphatase (as might be expected) was not. That there is a similarity between kidney and bone phosphatase is also suggested by the work of King and Hall (1930), who showed in the hen that the phosphatase activity of both these organs was reduced by feeding irradiated ergosterol. On the other hand, a similarity between kidney and intestinal phosphatases was shown by Page and Reside (1930) who found that irradiated ergosterol fed to dogs produced a reduction in the activity of both types of phosphatase. Cloetens (1939) found that the inhibition by CN^- takes place in such a way that the presence of two alkaline phosphatases is indicated and Monche, Jimenes-Vargas and Sols (1947) claimed that the inhibition produced by the methyl ester of the two tautomeric forms of HCN differed with purified phosphatases derived from a variety of different tissues. There is also evidence that metals such as magnesium, manganese, cobalt and zinc produce activating effects in phosphatase extracts of different tissues (see Kay, 1930, and Massart and Vandendriessche, 1944). Although these results give no clear-cut answer to the identity of various phosphatases it seems reasonable to suggest that the phosphatases of various organs like many of their constituent proteins have a certain degree of specificity (see review by Roche, 1950). The present author has studied histochemically the distribution of the phosphorolytic activity of soft tissues towards a number of phosphate esters (Bourne, 1954a, b, and c). These include riboflavin-5-phosphate, pyr-

idoxal phosphate, various sugar phosphates, naphthohydroquinone phosphate, hexestrol, stilbestrol and estrone phosphates, two chalcone phosphates and glycerophosphate.

In most organs the pattern of activity was similar to glycerophosphate in the case of the sugar phosphates and the naphthohydroquinone phosphates. But even in the case of the former there were in some organs, e.g., the adrenal cortex, differences between the reaction with glycerophosphate and with sugar phosphates. With the latter, for example, there was much greater nuclear activity. The two vitamin phosphates, the estrogen phosphates and the chalcone phosphates were all dephosphorylated in a pattern which was in most cases quite different from that of the conventional alkaline phosphatase preparation. The conclusion derived from this work and which is indeed supported by a considerable amount of biochemical work which has gone before is that the body contains a spectrum of phosphatases with overlapping substrate preferences. The various substrates used above have not been applied yet to the study of the dephosphorylating activity of bone but it seems reasonably certain that there are a number of non-specific phosphatases present in this tissue. It is of interest to record that Huggins (1931, 1933) found that pieces of urinary and gall bladder epithelium which contain appreciable amounts of glycerophosphatase, when transplanted into the sheath of the rectus muscle, produced bone. If phosphatase plays any part in the formation of bone, therefore, one might well ask why it does not form bone in all parts of the body where it occurs. Huggins and Sammet (1933) found that the artificially produced areas of bone formation were rich in phosphatase. Although bone does not normally form in soft tissues we must remember that occasionally ectopic bone does sometimes occur. For example, Keith (1928) has described the formation of such bone in laparotomy scars, in the choroid coat of the eye, in the breast, the thyroid gland, the aorta, and in the uterus. Kohno (1925) has found ectopic bone in the adrenal. Phosphatase has been found histochemically in all these sites although in some, e.g. the adrenal cortex, it is restricted to the walls of the sinusoids (at least in rats). Huggins and Sammet point out that probably the reason why urinary epithelium (for example) does not normally produce bone is that it needs a different type of connective tissue from that usually in contact with it. So perhaps some fibroblasts can be stimulated to form bone in the presence of phosphatase and some can not. Presumably most of the former are present in association with the various bones which constitute the skeleton, but in addition, they may occur elsewhere, e.g., rectus muscle sheath. If this is so then the occasional migration of these fibroblasts into regions where phosphatase is present (perhaps only in

the capillary vessels which in all organs and tissues appear to be rich in this enzyme) may be the reason for the occurrence of ectopic bones in unusual sites. That different races of fibroblasts do exist in the body has been shown by various authors (see Weiss, 1934).

III. Histological Distribution of Phosphatase in Mature and Developing Bone

1. METHODS

The experiments of Robison and his colleagues in which the ability of bone phosphatase to precipitate calcium phosphate from solutions of calcium salts of hexose monophosphate and its subsequent demonstration with silver nitrate, served to adumbrate the histochemical method for the localization of phosphatase activity in bone and soft tissues which was devised by Gomori (1939) and Takamatsu (1939). Kabat and Furth (1941) and Gomori (1943) subsequently described the localization of the enzyme in fetal bones and a description of the application of the technique to adult bone was given by the present author (Bourne, 1943). Most of the latter author's preparations were made on rather thick non-decalcified sections of femur, humerus, and parietal bone and of costochondral junctions of rat and guinea pig. One of the difficulties of applying the histochemical test for phosphatase to bone is that in most cases good histological preparations cannot be obtained without decalcification and most decalcifying fluids inactivate the phosphatase.

Kabat and Furth, however, (1941) studied the distribution of phosphatase in the developing bones of human fetuses after decalcification with diammonium citrate (which did not appear to interfere with the activity of the enzyme).

Lorch in 1947 produced a method of decalcifying adult mammalian bones with sodium citrate-HCl buffer at pH 4.5, subsequently regenerating the phosphatase with barbitone and then demonstrating it in wax sections of the bone obtained in the usual way. Similar techniques were also introduced by Morse and Greep (1948) and by Zorzoli (1948).

2. MATURE BONE

In mature bone the phosphatase reaction appeared to be restricted to the periosteum (osteoblasts, capillaries, and some fibers) and endosteum (including that of the Haversian canals) and to the more superficially placed osteocytes, see Bourne (1942) and Lorch (1947). See Plate I, Figs. 3 and 5. These results were confirmed by those of Mäjno and Rouiller (1951) and by other authors, e.g., Morse and Greep (1951). These latter authors found that at edges of bones where bone

was actually being formed, not only were the bone-enclosed osteoblasts (including their nuclei, cytoplasm and canaliculi) positive but the matrix was positive too. They also noted a positive reaction in the osteoclasts, confirmed by Mäjno and Rouiller (1951).

Mäjno and Rouiller (1951) showed that osteoblasts have considerable phosphatase activity and that as bone substance encloses them they show less activity and as they become more deeply embedded, and thus older, they become more and more enzymatically inactive until finally they become negative. However, they have not lost their ability to synthesize phosphatase because in onkosis (which the authors describe as a special form of osteocytic necrobiosis) they show signs of intense phosphatase activity again. See Plate III.

3. BONES OF EMBRYOS AND NEW-BORN ANIMALS

A number of studies have been made on the distribution of alkaline phosphatase in embryonic bone; these include those of Horowitz (1942), Gomori (1943), Zorzoli (1948), Lorch (1949a and b), Bevelander and Johnson (1950), Pritchard (1952), Rossi and coworkers (1951, 1952), Borghese (1952, 1953), see Plate I, Figs. 6 and 7. The findings of these authors are substantially in agreement with each other but individual reference will be made to some of the results.

Gomori (1943) studied the embryos of mouse, rat, guinea pig, rabbit, dog, pig, chicken and man and found that the first signs of phosphatase activity in developing bone was in the perichondrium of the vertebrae and the ribs. Zorzoli (1948) also found in mouse embryos that the first signs of ossification occurred in the "connective tissues surrounding the cartilage in a localized region which was destined to become a centre of ossification." Similar findings were described by Borghese (1953) in the mouse embryo; Lorch (1949a) in *Scyliorhinus canicula* (an elasmobranch) and (1949b) in the trout embryo.

Subsequently, according to Gomori, the perichondrium of practically all cartilage that will later ossify showed the presence of the enzyme. Later still the cartilage (usually about $20\text{--}50\mu$ across) near the positive portions of the perichondrium gave an intense positive reaction in the cell nuclei and in the matrix. It was in the region of localization of the first reaction within the cartilage that, according to Gomori, the first signs of calcification appeared. The first granules of bone salt were always found at the centers of the phosphatase-positive areas. Borghese (1953) described and figured similar results, see also Zorzoli (1948) and Lorch (1949a and b). In the development of membrane bones Gomori recorded the development of phosphatase activity in strands of connective tissue which later became bone. Within these strands calcium salts,

at first granular, and later coalescing, were deposited. Bevelander and Johnson (1950) showed in the development of membrane bones in heads of embryo pigs that phosphatase was present in the embryonic mesenchyme in osteoblasts, osteocytes and in the young spicule of the fibrous matrix prior to calcification. With the progression of mineralization the activity of the enzyme became greatly reduced in the matrix although it persisted in the cells. They noted also that glycogen was present in the periosteal fibroblasts and in osteoblasts and osteocytes. Also mucopolysaccharide was present in the periosteal fibers and fibroblasts and later in the bone matrix. It appeared to increase in amount as mineralization of the bone progressed (Plate I, Figs. 8 and 9). Pritchard (1952) studied the first development of phosphatase in the embryo rat (see Chapter VII for some details of his results).

Siffert (1951) also made a study of phosphatase in the bones of growing rabbits and new-born human beings. In general his results agree with those already described but there were some differences and simultaneously with his phosphatase studies he examined his sections for the presence of free phosphate and chondroitin sulphate (the latter by metachromatic staining with toluidine blue). Siffert also classified cartilage cells approaching zones of ossification into three groups but separated the aligned cells from the hypertrophied cells. In the undifferentiated cells (in agreement with all other authors) he found phosphatase only in the nuclei. He found a complete absence of phosphatase from cytoplasm and matrix and like many other authors found considerable metachromatic staining material in the matrix.

In the next zone (that of aligned cartilage cells) the nuclei of the cells and the matrix were positive; no phosphates were present in the matrix but it still stained metachromatically. In the region of hypertrophied cells, at the inner zone of which provisional calcification is beginning, phosphatase was found at first to be intensely active in the cells and matrix and then present in the matrix but not in the cells; the metachromatic substance was still present in the matrix. The simultaneous presence of metachromasia and alkaline phosphatase activity is claimed by Siffert to be in contradistinction to the findings of Sylvén (1947) who suggested that chondroitin sulfate disappeared from cartilage matrix before phosphatase made its appearance and that the removal of the former permitted an alkaline milieu to develop which was favorable to alkaline phosphatase activity.

Siffert (1951) in this study of endochondral ossification pointed out that alkaline phosphatase, phosphates and chondroitin sulfate were present in relatively large concentrations in remnants of cartilage present in the metaphysis. These remnants he said appeared to become calcified

without further apparent morphological change almost, as he puts it, as if the remaining constituents were reorganized to form bone matrix. The metachromatic staining ability of these remnants disappeared as calcification took place. During this process they became surrounded by osteoid in which both matrix and cells showed considerable alkaline phosphatase reaction. That free phosphates and phosphatase were found in cartilage cells and fibrocallus in the absence of demonstrable calcium phosphate suggests, according to Siffert, that the enzyme may be basically concerned with cellular functions associated with the elaboration of matrix. It is of interest that Follis (1949) found that costochondral cartilage and periosteum contained not only alkaline and acid phosphatases but also an enzyme which attacks depolymerized yeast nucleic acid at neutrality.

Lorch found that in the ribs of a new-born kitten the cartilage at a distance from the costochondral junction was negative. Nearer to the junction the nuclei of the chondrocytes began to give a positive reaction—those of the periphery more strongly than those of the center, but no reaction was given by the matrix or by the perichondrium. In the region of alignment of the cartilage cells the perichondrium became strongly positive, the inner layer being much more intense than the outer layer. A reaction was also present in the nuclei and cytoplasm of the chondrocytes.

At the junction itself the hypertrophic cartilage cells gave no reaction and only a slight one was given by the matrix.

In long bones of the kitten the endosteum and bone marrow of the epiphysis were positive but the matrix negative. In the diaphysis the matrix was uniformly negative but the linings of the Haversian canals stood out as black (positive) areas. In the older bone the osteocytes were negative but nearer the epiphysis some cells (including their processes) were obviously positive. In the epiphyseal plate the small-celled cartilage gave no reaction but in the hypertrophic regions the nuclei of the cells and the matrix did show signs of enzyme activity.

The cells of the articular cartilage also had positive nuclei and their capsules also contained phosphatase—a similar reaction was given in the epiphyseal plate at the region of transition between the small-celled and the hypertrophic cartilage. Morse and Greep (1951) found no evidence of enzyme activity in the outer regions of articular cartilage; they speculated on the possibility of the enzyme being present in the outer region but being not demonstrable. In membrane bone it is of interest that the periosteum on the outer side was positive, but in that on the inner side no evidence of enzyme activity could be seen. The endosteum lining the marrow spaces was uniformly positive.

It is of interest that, besides Lorch, other authors have shown that in the three characteristic zones of cartilage approaching a region of ossification (e.g. at costochondral junctions and at epiphysial junctions) there are three characteristic zones of phosphate distribution which correspond with these. Morse and Greep (1951) for instance, classified these zones as (1) proliferative zone, (2) hypertrophic zone, (3) zone of provisional calcification (compare with zones listed by Siffert, 1951). In the first of these zones where the cartilage cells are small and unorientated they found phosphatase only in the nuclei of the cells. In the second zone where the cells are aligned and hypertrophied the cells become progressively richer in enzyme activity as the junction was approached. The increase in activity began first in the nuclei, then in the cytoplasm and finally in the matrix. The third zone, Morse and Greep found to be relatively free of phosphatase. Similar results have been recorded by Zorzoli (1948) and Borghese (1952, 1953) in the mouse, by Greep, Fisher, and Morse (1948) in the rat, by Zorzoli and Mandel (1953) in the guinea pig, and in man and dog by Follis (1949).

4. TUMORS

The localization of the enzyme in osteogenic sarcomas was described by Gomori (1943). He examined four tumors and found them all to be positive for phosphatase and that the reaction was present both in the cells and in the "fibrillar-intercellular substance." In those tumors which showed areas of sclerosis the sclerotic areas were found to be enzymatically much less active. In an osteoblastic metastasis of the breast, Gomori found the tumor cells to be negative but the stroma to show a strong positive reaction. He also showed that in two giant cell tumors and two fibrosarcomas of bone which showed no signs of bone cell formation there was no phosphatase.

5. DEVELOPING BONES OF FISH

Lorch has examined the distribution of phosphatase in the developing bones of cartilaginous (Elasmobranchs) and bony fishes.

The representative of the Elasmobranchs used (Lorch, 1949a) was *Scyliorhinus canicula* (Plate II, Figs. 11 & 12). This is of particular interest because the skeleton of this animal, as of other Elasmobranch fishes, is composed of calcified cartilage, not of bone. In these animals, according to Lorch, calcification always starts at the periphery of a piece of cartilage and is usually remote from and not associated with the cartilage cells. The granules of calcium which are first formed later coalesce and form a series of crystalline plates. The relation of phosphatase to this process had previously aroused the interest of other authors. For example,

as long ago as 1931 Bodansky, Bakwin, and Bakwin had shown that alkaline phosphatase was present in the elasmobranch skeleton and Roche and Bullinger (1939) had shown that it was present in both cartilaginous and bony fishes and that it was similar to the bone phosphatase of mammals. Furthermore, these authors found by biochemical means that the enzyme was present in the calcifying portions of cartilage and not in those parts which did not calcify. They also found the enzyme to be present in association with the calcification of scales and teeth. Some important points from Lorch's work can be obtained from the following quotation from her paper:

"Phosphatase is absent from the cartilages of the young embryos and appears first in the chondrocyte nuclei and in the perichondrium. At a stage just prior to calcification the enzyme is also detected in the cartilage matrix. The maximum extracellular phosphatase activity occurs during the first stage of calcification. The phosphatase positive zone then retreats in front of the wave of calcification."

It seems reasonable to claim from these results that phosphatase is concerned with calcification here, as it is in ossification in mammals and (Pritchard, 1950) in frogs and lizards. One important point noted by Lorch was that calcification was found only to occur where *extracellular* phosphatase was present. Also there was a lag from the time of first appearance of phosphatase to the first signs of calcification. In the Cyclostomate fishes, of which the lamprey is a characteristic representative, the skeleton is cartilaginous and never calcifies, although the nuclei of the peripheral chondrocytes gave a positive reaction phosphatase was never found in the matrix. Lorch concluded that in the case of the Elasmobranch fishes the only interpretation of the function of phosphatase was that it is concerned directly with calcification and not much with the formation of an organic matrix. See Plate II, Figs. 11 and 12.

In a later paper on the development of the skull of the trout, Lorch (1949b) found that in the early stages of development as in mammals alkaline phosphatase was present in most cells though confined mostly to the nuclei. In mesenchyme and cartilage which was to form bone the activity of the enzyme increased in the nuclei and later spread to the cytoplasm of the cells and to the matrix, while in cartilage which was not to ossify, and in undifferentiated mesenchyme, enzyme activity decreased. Bone was found never to form in the absence of extra-cellular phosphatase. This is in keeping with the findings in mammalian bone and in general the relationship of alkaline phosphatase to the processes of ossification observed by Lorch follows in all important respects that seen by her and by other authors in mammalian skeletal development.

6. CALCIFICATION IN OTHER ANIMALS

The possibility that alkaline phosphatase may be concerned with the deposition of calcium salts in other animals has been investigated histochemically by Bourne (1943) and Wagge (1951).

Manigault in 1939 and 1941 had shown biochemically that there was a direct correlation in Molluscs between liver, mantle and blood phosphatase in the snail *Helix pomatia* and calcium precipitation in the shell. The present author investigated histochemically the distribution of alkaline phosphatase in the mantle edge of two representatives of the Mollusca. In these animals the mantle is an organ which is responsible for the production of the shell, and the mantle edge is believed to be the most active portion in this respect. It is by constant deposition of calcium salts from this part of the mantle that the shell grows in size. The two genera of molluscs used for this study were a bivalve mussel, *Mytilus*, and a gastropod, *Calliostoma*.

In both these species the mantle edge gave a very strong positive phosphatase reaction and there was also a good deal of pre-formed phosphate present which appeared to be in the form of granules of shell substance. Whether phosphatase plays a direct part in the formation of these granules is not known for certain. If it does one would expect, on the precipitation theory, the shell of molluscs to contain appreciable quantities of phosphate, but Pelseneer (1906) states that in most molluscs only about 1-2.5% of the shell is calcium phosphate. On the other hand, Plate (1922) claims that the calcium of the mollusc shell is secreted first as the phosphate and that it later changes to the carbonate. Wagge (1951) studied the distribution of alkaline phosphatase in the repair of the damaged shell of the snail *Helix aspersa*. She found that amebocytes played an important part in the repair of such damage by transporting calcium carbonate and proteins from other parts of the shell or from the digestive gland (which stores calcium) to the damaged area. After injury the mantle became applied to the area and exuded a fluid containing many amebocytes over it. The amebocytes secreted at first a protein membrane which rapidly became calcified. Wagge found that abundant alkaline phosphatase was present during shell repair, both in the injured area and in the calcium-storing cells of the digestive gland. Small amebocytes were massed around these cells and their nuclei were strongly phosphatase positive, a good deal of extracellular phosphatase was also present. It is of interest that the larger amebocytes which carry a good deal of calcium both within their nuclei and within the cytoplasm did not appear to contain phosphatase. The enzyme was also found to be present in the small amebocytes and extracellularly in the region where the shell was being regenerated.

The present author has also studied (1943) the phosphatase activity

of the shell-secreting membrane of the lobster (*Homarus*). However, the animal was not in a state in which active shell secretion was occurring and phosphatase was found to be present only in the nuclei of the cells of the membrane and not in the cytoplasm. Further investigations on the relation between phosphatase and calcification have been carried out by the present author on the oviduct of the fowl.

It is of interest that in the laying hen the alkaline phosphatase activity of the serum is increased by 30% during the period of shell formation. The egg shell appears to be secreted by the uterus, which showed more phosphatase activity than other parts of the reproductive tract. Conrad and Scott (1938) state that shell deposition begins the moment the egg reaches the uterus. There was some variation in the amount and distribution of phosphatase in this region according to the physiological state in relation to egg laying. In a hen which was killed 36 hours after having laid a well-shelled egg, the walls of the blood vessels in the uterus gave a strong reaction (mainly in the interna) and there was a slight diffuse reaction in the epithelium which was most intense at the brush border. In a hen that had a soft-shelled egg in the uterus and other smaller ones in the upper part of the oviduct the epithelium of the uterus was definitely more positive than any of the other uterine tissues. This reaction was diffuse and was particularly intense at the cuticular borders. The nuclei were also positive. Below the epithelium the cells of tubular glands showed positive nuclei and the outermost portion of the distal regions of the cells contained numerous granules of positive material. It appears therefore that phosphatase may play some part in the secretion of the egg shell in the hen but when one considers the rapid and spectacular way in which this shell is laid down it is surprising, if phosphatase does play a part, that its activity in the uterus is not very much greater.

The consideration of the calcificatory activities of other animals has shown in general that in these animals, even where, not calcium phosphate but calcium carbonate is being laid down, phosphatase seems to be associated with the process. If the enzyme therefore plays a part in these processes it seems unlikely that it is responsible for the direct precipitation of calcium salt on the lines suggested originally by Robison for bone. However, this problem will be discussed later in the chapter.

For biochemical studies on the association of phosphatase with calcificatory processes in a variety of animals, see the review of Roche (1950).

IV. Phosphatase and Bone Repair

The association of phosphatase with normal bone formation has been demonstrated in the preceding sections and it remains now to be shown

that phosphatase is also associated with the processes of bone repair.

It has been shown (Bourne, 1943a, b, 1948) that phosphatase is involved in the process of healing of 1 mm holes bored in the femur, and in the skulls of guinea pigs, with a dentist's drill. Within 24 hours intense activity was shown by the periosteum surrounding the injured area. This reaction was mainly due to the strong reaction of the osteoblasts and the capillaries in the osteogenetic layer. The fibrous layer, as has been shown by other authors in ordinary endochondral ossification, gave very little reaction. The osteoblasts showed a diffuse reaction in the cytoplasm and in the nucleus, of about the same intensity. In some preparations a small amount of cartilage had been formed near the hole by the periosteum by the end of one week, and osteoblasts near such regions gave a very intense reaction. It was noticed that where hypertrophied cartilage cells, which were surrounded by only small quantities of matrix, occurred, they often showed intense enzyme activity. In some cases when such cells were surrounded by abundant matrix which had itself given a positive phosphatase reaction, the cell appeared to contain very little of the enzyme. A site of strong activity was seen to occur at the boundary of the cell capsule and the matrix. The impression given by these observations was that the cartilage cell had charged itself with phosphatase presumably by synthesis and then as it elaborated and excreted the cartilage matrix it excreted the phosphatase with it. Similar observations have been made by some of the other authors mentioned earlier. Some specimens of cartilage were found, however, in which the cartilage cells, embedded in a matrix containing phosphatase, themselves demonstrated a high enzyme activity. In some preparations, and rather more noticeable in animals receiving a little less than the normal amount of vitamin C, "fibroblasts" were seen which were outlined by a black border indicating phosphatase activity. The presence of phosphatase at this site was probably due either to the synthesis of the enzyme by that region of the cell membrane or by its absorption there from the phosphatase-containing medium in which the cell was bathed. Other "fibroblasts" were found with one process stretched out until it had become an elongated thread which gave a positive phosphatase reaction. This suggested the spinning of a fiber made of phosphatase-impregnated protein.

Masses of phosphatase-active fibers were also seen bunched together to form trabeculae (Plate I, Fig. 4) and in many cases these appeared to be aggregated around phosphatase-active capillaries. All fibers in the repair tissue of many of these holes gave a positive reaction for phosphatase (Plate I, Fig. 2). It should be noted that although in some cases bone regenerated in these holes in association with the production of small quantities of cartilage, in most cases regeneration was obtained by the process of intramembranous ossification.

Theoretically, therefore, there should be little difference between the changes seen in the repair of these small holes in a bone such as the femur and in a membrane bone such as the parietal. In practice this is so, the main difference being that repair is a very much slower process in the parietal. The present author has studied the phosphatase reaction in healing parietal bones at various periods after the injury. Twenty-four hours after 1-mm holes were bored in the parietals, an accumulation of cells with phosphatase-positive nuclei was found in the injured area and in the periosteum and endosteum near the region of the injury. These cells were similar to those found by Fell and Danielli (1943) in injuries to the skin and described by them as polymorphs. Those polymorphs present in the preparations now being described had come probably from the blood vessels in the periosteum, though some may have come also from the blood vessels within the bone itself. It is of interest that it was only when they were relatively close to the area of injury that they gave a strong phosphatase reaction, further away from the hole it was slight or moderate. It appeared that something was diffusing from the injured area which was inducing phosphatase synthesis or phosphatase absorption by polymorphs when they had migrated close enough to the injured area to come under its influence. Botterell and King (1931) claimed weak phosphatase activity in a fracture callus by the fourth day and strong activity by the eighth day, but Mäjno and Rouiller (1951) claimed that phosphatase-rich cells were present in the periosteum of a fracture callus within 48 hours and that they persisted until the end of the repair processes. In addition to the polymorphs a second type of cell was present at the site of injury after 24 hours. In this type there was a strong positive reaction in both cytoplasm and nucleus and many possessed elongated processes which were rich in enzyme activity. These cells appeared identical with those which could be seen in healing holes in the femur and with those described by Fell and Danielli (1943) in healing skin wounds: it seems fairly certain that they were fibroblasts. Towards the central part of the hole phosphatase-positive cells of a similar appearance seemed to be joining up to form phosphatase-positive capillaries. From many of these cells greatly elongated phosphatase-positive processes could be seen which at their distal ends were of the same size and appearance as the few fibers present in the hole by this time. These cells appeared similar to those found in the healing holes of the femur. At 3 days after injury many phosphatase-positive fibers were seen but quite a number were also present (probably the more mature fibers) which gave no signs of enzyme activity. Positive osteogenetic fibers could be seen both in the injured area and also in the cellular layers of the nearby periosteum. Most of the nuclei of the periosteal cells gave a positive phosphatase reaction.

After a week of healing two massive concentrations of phosphatase appeared in the repair tissue, one on either side of the hole, and there were histological signs that in these regions formation of osteoid trabeculae was beginning and associated with them were a number of strongly positive and typical osteoblasts.

After 2 weeks phosphatase-positive osteogenetic fibers were still forming but a lot of negative fibers could also be seen. The greatest concentration of enzyme activity was where the trabeculae were forming and enlarging. None of the enzyme-positive polymorphs could be seen at this stage.

The injured area was invaded during the early period not only by polymorphs and fibroblasts but by masses of histiocytes. These appeared to flock to the injured area along the blood vessels and along the fibrous layer of the periosteum. Trypan blue preparations showed enormous numbers of them aggregated in the injured area by 24 hours. Phosphatase reactions made on such trypan blue preparations showed, however, that none of the cells containing this dye possessed any phosphatase activity. From these results it is possible to list the apparent cycle of events in such an injury and the association of phosphatase with them.

Following the injury, the hemorrhage, and the clotting of the blood, there was an invasion of the clot by polymorphs (phosphatase-positive), histiocytes (phosphatase-negative), and fibroblasts (phosphatase-positive, at first in the nuclei and around the cell membrane). The fibroblasts then appeared to spin a series of phosphatase impregnated fibers through the area of injury (see Plate I, Fig. 1). This continued for some days and then after a period, presumably at maturation of these fibers, the latter lost their enzyme activity. This is in keeping with the findings of Fell and Danielli who found that the first-formed fibers in healing skin wounds were phosphatase-positive and that as they matured they lost this activity.

The loss of phosphatase activity by these fibers represents the end of the first stage of repair of bone injury and it seems to follow essentially the same pattern as in skin. In skin, repair was effected by this time, in bone however, a second cycle of phosphatase activity soon began and it appeared to be initiated in, and by, obvious osteoblasts. Where these cells were aggregated a great deal of extracellular phosphatase appeared on or among the osteogenetic fibers and in these regions bony trabeculae began to form. Apparently fresh phosphatase-containing fibers were laid down (see Bourne, 1943a and b) which already contained bone salt: the two processes of production of a phosphatase-impregnated fiber and deposition of bone salt seemed to occur simultaneously. The simultaneous production of matrix and bone salt has also

been observed by Urist and McLean (1941). Following the formation of bone, phosphatase activity was again lost from the bone cells and from the matrix. This is in keeping with the results of other authors (see particularly Mäjno and Rouiller, 1951). McKelvie and Mann (1948) have suggested that there are two types of osteoblasts, both of which contain alkaline phosphatase. One group, in their opinion, includes the osteocytes of mature bone and the other comprises "matrix cells" which are concerned with matrix formation and which undergo a process of morphological disintegration and physiological death. Both types of cell eventually lose their phosphatase.

Pritchard has made a study of fracture repair in rat, lizard, and frog as representatives of three groups of vertebrates. He found very little phosphatase activity associated with the healing of fractures in a frog. He suggested that a reason for this might be that the enzyme in the Amphibia was excessively thermolabile and had been largely destroyed by heat during the process of embedding. However, he suggests that the enzyme may in fact be lacking in repair processes in the frog's skeleton and that this might be the explanation for the virtual absence of calcification in the cartilage which forms in association with a healing fracture in this animal. It is of interest too that the author found a deficiency of the enzyme in the normal epiphyseal cartilage of the frog.

In the rat and lizard the relationship of phosphatase to the healing process was more or less the same. The enzyme was found to appear in the region of the fracture as soon as signs of proliferation by medullary and periosteal cells became evident. The reaction was found to be in the proliferating cells, in their nuclear membranes and nucleoli, and also in the intercellular fibers. Some osteoblasts showed a concentration of phosphatase in the Golgi region. In the periosteum it was found that the cells of both layers gave a positive reaction. In general, a reaction was found in the callus in osteoblasts, in the external zone of proliferating cells, and in a patchy manner in cartilage (some cells positive, others with positive capsules, matrix positive in parts). In the frog what small amount of activity appeared to be present was mainly in the nuclei of the osteoblasts and in the nuclei of certain cartilage cells.

The presence of phosphatase in osteoblasts has been confirmed by all authors who have described the relationship of phosphatase to ossification (see Chapter VII). Pritchard has described the development of nuclear phosphatase in fibroblast-like cells as an indication that they are or are about to change into pre-osteoblasts. This became more intense as the osteoblasts became differentiated and the reaction sometimes spread diffusely into the cytoplasm or in some cases into the Golgi apparatus. It is of interest that Cappelin (1948) claims that phosphatase

in fact changes the distribution of the nucleic acids in the fibroblasts of the periosteum, which are undergoing evolution to osteoblasts. The coincidental development of cytoplasmic basophilia and alkaline phosphatase in the developing osteoblast is also recorded by Pritchard but he does not suggest a causal relationship. It is of interest that he also found glycogen in pre-osteoblasts but not in the fully differentiated osteoblast. (See Chapter VII.)

Mäjno and Rouiller (1951) have studied and figured the life cycle of the human osteoblast in relation to alkaline phosphatase in some detail. They showed the osteoblast to possess potential enzyme activity even in the resting stage and demonstrated that there was an increase of enzyme reaction with the onset of activity both in osteogenesis and in bone resorption such as that found in Paget's disease. Once the osteoblast became incorporated into the bone substance and turned into an osteocyte, phosphatase activity was retained for a time but it became progressively less and less until the cells appeared completely negative. Whether this was due to loss of the enzyme or to the enzyme becoming inactive cannot be said, but under certain conditions, e.g., in necrobiosis or onkosis, enzyme activity returned to the osteocyte, apparently developing mainly in the cytoplasm at first. During this stage the processes of the osteocyte in the canaliculi gave a positive reaction. As the onkotic activities developed both nucleus and cytoplasm of the osteocyte showed considerable enzyme activity which eventually became as intense as that found in osteoblasts. With this increase of activity the cell enlarged considerably. Finally there was a disappearance of the enzyme and a lysis of the cell.

In the ordinary process of bone resorption Mäjno and Rouiller also found phosphatase played a part because they found that osteoclasts in human beings and in rabbits were very rich in the enzyme, which they thought might be concerned with the synthesis of phosphoric esters, although the significance of this in bone resorption is not obvious. The authors also claim that osteoclasts have a positive tropism for bone salts. While phosphatase-positive osteoclasts have not been observed by the present author it is of interest that intense enzyme activity was found extracellularly in bone chips undergoing resorption in the repair tissue in the holes bored in guinea pig bones.

The evident association of phosphatase with ossification and calcificatory processes have led to some experiments in which the enzyme was used to stimulate ossification. Blum (1944) found that if experimental fractures were treated with a mixture of phosphatase and calcium glycerophosphate with or without alginate gel to hold the mixture in position the fractures healed more rapidly. However, Slessor and

Wyburn (1948) injected alkaline phosphatase solutions into leg muscles of rabbits and implanted pellets of the enzyme and substrate into muscle but obtained no sign of bone formation or calcification. The present author (unpublished results) has attempted to stimulate bone formation in drill holes in the femur and the parietal of rabbits by packing the hole with crude alkaline phosphatase powder obtained by the method described by Cameron (1930) and by covering such holes with pieces of fresh or alcohol fixed gall and urinary bladder: there was no evidence of increased bone formation. Also no signs of bone formation or of calcification were obtained when similar pieces of these bladders or of phosphatase powder or of filter paper impregnated with phosphatase were implanted in the sheath of the rectus muscle. It seems from these results that ossification and calcification are processes which are carefully timed *in vivo* and require a balanced reaction between the right types of cells and soft tissues and the initiating factor, whatever this may be.

V. Significance of Phosphatase in Bone Formation

Despite the widespread distribution of alkaline phosphatase in the soft tissues of the body, the intimate association of the enzyme with ossification indicates that it plays some special role in this process. What can this role be? Robison's original view that phosphatase liberated phosphate ions until their concentration exceeded the limits of solubility and they were precipitated in association with calcium is an attractive hypothesis, but there is no evidence that precipitation of bone salt actually occurs, and according to Neuman and Neuman (1953) there is in fact evidence against this occurring. X-ray crystallographic analysis of bone salt shows that it is crystalline and that the crystal structure is compatible with that of an hydroxyapatite. Neuman and Neuman point out that the simplest structural unit of such apatite would contain 18 ions and that such "a solid phase of apatite could not possibly form spontaneously by precipitation. It can only form by crystallisation, either by a step-wise addition of ions to a nucleation centre or by a similar process involving the hydrolysis of secondary calcium phosphate." They go on to point out that they fail to see how a highly orientated crystal structure can be formed by precipitation.

In a personal communication Dr. Neuman has stressed that the normal serum values of ionic calcium and phosphate are very much below their precipitation points and that if a local factor such as phosphatase was to cause such precipitation it would have to increase the local concentration of phosphate by 300%. This has been demonstrated in a yet unpublished work in Dr. Neuman's laboratory both in theory and in fact.

Another argument against the precipitation theory was advanced quite early and was in fact anticipated by Robison and his colleagues, and this was that suitable phosphate esters were not present in bone. However, Robison and Martland (1927) (see Robison, 1932), as has already been mentioned, showed that bone phosphatase was able to synthesize phosphate esters from glycerol, glycol, and some sugars. That this ability may not have been due to the phosphatase but to the simultaneous presence of a phosphorylase was indicated by Gutman and Gutman (1941). It is also possible that the phosphatase might act as a transphosphorylase as they have also suggested. In the process of glycolysis one or more phosphate esters could be formed which would serve as substrates for the action of phosphatase. In particular, glucose-1-phosphate was suggested. The association of glycogen with osteogenic processes has been well established, e.g. Glock (1940), Gutman and Yu (1950), and Marks and Shorr (1952). Also Pritchard (1952) showed that both chondrocytes in the early stages of hypertrophy prior to ossification, and osteoblasts, contain considerable stores of glycogen.

Roche (1950) published a scheme of ossification in which phosphatase took part and which incorporated the conception of the provision of a phosphate ester from glycogen.

Roche pointed out that plasma contained only traces (0.1–0.2 mg) of esterified phosphorus per 100 ml and that since virtually no phosphate ester could be found in cartilage and bone its production from glycogen must be an integral part of the ossification processes.

Dallemagne (1947, 1948, 1950, 1951) has claimed that when $\text{PO}_4^=$ ions are released by the action of phosphatase during the period of polymerization of matrix to form ossein there are not sufficient calcium ions brought in by the blood to secure precipitation of all of the phosphate ions. Some, therefore, become temporarily attached to the pre-osseous organic substance. Later, they are freed to combine with calcium to form more bone salt. As evidence of this, Dallemagne cites the fact that first-formed bone has a calcium:phosphorus ratio of 1.29, which later rises to 2.20.

In support of the agency of phosphatase in causing precipitation of bone salt are the results of Lorch on calcification in the cartilaginous fish, *Scyliorhinus*. In such an animal she could not find any demonstrable change in matrix or cells prior to calcification other than that of phosphatase distribution, although this is not to say that some changes did not occur at a submicroscopic level. Crystalline bone salt, however, appears to be deposited straight into the cartilage matrix. Lorch cannot see that phosphatase can have any role in this instance except that of causing precipitation of bone salt.

That such precipitation can in fact lead to the formation of crystalline calcium phosphate is suggested by recent work on soft tissues using Gomori's histochemical technique. It is known that in this technique precipitation of calcium phosphate takes place by liberation of phosphate ions from the substrate (in most cases glycerophosphate). The resultant precipitate, which is subsequently made visible by the cobalt chloride and ammonium sulfide technique, appears to be amorphous. In preparations of small intestine a deposit of calcium phosphate can be detected by these means in a matter of seconds after incubation, and by one minute quite a heavy deposition of the phosphate can be seen in the brush border of the epithelial cells after visualization with cobalt chloride and ammonium sulfide. However, if incubation is continued for some hours the calcium phosphate appears to have disappeared from this region which now gives a negative reaction. Although the cobalt chloride/ammonium sulfide reaction is negative in this case, if the sections after incubation are examined under phase contrast illumination or are treated with silver nitrate and exposed to light, a considerable amount of calcium phosphate is seen still to be present. It can also be shown that this difference is due to a change of the calcium phosphate from the amorphous to the crystalline condition. Now it is also of interest that the cobalt/ammonium sulfide technique appears to stain recently deposited bone salt but will not stain older bone. On the other hand, silver nitrate will stain mature crystalline bone salt. Can we assume therefore that bone salt is in fact originally deposited in an amorphous condition and changes subsequently to the crystalline form? On the other hand Carlsen, Hensen and Johansen (1953) have shown that the calcium phosphate precipitated in a test-tube in the Gomori incubation medium used for the histochemical localization of alkaline phosphatase gives the X-ray diffraction pattern of apatite. Thus, although the precipitation theory does not bar the possibility of crystalline bone salt being formed in this way, the strictures of Neuman and his colleagues based on the ionic concentration of Ca^{++} and $\text{PO}_4^=$ in normal serum still apply.

The presence of glycogen in pro-osteoblasts and pre-hypertrophic chondrocytes may not necessarily be connected with the synthesis of phosphoric esters directly concerned with the formation of bone salt. Many cells accumulate glycogen before entering into a phase of rapid multiplication and/or synthesis or differentiation (the cells of the basal layers of the skin are one example) and it may be that the accumulation of glycogen in bone-forming cells is associated with the secretion of mucopolysaccharides or protein which forms part of the osteoid substance or, as Pritchard has suggested, with differentiation of these cells

(see also Follis, 1949; Borghese, 1952). In other words, the glycogen may simply be acting as an energy source and its phosphorolytic breakdown may simply be part of this process.

Despite the earlier claims which denied the existence of phosphate-donating esters in cartilage, recent work by Allbaum, Hirshfeld, and Sobel (1952) has confirmed the presence of adenosine triphosphate in pre-osseous cartilage. As they point out, the presence of glycogen and some of the enzymes necessary for its breakdown have already been demonstrated but the presence of A.T.P. had not. They quoted the work of Cartier (1950) who found that calcification of the cartilage of the bones of embryonic sheep was enhanced if A.T.P. was added to the medium. Allbaum and his colleagues found that pre-osseous cartilage contained A.T.P. at the level of brain, kidney and liver; however, they were not able to say whether it was contained primarily in the matrix or in the cells. The presence of A.T.P., however, does not mean necessarily that it provides the phosphate actually used in the formation of bone salt; it is more likely to be concerned in the energy exchanges associated with the oxidation of the glycogen.

The apatite crystals of bone (about 350-400 Å long and broad, and about 25-50 Å thick) have been shown by various authors (see Robinson and Watson, 1952 and Chapter VI) to be associated with the collagen of bone and to be distributed between the characteristic bands which can be seen in collagen fibers under the electron microscope, the longitudinal axis being parallel to the long axis of the fibers. Neuman and Neuman state that, "The organic phase of osteoid or endochondral cartilage may bind either calcium or phosphate ions in the proper space relationships of the apatite lattice." DiStefano, Neuman and Rauser (1953) have searched for specific chemical groups in the matrix which acting as a template, would bind either calcium or phosphorus. Chondroitin sulfate is usually associated with collagen, and calcium ions are known to form complexes with it, but Boyd and Neuman (1951) have shown that such complexes have a high dissociation and that this would reduce the value of the chondroitin sulfate as a template. However, DiStefano and his colleagues discovered in calcifiable cartilage a phosphate ester associated with a matrix substance insoluble in trichloracetic acid. The presence of this, they say, "correlates well with the ability of the cartilage preparation to calcify *in vitro*." The evidence at present available indicates that this ester is a "five or six membered heterocyclic compound with ester phosphate and amine groups present but carbonyl groups absent." There is some evidence that it is a galactose derivative possibly 2-amino galactose-6-phosphate (see Plate II, Fig. 10). The authors found that if fresh cartilage is given an overnight

incubation in saline (which destroys its calcifiability) the amount of this ester is reduced to negligible amounts. They suggested that the ester may form part of the proposed template which is thought to aid the crystallization of bone salt. If this compound is in fact able to function partly or completely as a template for the crystallization of bone salt the role of phosphatase becomes somewhat obscure. The compound is in fact actually attacked by phosphatase, an activity which would certainly destroy its value as a template. This suggests however that Gutman's suggestion of a transphosphorylase activity for the enzyme concerned perhaps in the reaction chains associated with the synthesis of such an ester is a more likely field of activity than that of the liberation of phosphate.

In the template theory of bone salt crystal formation in which a phosphate ester forms part of the template the phosphate moiety is already present, and its position controls the point of deposition of the calcium ions which come down little by little in step-like fashion to build up characteristic crystals. In this theory there is no need at all for phosphatase to split off the phosphate in order to secure *precipitation* of the calcium ions. Dallemande's theory of the binding of phosphate to the matrix fits in well with the template theory—the only point at issue is that he conceives of a subsequent release of the phosphate to *precipitate* the calcium ions, but by the template theory no such release is necessary.

The template theory of calcification may help to explain the deposition of crystalline bone salt in the cartilage of *Scyliorhinus* as described by Lorch. Although some sort of template, probably protein, but not necessarily containing phosphate, is probably concerned with laying down of the shell in molluscs and the shell in birds' eggs it is difficult to see where phosphatase is involved in this process.

Although chondroitin sulfate has been said to be not suitable as a template, recent work suggests that it may play an important part in calcification. Toluidin blue combines with chondroitin sulfate to form a metachromatic staining substance, and Sobel and Berger (1954) have shown that when toluidin blue is present in a calcifying medium the degree of innactivation is a function of the concentration of the dye. This suggests that chondroitin sulfate is responsible in some way for the deposition of bone substance. In addition Sobel (1954) obtained a complex of chondroitin sulfate and collagen which, when placed in a test tube in solutions employed for *in vitro* calcification, produced 20% ash against control collagen giving only 0.1% ash. Here then, is a preparation which catalyzes deposition of bone salt without the mediation of an enzyme at all.

Where again does phosphatase come in?

Neuman, DiStefano, and Mulryan (1951) have obtained some results that suggest another possible function for the enzyme. They found that traces of ester phosphates are strongly adsorbed by the bone mineral. The phosphate added on to the crystal and the organic part of the molecule inhibited further crystal growth. Phosphatase, therefore, by hydrolyzing the ester phosphates would permit crystallization to occur on a template which would not otherwise take place in the presence of the latter type of compound.

One other complicating factor, they say, is that if the template itself contains phosphate esters then the phosphatase is capable of destroying it, but does not do so. However, Neuman has shown that in *in vitro* calcification at unphysiological concentrations of the ester the enzyme can be kept busy preferentially hydrolyzing other phosphate esters during the supposed formation of the template; in theory once this is formed and the first bone crystals deposited on it, the template is then protected from attacks by the enzyme. One cannot, of course, say that this is what happens *in vivo*.

It is of interest that bone contains a relatively large amount of citrate (see Chapter XI) and that this has an inhibitory effect on alkaline phosphatase (Baccari and Quagliarello, 1950).

However, another function of phosphatase is possible, and that is that it is concerned with the production and maturation of the protein matrix on which the bone salts are received. There are some findings which might be interpreted as providing evidence in favor of this. Fell and Danielli (1943) and Danielli, Fell and Kodicek (1943) showed that the first formed fibers in repair of skin wounds always contained phosphatase. Danielli (1951) has suggested that as this phosphatase disappears when the scar contracts, that is, at the time of maturation of the collagen fibers, the enzyme is concerned with the maturation of these fibers. The present author (1943, 1948) has shown that the first formed fibers in bone regeneration are also impregnated with phosphatase and that in fact if the fiber is formed at all it also contains the enzyme. Dietary experiments in which animals were deprived of vitamin C (see Chapter XVIII) showed that such deficiency could inhibit the production of fibers, but any which formed contained, as far as could be estimated by their histochemical reactions, a normal quota of enzyme activity. In this connection it should be remembered that Bloom and Bloom (1940) maintained that the property of calcifiability was conferred upon osseous tissue as it was deposited and that the actual process of deposition of calcium salts was simply a matter of these salts being deposited

into the osteoid when it had reached the appropriate stage of acceptability. Bradfield (1946) has also shown that a very active alkaline phosphatase is always present in the silk-spinning glands of insects. That the enzyme is associated with protein synthesis may also be suggested from its frequent presence in nucleoli which are important centers, if the available evidence is interpreted correctly, of protein synthesis. Casperson (1947), for example, has claimed that an increase in cytoplasmic basophilia associated with an increase in nucleolar size is a diagnostic feature of cells engaged in active protein synthesis. At the time of matrix formation in bone there is also a great increase in the alkaline phosphatase activity of the osteoblast (see Cappelin, 1948) and of cytoplasmic basophilia. McKelvie and Mann (1948) have found accumulations of alkaline phosphatase in periosteal fibrosarcomata in which bone was never formed and in polyostotic fibrous dysplasia in which there is a great overproduction of fibrous tissue which is rich in alkaline phosphatase activity (but see contrary results of Gomori, 1943). Also, Siffert (1951) has pointed out that the frequent association of alkaline phosphatase with the matrix in both cartilagenous and fibro-callus would indicate an association with the elaboration of cartilage and bone matrix rather than with their calcification. Furthermore, the presence of phosphatase in the uterus of the hen and the mantle of molluscs (Bourne, 1943; Wagge, 1951; Bevelander, 1952), the shell of which contains no significant amount of phosphates (Pelseneer, 1906; Turek, 1933), also suggests some connection with the formation of the protein matrix on which the calcium salts of the hen's egg and the mollusc shell are deposited, rather than with the deposition of these salts. In further support of this thesis is the work of Moog (1944), Rossi, Pescetto, and Reale (1951a, b & c) and Engel and Furuta (1942) who showed the widespread diffusion of phosphatase in embryonic soft tissues during the process of growth and differentiation; and that of Jeener (1947) who showed that alkaline phosphatase was associated with cell proliferations in organs stimulated by sex hormones.

A suggestion, purely speculative, regarding the role of phosphatase in fiber formation has been made by Neuman (personal communication). He points out that collagen fibrils can be dissolved up in acetic acid and that following removal of the acid the small units so formed may re-combine to form characteristic collagen fibrils. In this case it is necessary to ask why these small units do not combine within the cell itself. He suggests that they are synthesized and secreted as phosphate esters and that as they pass to the cell surface the phosphate is hydrolyzed off by extracellular phosphatase and the units then combine to form fibrils.

VI. Summary

The facts regarding the distribution of phosphatase in bone are summarized as follows:

In mature bone, the endosteum and the periosteum are phosphatase positive and in the latter nearly all of this reaction is localized in the inner layer of the membrane. The superficial osteocytes are positive and so is recently deposited bone matrix. Older osteocytes and bone matrix are negative.

In developing embryos condensations of mesenchyme destined to form membrane bone show phosphatase activity, those destined to form cartilage do not. Phosphatase does not appear in cartilage until just prior to ossification, in cartilage which never ossifies phosphatase is never found.

When cartilage ossifies the first phosphatase appears in the perichondrium near the regions where ossification of the matrix will first appear. The cartilage cells show signs of enzyme activity when they begin to hypertrophy; phosphatase appears at first in the nuclei and then spreads to the cytoplasm and the matrix. Once the matrix shows activity the cytoplasmic reaction decreases. Calcification appears to occur only in the presence of *extracellular* phosphatase. Periosteal osteoblasts appear to go through a cycle of phosphatase activity in growing bone. Originally possessing considerable phosphatase activity, they retain this activity when they eventually become surrounded by bone matrix and become young osteocytes; as they become older osteocytes they lose their phosphatase but under certain circumstances, particularly in breakdown of bone, they may show signs of renewed activity. Osteoclasts also contain some phosphatase (see Chapter VIII).

In the regeneration of bone and of skin wounds after injury, large numbers of phosphatase positive polymorphs migrate into the injured area within 24 hours. They are accompanied and followed by phosphatase positive fibroblasts which appear to produce a mass of phosphatase positive fibers. There is then a loss of phosphatase activity in the fibers. In the skin wounds this signals the virtual completion of the repair process; in bone injuries this is followed by a second cycle of phosphatase production associated with the production of calcified osteoid.

In addition to the association of phosphatase with bone formation, the enzyme is also present when the shell of molluscs and of hens' eggs are being laid down.

The views regarding the significance of phosphatase in bone formation are: (1) it is associated with production of phosphate ions which secure the precipitation of calcium as bone salt; (2) it is associated with the formation of the organic matrix of bone; (3) it is concerned with

the formation of a phosphate ester which acts as a template or part of a template for the catalytic crystallization of bone salt; (4) it keeps the surface of bone crystals free of ester phosphate, thus permitting continued growth of the crystals.

ADDENDUM

Sobèl (*3rd Intern. Congr. Biochem. Brussels*, 1955) showed that if a bone is decalcified in ethylenediaminetetraacetic acid then treated with calcium chloride and sodium hydrogen phosphate and finally placed in calcifying fluid it will recalcify. This mineralization appears to be as complete as in the normal bone. Sobèl believes that the first two processes (following decalcification) result in the formation of nuclei for initiating crystallization processes and that the calcifying fluid then deposits calcium phosphate on these nuclei. It appears from this that the actual process of calcification *in vivo* can, and probably does, take place without the intervention of phosphatase and adds further support to the conception that the enzyme is concerned with the production of the organic matrix.

PLATE I

FIG. 1. Repair tissue in healing hole in rat parietal. Note fibroblast-like cell in which both cytoplasm and nucleus give a very strong phosphatase reaction. An elongated phosphatase positive process (? fiber) can be seen extending a considerable distance from the cell.

FIG. 2. Mass of phosphatase-positive fibers in first stage of repair of damaged rat femur.

FIG. 3. Wave of phosphatase activity in cartilage matrix prior to ossification in association with damaged rat femur.

FIG. 4. Phosphatase-positive osteoid trabecula in second stage of repair in damaged rat femur.

FIG. 5. Phosphatase-positive osteocytes in bone. Note positive processes and also positively staining fibers extending from endosteum; taken from the compact bone of rat's rib near the marrow cavity. (Lorch, 1947 and 1949c. By courtesy of the author and the *Quarterly Journal of Microscopical Science*.)

FIG. 6. From 17 day mouse fetus. Sagittal section of the vertebral column, showing phosphatase in the hypertrophic cartilage at the stage of onset of ossification.

FIG. 7. From a 17 day mouse fetus. In the right hand bottom corner is the humerus and the radius; the ulna is to the left. Phosphatase can be seen in hypertrophic cartilage and in the perichondrium.

(Figures 6 and 7 are from Borghese (1953), by permission of the author and the *Zeitschrift für Anatomie und Entwicklungsgeschichte*.)

FIG. 8. Phosphatase in membrane bone of embryo pig head. Regions negative for phosphatase are those which have become calcified.

FIG. 9. Phosphatase in section of membrane bone of embryo pig head (decalcified and enzyme re-activated). Note positive osteoblasts lining the bone trabeculae and some positive osteocytes.

(Figures 8 and 9 are from Bevelander and Johnson (1950), by permission of the authors and the Wistar Institute of Anatomy and Biology.)

PLATE I

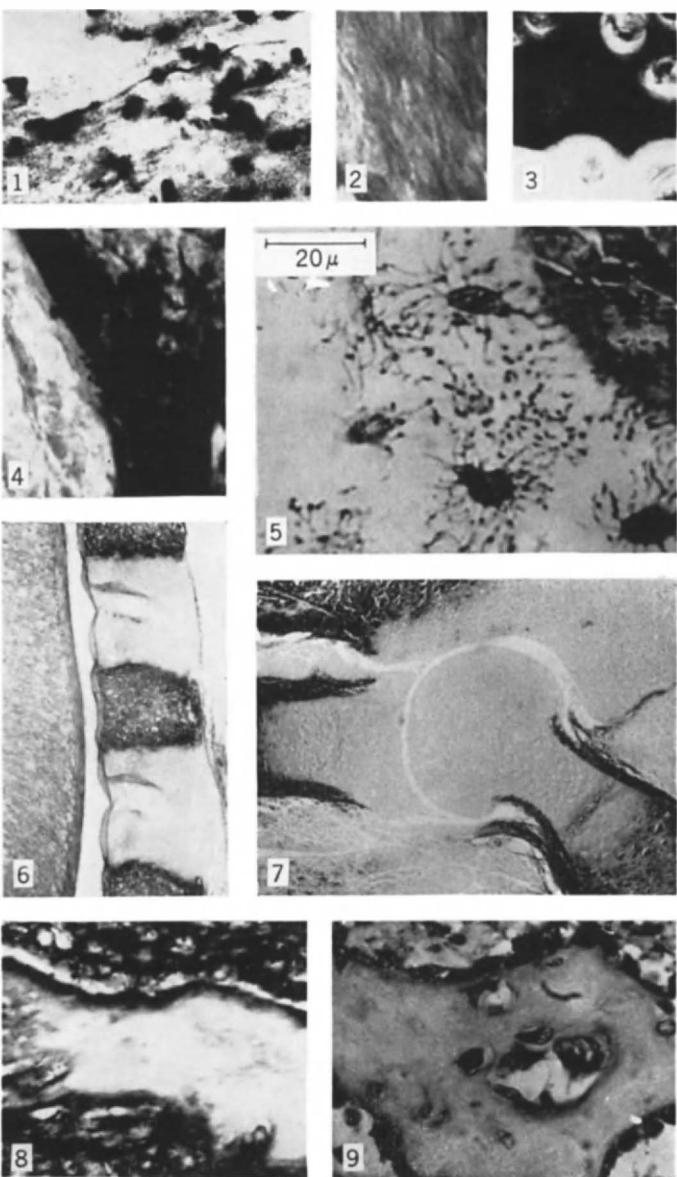


PLATE II

FIG. 10. Two dimensional chromatogram of an acid hydrolyzate of rachitic rat cartilage sprayed with ninhydrin. (Similar results have been obtained by the authors with trichlor acetic acid-extracted material and embryonic chick cartilage.) The arrow indicates the presence of a previously undescribed compound. If the hydrolyzate is treated with phosphatase this spot disappears. It appears to be a galactose amine phosphate ester and it is discussed on page 272 of the text.

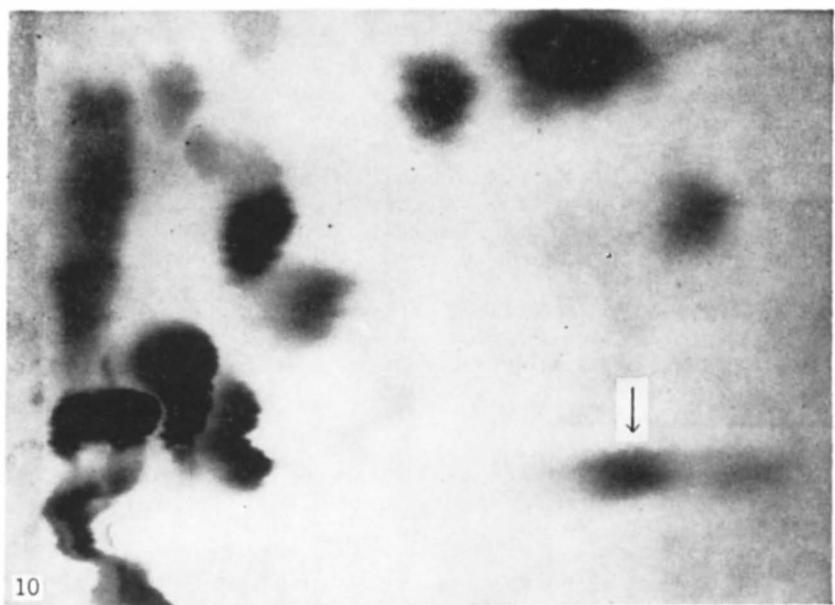
(By permission of Drs. DiStefano, Neuman, and Rouser, and the *Archives of Biochemistry and Biophysics*.)

FIG. 11. Phosphatase in a transverse section through the vertebral column of a 75-mm Scyliorhinus embryo (decalcified and re-activated specimen). The positive ring at the bottom of the photograph is the notochordal sheath. Note phosphatase is absent from the interior of the neural plates (enclosing the spinal cord) which has become calcified but is still present at the outer edges. This should be compared with the next figure.

FIG. 12. Phosphatase in a transverse section through the vertebral column of a 58-mm Scyliorhinus embryo. Calcification of the neural plates is just beginning and phosphatase can be seen extending through most of the thickness of the portion enclosing the spinal cord.

(Figures 11 and 12 are from Lorch (1949b), by permission of the author and the *Quarterly Journal of Microscopical Science*.)

PLATE II



10



11



12

PLATE III

The human osteocyte cycle in relation to alkaline phosphatase.

The scheme illustrated in this figure is a resumé of the evolution of the human osteocyte from the osteoblastic stage to that of necrobiosis (onkosis, see text). The distinction between the six stages is based on the morphological aspect of the cell and its alkaline phosphatase reaction. In each of the vertical columns the first three photographs represent the result of applying the Gomori technique to bone tissue decalcified by the Lorch method. The bottom figure in each column shows an osteocyte in the same stage as those above it but stained with hematoxylin and eosin.

I. Osteoblasts.

- (1) Resting. Three osteoblasts can just be distinguished.
- (2) Active. Showing intense phosphatase activity. Individual osteoblasts cannot be distinguished; black and white line represents a layer of osteoid.
- (3) In Paget's disease. Phosphatase is present in both nucleus and cytoplasm.
- (4) Appearance of osteoblasts with hematoxylin and eosin (bone is below them and marrow above).

II. Young osteocytes.

- (1) (2) (3) Young osteocytes are phosphatase-positive.
- (4) Stained hematoxylin and eosin.

III. Adult osteocytes.

- (1) (2) (3) Show loss of phosphatase activity by older osteocytes.
- (4) Appearance with hematoxylin and eosin.

IV. Beginning of necrobiosis. Reappearance of alkaline phosphatase.

- (1) Only slight morphological change in the osteocyte.
- (2) (3) More advanced stages. There is enlargement of the lacunar space in which the osteocyte lies and beginning of phosphatase reaction in the canaliculi.
- (4) Morphological changes and enlargement of the lacuna can be seen in hematoxylin and eosin preparation.

V. Established necrobiosis.

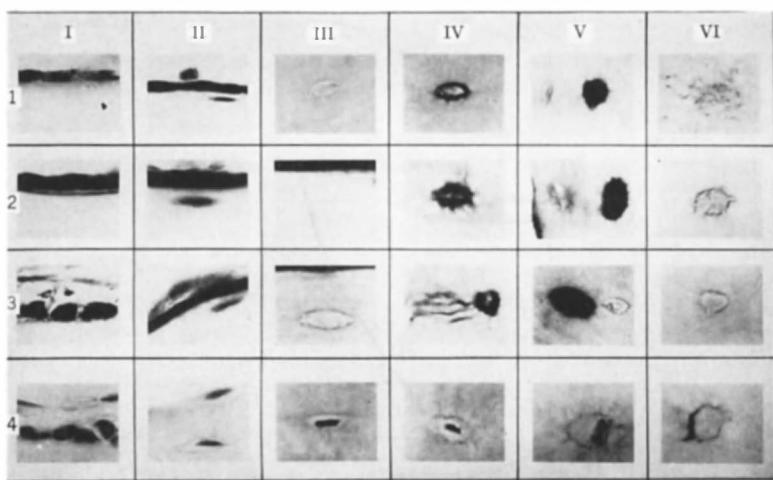
- (1) (2) (3) The phosphatase activity is now as intense as it was in the osteoblasts.
- (4) The hematoxylin and eosin preparation shows considerable enlargement of the lacunar space.

VI. End of necrobiosis.

- (1) At this stage the osteocyte has undergone lysis and phosphatase has disappeared except in the small amounts in the canaliculi.
- (2) (3) Empty lacunae.
- (4) The remains of the nucleus can be seen in the left of the lacuna.

(Reproduced by permission of Drs. Mäjno and Rouiller and *Virchow's Archiv, für pathologische Anatomie und Physiologie und für klinische Medizin.*)

PLATE III



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CHAPTER X

THE CHEMISTRY OF CALCIFICATION

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I. Nature of Bone Salt

1. CALCIUM AND PHOSPHORUS

Although most of the evidence (chiefly X-ray diffraction studies) indicates that bone salt has the general basic structure of an apatite its precise structure is still being debated. Whether the bone mineral is hydroxyapatite $3 \text{Ca}_3(\text{PO}_4)_2 \cdot \text{Ca}(\text{OH})_2$ with adsorbed carbonate, or tricalcium phosphate hydrate $3 \text{Ca}_3(\text{PO}_4)_2 \cdot 2\text{H}_2\text{O}$ with adsorbed calcium carbonate, or if the carbonate substitutes for phosphate at intervals in the crystal lattice, is still not clear. One of the difficulties is that the composition of bone salt and its quantitative relationship to the organic matrix carrying it is quite variable in different areas, species, and circumstances. Another is that the bone salt first formed may have the composition of secondary calcium phosphate (Hodge, 1950) or of pyrophosphate (Cartier, 1951a) and then may subsequently change on remodelling to the above salts. Again, it is possible that in bone part of the calcium and phosphate are not actually combined together at all but are in reality fixed in some periodic arrangement on the pre-osseous matrix, becoming joined and therefore demonstrable as apatite, only after the destruction of organic material which is usually carried out before any X-ray investi-

gations. Certainly bone has a very active metabolism, not as regards its gaseous exchange such as is measured by tissue respiration techniques, which give low values compared with other tissues, but as regards replacement of its mineral phase determined by isotope studies. For example, according to experiments with P^{32} in the rat, about 29% of the inorganic phosphate of epiphyses is renewed every 50 days. Nor is the deposition of bone salt, in all probability, the simple precipitation to which numerous investigators have attempted to apply the classical methods of physical chemistry, on the assumption that deposition occurred when the solubility product of calcium and phosphate in the circulating fluids was exceeded. The widely divergent views held on the composition of bone mineral are given in Table I, although it may be inferred from the above remarks and from the essentially dynamic state of bone salt that no empirical formula is likely to be found which will adequately express the complexity of combinations present in different sites under different conditions.

TABLE I
CONCEPTS OF BONE SALT COMPOSITION

Early concept	$Ca_3(PO_4)_2$ admixed with $CaCO_3$, CaF_2 , $Mg_3(PO_4)_2$, etc.
Berzelius, 1845	$Ca_5H_2(PO_4)_2$
Hoppe-Seyler, 1862	$Ca[(O \cdot PO_3Ca)_2 Ca]_2 CO_3$
Klement, 1929	$Ca(OH)_2[Ca_3(PO_4)_2]_3$ (with $NaHCO_3$, $KHCO_3$, $MgCO_3$)
Bogert and Hastings, 1931	$CaCO_3[Ca_3(PO_4)_2]_n$, $n = 2$ or 3
Bale, LeFevre and Hodge, 1936	$Ca(OH)_2[Ca_3(PO_4)_2]_3$ with $CaCO_3$
Gruner, McConnell, and Armstrong, 1937	$(OH_2Ca_6 \{ [(P.C)O_4]_6 (Ca \cdot C)_4 \})$
Hendricks and Hill, 1942	$Ca_{8.8}Mg_{0.23}Na_{0.19}[PO_4]_{5.07}(CO_3)_{1.24}2H_2O$
Sobel, Rockenmacher, and Kramer, 1945	$mCaHPO_4 \cdot n[Ca_3(PO_4)_2]CaCO_3$
Cartier, 1948; Dallemagne, 1952	$3Ca_3(PO_4)_2 \cdot H_2(OH)_2$ admixed with $CaCO_3$
Hendricks and Hill, 1950	$Ca_{10}(PO_4)_6(OH)_2$ with PO_4, CO_3, Mg , Na citrate

Before discussing the chemical composition of bone salt the properties of tricalcium phosphate in particular should be considered. Recently the nature of calcium phosphate itself has been debated and even the existence of the salt as a crystalline entity has been questioned. This uncertainty probably derives directly from the properties of calcium phosphate. For example, the smallness of its crystalline particles allows it to adsorb many other elements, particularly the reagents from which the salt is prepared in an aqueous medium. Since the substance diffracts

X-rays, its crystalline character is not questioned, although again, due to the smallness of its crystallites, the interference lines are very diffuse. Other confusing points are the actual definitions. Thus when a molten mixture of CaO and P₂O₅ in amounts approximating to Ca₃(PO₄)₂ is allowed to cool, the first crystals to separate out are called α -tricalcium phosphate and these have a characteristic diffraction pattern and refractive index. The second batch of crystals separating below 1350° C has again a different X-ray pattern and refractive index and is referred to as β -tricalcium phosphate. Also when the above mixture is heated the first crystal developing is the β -form and this changes on further heating into the α -form again. The term "hydroxyapatite" is sometimes applied not only to the true natural compound but also to tricalcium phosphate which has absorbed calcium hydroxide in about the same proportions as in the molecule of hydroxyapatite. The natural hydroxyapatite is a complex salt composed of tricalcium phosphate and calcium hydroxide, the relative proportions of which are indicated by 3 Ca₃(PO₄)₂ • Ca(OH)₂ or Ca₁₀(PO₄)₆(OH)₂, i.e. three molecules of tricalcium phosphate for one of calcium hydroxide. The naturally occurring substance gives the same diffraction pattern as tricalcium phosphate but the interference lines are fine, indicating good crystallization. Although hydroxyapatite is thermostable up to 1300° C, at this temperature water representing 1.79% of the original substance is eliminated. The refractive index of hydroxyapatite is high (between 1.64 and 1.65) and the Ca/P ratio is 2.14. The difficulty regarding the structure of synthetic tricalcium phosphate is that when calcium hydroxide and phosphoric acid are mixed in aqueous solution to give a Ca/P ratio corresponding to Ca₃(PO₄)₂, i.e. 1.94, the precipitate obtained has a Ca/P ratio which according to circumstances may vary between 1.94 and 2.79 (Greenwald, 1944). This has been explained by Klement (1938) and by Hodge, LeFevre, and Bale (1938) as being due to the non-existence of Ca₃(PO₄)₂ as such, the main substance being 3 Ca₃(PO₄)₂ • Ca(OH)₂, i.e. hydroxyapatite with a Ca/P ratio of 2.14. Lower values down to 1.94 indicate adsorption of phosphate ions and higher values are due to calcium hydroxide adsorption. This is supported by the following facts: (1) X-ray diffraction patterns of synthetic tricalcium phosphate and natural hydroxyapatite are identical, that of the former being unchanged after heating to 900° C. (2) Only occasionally does the analysis of the precipitate give values corresponding to the theoretical for Ca₃(PO₄)₂.

Another view (Dallemande, 1952) is that a precipitate with a Ca/P ratio of 1.94 would be pure Ca₃(PO₄)₂. A higher ratio would indicate some adsorption of Ca(OH)₂ but it would not be fixed in the crystal lattice unless the precipitate had been brought to a high temperature.

Now mineral hydroxyapatite is thermostable up to a high temperature whereas precipitated tricalcium phosphate loses water from 100 to 700° C until complete dehydration has occurred. The amount lost is variable, but 5% is a fair approximation in most cases. The X-ray pattern of this heated precipitate is very different from that of the original substance, indicating an important change in crystalline structure (Schleede, Schmidt, and Kindt, 1932). The conclusion has been reached by Dallemagne that tricalcium phosphate contains 2 molecules of water for every 9 atoms Ca, corresponding to 3.87% H₂O. This explains the isomorphism of the α -form of hydrated tricalcium phosphate [Ca₃(PO₄)₂]₃H₂(OH)₂ and the apatites [Ca₃(PO₄)₂]₃Ca(OH)₂. Analysis of the dehydration curve obtained between 100 and 700° C seems to indicate that there are two types of attachment of H and OH in α -tricalcium phosphate. Thus between 600 and 700° C the substance loses half a molecule of H₂O for every 9 atoms Ca and between 100 and 600° C about 1½ molecules or three times as much. The precipitate partly dried at 600° C is able spontaneously to recover the water which it has lost, whereas the completely dry substance obtained at 700° C is stable. The refractive index of α -tricalcium phosphate (1.590) differs from that of hydroxyapatite (1.64–1.65). When either α - or β -tricalcium phosphate is calcined at 900° C in the presence of the stoichiometric amount of Ca(OH)₂ one obtains hydroxyapatite with the same interference lines and refractive index as the natural substance. Also by mixing calcium hydroxide and phosphoric acid with an amount of water several times greater than that required to dissolve all the calcium hydroxide, the adsorption of Ca(OH)₂ is reduced or prevented and the precipitate obtained has a Ca/P ratio of 1.94 and consists of pure tricalcium phosphate. This view of Dallemagne that the main salt is α -tricalcium phosphate with adsorbed calcium hydroxide is not incompatible with the fact that β -tricalcium phosphate can be obtained by calcining at 900° C in the presence of dicalcium phosphate (CaHPO₄) or tricalcium phosphate with some adsorbed calcium hydroxide.

Various methods of isolating bone salt have been employed. Calcination has been used, but cannot be recommended since it always gives the same result no matter what temperature is used, apatite being formed from the combination of phosphate and carbonate. According to some calcification theories to be discussed later, calcium and phosphorus in bone may be separately fixed at periodic intervals on the pre-osseous matrix, and if this were so it would obviously not be possible to isolate bone salt from the organic component at all. Slices of sheep cartilage separately impregnated with both calcium and pyrophosphate do not in fact diffract for this reason. The classical method of Gabriel (1894),

which involves heating bone to 290° C with anhydrous glycerol containing 6% KOH, and then washing with methyl alcohol has the advantage that the mineral elements are not dissolved although calcium citrate may be changed to carbonate. Quantitative analyses, X-ray diffraction patterns, and refractive indices agree with the view that bone salt isolated by Gabriel's method is α -tricalcium phosphate. Calcination of bone salt at 900° C results in the formation of either hydroxyapatite or carbonate apatite, depending on whether CO₂ can or cannot escape. In the latter case it becomes impossible to separate the carbonate fraction from the phosphate fraction by any chemical method. Dehydration curves which relate the water loss of bone salt and of tricalcium phosphate after heating similar samples to various temperatures, are similar in the range 100 to 500° C but in the range above 600° C there is a discrepancy between the two curves. Tricalcium phosphate loses some more water, whereas bone salt does not. The probable explanation for this is that at 600° C calcium carbonate is decomposed, forming calcium oxide (a process which is complete in a ventilated oven.) The water released from tricalcium phosphate then combines with calcium oxide forming calcium hydroxide and thus the synthesis of hydroxyapatite is made possible.

Treatment of bone residue, prepared by Gabriel's method and containing the mineral part, with dilute 0.1N HCl was found by Logan and Taylor (1938) to release 50% or more of its carbonate with most of its sodium and magnesium and only 10% of its phosphate. If the same bone residue be heated to 600° C for 24 hours and then immersed in CO₂-free water, the aqueous phase immediately becomes strongly alkaline (pH 12) and after it is neutralized and separated from the solid it is found to contain all the sodium and magnesium, but little calcium and no phosphorus.

Analogies have been drawn between the composition of bone salt on the one hand and various natural and synthetic carbonate fluorapatites on the other. The advantage of studying these natural substances is that the processes of their formation have been much slower than the biological process for the formation of bone salt, as a consequence of which these materials can be obtained in much larger and better defined crystals whose properties can be more precisely determined. On the basis of such studies on francolite, a natural carbonate-fluorapatite, McConnell and coworkers have put forward arguments against the view that bone salt is tricalcium phosphate with some adsorbed calcium carbonate (McConnell and Gruner 1937, 1940; Gruner, McConnell, and Armstrong, 1937; McConnell, 1952). If francolite were merely a mixture of submicroscopic or colloidal particles of calcium carbonate and fluorapatite,

subtraction of the calcium oxide and carbon dioxide from the molecular ratio of francolite should give that of fluorapatite, which is not the case. Neither does francolite give any of the X-ray diffraction lines associated with calcium carbonate. Instead the carbonate is thought to substitute for phosphate at intervals in the crystal lattice although the fluorine positions of fluorapatite have been completely filled by fluorine in francolite.

Bone mineral is certainly microcrystalline and presents a surface area estimated to be between 100 and 200 square meters per gram of bone (Neuman, 1950). This very large surface of the microcrystallites helps to explain the rapid replacement and exchange of Ca and PO₄, which have been extensively studied by the use of radioactive calcium and phosphorus (see Chapter VII). When such materials are introduced into a living animal, two processes are responsible for their subsequent appearance in bone. Firstly, the circulating calcium or phosphorus, radioactively labelled, may exchange by non-vital mechanisms with atoms of the same elements already present in the bones, and secondly, any newly deposited bone salt will also contain a proportion of labelled atoms. *In vitro* experiments have shown that about 20% of the calcium in bone is rapidly and reversibly exchanged (Falkenheim, Underwood, and Hodge, 1951) and in a similar way phosphorus is also absorbed by bone *in vitro* (e.g. Falkenheim, Neuman, and Hodge, 1947). There is considerable evidence that this type of exchange is related to the inherent calcifiability of the part of the bone concerned, newly calcified regions, or those surrounding Haversian canals, showing the greatest uptakes. It seems likely that the mucopolysaccharide level of the organic matrix is closely related to the readiness with which the tissue will take up mineral elements. Furthermore, although cells and enzymes no doubt play a part in producing this calcifiability, they are not essential to the final stages of the process (Amprino, 1952).

2. OTHER ELEMENTS IN BONE

a. Sodium

From earlier analyses quoted by Shohl (1940) it had been known that there was a considerable amount of sodium and water in bone. Thus a 70 kg man was estimated to have in his bones about 23 g of sodium equal to 30% of his body sodium and about 4 liters of water equal to 8% of total body water. These earlier figures of bone sodium are probably low since sodium is partially co-precipitated with calcium oxalate during the removal of calcium prior to the determination (Edelman, James, and Moore, 1952; Davies, Kornberg, and Wilson, 1952). More recent figures by the latter authors particularly by the isotope dilution method have indicated up to 7 g of sodium per kg dry bone. In order

to find out how much of this bone sodium was exchangeable Edelman *et al.* used as an index the ratio of the specific activity of tissue sodium (^{24}Na) to serum sodium after distribution equilibrium had been obtained. This ratio was unity in 24 hours in all tissues except bone wherein it was one half, showing that tissues other than bone exchange sodium freely with blood. The possibility that the low result with bone was due to a slower rate of reaching equilibrium was ruled out by repeating the experiments for longer periods with ^{22}Na which has a 3-year half-life as compared with the 15-hour half-life of ^{24}Na , since a falling serum sodium activity could introduce an artefact in the tissue-serum ratio. It was thus shown that only one half of bone sodium is in fact freely interchangeable and can contribute to the metabolic sodium pool. Thus in dogs and man 45% of bone sodium exchanged in 4 hours, but during periods up to 1 month there was little further change. On the other hand, bone water had exchanged completely with serum water in 4 hours (Edelman *et al.*, 1954). The ratio of sodium in association with chloride to total bone sodium was between 0.1 and 0.2; thus free extracellular sodium based on chloride does not account for all the exchangeable sodium, some of which must therefore be in the matrix or combined for example with citrate. There is probably a large proportion of the bone sodium on the surface of the apatite lattice.

b. Fluorine

The administration of fluoride to the body by various routes leading to its absorption into the blood stream causes a prompt disposal either by urinary excretion or by skeletal deposition in nearly equal quantities and in 2 or 3 hours the process is practically complete. Whereas the fluoride content of blood and soft tissues is normally of the order of a few micrograms per 100 g, that of the skeleton may reach 100 mg/100 g. The body fluid fluoride concentration may be the controlling factor in the hypoplastic changes shown by ameloblasts associated with the fault in enamel formation known as mottled enamel, and the amount of fluoride deposited in the tooth mineral may be a critical factor in dental caries. In contrast to tooth defects, in the development of osteosclerosis from excessive fluoride intake the concentrations of both body fluid fluoride and skeletal fluoride may be important, since on the one hand fluoride may influence the production of osteoid and the enzyme processes occurring in calcification and on the other the mineral crystals containing fluoride may differ sufficiently in their physicochemical properties from those of hydroxy or carbonate apatite to contribute materially to the condition.

In some respects the deposition of fluoride in the skeleton may be analogous to that of other substances such as heavy metals like lead, and may be regarded as a detoxification mechanism whereby these sub-

stances are removed from the general circulation. Although the development of an equilibrium state would be expected on low intakes of fluoride, the very low solubility of calcium fluoride and fluoroapatite causes skeletal retention on all except the lowest intakes (Machle and Largent, 1943) and a gradual increase in fluorine content has been demonstrated in human bone with increased age. Thus Smith, Gardner, and Hodge (1952) found 20 mg/100 g in the 70-80 year group. The rapid deposition may be regarded as an exchange of fluoride for hydroxyl or bicarbonate on the surface of the mineral crystal (Neuman *et al.*, 1950; Megirian and Hodge, 1951). A slower process of incorporation of fluoride ions into the crystal lattice may be due to recrystallization or to resorption and replacement of bone occurring during growth by osteoblastic and osteoclastic activity. This accounts for the gradual storage of fluoride over periods of years on quite low intakes. The fluoride once incorporated into this crystal lattice is comparatively stable and not readily mobilizable. In this respect its behavior is analogous to that of sodium and calcium as determined by radiosodium and radiocalcium retention. There is no doubt that raised fluoride intakes are harmful. A case described by Linsman and McMurray (1943) had mottled tooth enamel and fluoride osteosclerosis, abnormal Haversian pattern and osteoid matrix due to ingestion of drinking water from an area in Texas containing 12 ppm fluoride compared with the more usual concentrations of around 0.1 ppm. Other cases of excessive fluoride intake may occur in workers in the cryolite (sodium aluminum fluoride) industry by dust inhalation resulting in deposits in tendons and muscle sheaths, arthritis and rigidity of the spine, and marked osteogenesis with some resemblance to advanced Paget's disease. Since, as described later, fluoride at a concentration of $10^{-4} M$ (1.9 ppm) blocks a stage in the glycolytic cycle concerned in calcification, it is probable that some of the effects of fluoride may well be due to interference with the enzyme systems in addition to its entry into the apatite lattice.

c. Magnesium

The presence of magnesium in bone has been mentioned above in the analyses of Hendricks and Hill (1942, 1950), and animal experiments have shown that variations in dietary intake produce changes in the bones as well as in other tissues. Sometimes diets low in magnesium which have no lasting effect on the behavior of other tissues are found to produce brittle bones depleted in magnesium, accompanied by calcification of the kidneys (Watchorn and McCance, 1937). The mode of influence of magnesium may be related to its effect on the deposition of bone salt. Thus Kuyper (1945), in experiments without the use of tissues, showed that magnesium increased the solubility of a precipitate similar

in composition to bone salt. Also, magnesium is well known as an activator of alkaline phosphatase, which is therefore another agent by which its influence could be exerted.

There seems to be no doubt that magnesium slightly inhibits the *in vitro* calcification of rachitic tibia slices (Sobel and Hanok, 1952). In addition, however, it has a profound influence on the effectiveness of other inhibitors, which will be discussed later. It was found by Subrahmanyam, Duckworth, and Godden (1939) that hydrolysis of bone matrix by trypsin did not alter the magnesium content, which suggests that bone magnesium belongs to the inorganic component, rather than being associated with the proteins.

II. Relation of Bone Composition to Diet

Reference has already been made to the variation in bone composition which can be produced by dietary means. Although the work most of this work has been on animals and does not necessarily apply directly to human calcification and bone salt formation, there is a clear implication that the various factors may have an influence in man. For example, Sobel, Rockenmacher, and Kramer in a series of papers (1944, 1945a, b) have shown quite clearly in rats that variations in calcium, phosphate, and carbonate in the diet affect the concentrations of these substances in the blood plasma, which in turn affect the amounts present in the bone. These authors consider that the bone Ca/P ratios on the high phosphate low calcium diets indicate a proportion of the bone salt in the form of CaHPO_4 , but they have nevertheless been unable to demonstrate the presence of this substance (Hirschman, Sobel, and Fankuchen, 1953). However, CaHPO_4 might still be the first aggregate formed since it is converted to apatite when placed in solutions used for *in vitro* calcification. The variations in bone composition influenced by vitamin D which are described in detail in another section involve both the composition of the bone salt itself and the proportion of inorganic substance and organic matrix present. Vitamin D affects the plasma levels of calcium and phosphate by influencing absorption of these substances from the gut and by improving the reabsorption of phosphate from the kidney tubule. There is little evidence that vitamin D directly affects the activity of the osteoblasts and osteoclasts as has been shown with vitamin A (cf. Chapter XVII).

III. Cartilage and Bone Matrix

The material between the cartilage cells and the bone cells is usually referred to as cartilage matrix on the one hand and bone matrix on the other. It consists of fibers and interfibrillar material and its composition

has a profound influence on calcification processes. There appear to be two principal components of cartilage matrix, a protein, collagen, and a mucopolysaccharide, chondroitin sulfate. The collagens derived from skin, cartilage, and bone have a similar amino-acid composition characterized by high glycine and proline-hydroxyproline contents, and appear to be related. Some justification thus exists for applying observations made on skin collagen to cartilage and bone collagen. Much information on the molecular structure of skin collagen has been obtained by Bear (1942) by the use of X-ray diffraction, and a periodicity of 640 Å has been demonstrated. This was subsequently shown by the electron microscope as alternate light and dark bands across the longitudinal axis of the fibers by Schmidt, Hall, and Jakus (1942). These varying techniques can be interpreted as showing that the fibers as seen microscopically consist of aggregates of polypeptide chains each having a thickness of 10-11 Å and about 800 Å long. Nageotte and Guyon (1930) and Faure-Fremiet and Garrault (1937) first showed that certain types of collagen, for example rat tail tendon and fish swim bladder, are soluble in dilute acid. When such clear, viscous solutions are neutralized or when salt is added, a fibrous precipitate occurs which microscopically has a strong resemblance to natural collagen fibers. If such dilute acid collagen solutions are dialyzed before precipitation, a structureless gel with no fibrils is obtained. If, however, various mucoprotein solutions are added before precipitation, collagen fibrils are formed with either the 640 Å spacing or with a periodicity of 2200 Å, called by Highberger, Gross, and Schmidt (1950, 1951) "long spacing" fibrils. It seems likely that collagen and mucopolysaccharide occur loosely bound in tissues and that the latter is responsible for the presence of the bands seen under the electron microscope.

Cartilage has one property in common with other tissues in which there are mucopolysaccharides, that is the property of being metachromatic. Thus if formol-fixed cartilage sections are stained with certain basic dyes (usually toluidine blue) some components of the tissue stain blue but the cartilage on the whole takes up red stain. On staining a fresh-frozen unfixed cartilage section with toluidine blue there is a little different distribution, in that the metachromasia or blue stain tends to be more aggregated about the cartilage cells. This tendency can be changed by enzymatic digestion with proteolytic enzymes so that the matrix loses the metachromatic stain. Bone is also metachromatic, showing that its constituents are related to those of cartilage. The polysaccharide of bone responsible for the metachromasia is probably chondroitin sulfate and has not been studied so extensively as that from cartilage, due to the difficulties of isolation in the presence of the large

amount of inorganic material. It had been originally thought by Lison (1936) that a substance needed to have a high molecular weight and to contain sulfate ester groups such as are present in chondroitin sulfate, heparin, and the mucoitin sulfates of cornea and gastric mucosa, in order to stain metachromatically. However, it has recently been found that a sulfate group is not necessary since highly polymerized hyaluronic acid shows metachromatic staining which immediately disappears on treatment with hyaluronidase (Lillie, Emmart, and Laskey, 1951). Sylvén (1945) has shown that metaphosphate is metachromatic and that cellulose which has been partly oxidized to glucuronic acid units is also. Cobb (1953) demonstrated glycoprotein in regions of normal bone and cartilage from which glycogen had previously been removed. The glycoprotein is considered to be relatively less polymerized in areas in which initial deposition of calcium salts occurs. Thus the ground substance of rachitic osteoid contains much slightly polymerized glycoprotein.

Siffert (1951) advanced the view that cartilage matrix is used in the formation of bone matrix. Rubin and Howard (1950) suggested that chondroitin sulfate plays a specific and important part in calcification in which its acidic groups help to fix calcium prior to the formation of bone. Dziewiatkowski (1949, 1951a, 1951b) injected S^{35} as $Na_2S^{35}O_4$ into rats and visualized by the radioautographic technique to obtain further supporting evidence. For example, after two hours S^{35} was present throughout the entire humeral epiphysis reaching the highest concentration in the epiphyseal-diaphyseal junction. The area of most intense deposition was associated with the more mature cartilage cells just prior to the stage when they disintegrate to give way to bone. Sulfate in bone can be separated into two fractions, (a) the major portion, insoluble in acid formol, representing chondroitin sulfate and (b) the minor portion insoluble in formol saturated with baryta, which is mainly inorganic sulfate. Since in the above experiments of Dziewiatkowski the tissue was fixed in acid formol the S^{35} observed was assumed to indicate labelled chondroitin sulfate. It can be inferred from these results that calcification is associated with an accelerated turnover of chondroitin sulfate. The S^{35} accumulating in the epiphyseal cartilage does not leave the cartilage quickly, in contrast to the inorganic fraction of the S^{35} , and this confirms that there is no non-vital exchange of sulfur.

IV. Calcium Binding of Cartilage

The calcium binding property of cartilage was demonstrated by Freudenberg and Gyorgy (1921) using cartilage with a low mineral content taken from areas relatively far from actively calcifying centers,

They concluded that calcium was bound first and phosphate only secondarily. Later Roche and Deltour (1943), using fairly well mineralized cartilage, found that the reverse was true and that phosphate was the main ion absorbed. The investigations of Neuman, Boyd, and Feldman (1952) were made on calf costal cartilage pulverized and almost completely demineralized with ethylenediamine tetraacetate. On passing solutions of various cations through columns of this demineralized cartilage powder, about 1 meq cation/g was bound whether calcium, sodium, or barium was used. The sulfate content of the powder was sufficient to account for exactly half the cation bound. They suggested that the carboxyl group of glucuronic acid accounted for the other half of the binding capacity. A reduction in chondroitin sulfate content by treatment with potassium carbonate solution produced a corresponding reduction in cation binding. Phosphate on the other hand was not taken up unless the cartilage had been previously treated with calcium chloride solution and then placed in phosphate buffer. The cartilage then took up phosphate giving a Ca/P ratio of 2/1, approximating that of bone salt. This same cartilage was again placed in calcium chloride solution when further calcium was taken up. When this preparation was again placed in phosphate buffer the phosphate uptake was much greater than expected and it seems that once cartilage has been mineralized it will absorb phosphate to a degree greater than can be accounted for by the molecule of apatite. The results of Roche and Deltour can thus be explained by absorption of phosphate by the preformed mineral present and a definite basis laid down for the role of chondroitin sulfate in calcification. It is clear from such experiments that bone salt can be made to deposit selectively under conditions which preclude enzyme action and cell participation. Thus Waldman (1950) has demonstrated that even after boiling or treatment with heavy metals rachitic cartilage will accumulate mineral deposits if immersed long enough in a fluid containing sufficiently high concentrations of Ca and P. It is significant that in these cases the bone salt is not precipitated diffusely but occurs in those areas where deposition would normally take place. The X-ray diffraction pattern of apatite even occurs after incubating tricalcium phosphate in the lumen of a chick femur which has been completely decalcified in nitric acid.

Even so, specific cellular activities may still be necessary for calcification in the living organism since deposition occurs only in areas of the organic matrix which are predisposed by virtue of their property of calcifiability, which is acquired during growth and cell differentiation. The concentration of calcium and phosphate in the incubating fluids in these *in vitro* experiments are also usually greatly in excess of those

obtaining in the tissue fluids of the living organism, and in order to effect calcification in the presence of the *in vivo* ion concentrations cellular activity might be expected to be needed. Thus while deposition of bone salt depends on the solubility characteristics of calcium phosphate, yet specific cell activities may be required to confer the calcifying property to the matrix, provide the necessary enzyme systems, and make available sources of energy.

V. In Vitro Calcification

Much valuable information on enzyme systems has come from the *in vitro* calcification technique used by Robison (1923) and by Shear and Kramer (1928) in which slices of rachitic rat tibial epiphysis are incubated in various media and the deposition of bone salt subsequently visualized by the von Kossa silver stain. It must be remembered, however, that this technique relates only to endochondral calcification with doubtful bearing on problems of endosteal, periosteal, and various pathological forms of soft tissue calcification. Its use even in the study of endochondral mechanisms has been criticized on the grounds that the test object, the rachitic rat epiphyseal cartilage, is already in a pathological condition and processes studied by its use are not necessarily parallel with those occurring in normal rat epiphyseal cartilage. Cartier (1950) has used slices of sheep embryo cartilage to overcome the latter objection and instead of the silver stain, which is really a test for inorganic phosphate, has weighed the slices and determined their Ca/P ratio so that an indication of the nature of the bone salt laid down is obtained as well.

Bone salt is derived from four species of ions present in the circulating fluids: Ca^{++} , H_2PO_4^- , HPO_4^{--} , and HCO_3^- and in accordance with the solubility rule calcium phosphate and calcium carbonate are precipitated from aqueous solutions whenever the products of the concentrations of the component ions exceed the values for their respective solubility products. Since calcium precipitation does not normally occur in the circulating plasma either the solubility product is not exceeded or the plasma is supersaturated with respect to the above ions. Only about half the calcium present in blood plasma is in the free ionic form, the remainder being mainly bound to protein. At the normal reaction of plasma most of the phosphate and carbonate are present as $\text{H}_2\text{PO}_4^- + \text{HPO}_4^{--}$ and HCO_3^- , the amounts of PO_4^{---} and CO_3^{--} being very low. For calcium to be deposited in growing bone some "local" mechanism must operate to raise the concentration of PO_4^{---} and CO_3^{--} . Robison (1923) found that calcifying cartilage is very rich in alkaline phosphatase (pH optimum about 9) and he was able to bring about calcification of

rachitic bone slices by immersing them in solutions of Ca^{++} and glycero-phosphate or hexosephosphate. According to his original theory calcification is due to the local liberation of PO_4^{---} ions from the organic phosphate esters of plasma by the action of the phosphatase (bone enzyme) secreted by the osteoblasts. In support of Robison's theory, rachitic cartilage slices calcify *in vitro* at the ossifying zone if immersed in solutions of calcium glycero- or hexosephosphate. Phosphatase appears in ossifying zones coincident with the beginning of ossification and is more abundant in these zones than elsewhere. Teeth of young animals also exhibit high phosphatase activity (associated with odontoblasts). In general non-ossifying cartilage, e.g., trachea, shows little phosphatase activity. In generalized bone disease such as hyperparathyroidism, Paget's disease, rickets, etc., plasma phosphatase rises markedly above the normal, presumably due to a diffusion of phosphatase from the bones, since the rise is roughly proportional to the severity. Other metals besides calcium which form insoluble phosphates such as lead, strontium and radium are deposited in, and mobilized from, bone by the same conditions as affect calcium.

The chief objections to this simple theory were that non-calcifying tissues such as kidney, lung and intestine are not deficient in alkaline phosphatase (although it is perhaps significant that they are known to calcify under pathological conditions) and rachitic cartilage itself is rich in phosphatase. The bone enzyme only attacks monophosphate esters which in blood are mainly located in the red cells whose content of these esters is raised during bone growth and lowered in rickets. In 1926 Shipley, Kramer and Howland observed that calcification of rachitic cartilage slices could occur when immersed in aqueous solutions containing calcium and phosphate showing that preformed organic phosphate esters were not essential, although in this case such calcification was inhibited by cyanide, iodoacetate, and fluoride. The effects of these inhibitors are described below in the discussion on the part played by glycogenolysis. Although the action of alkaline phosphatase is obscure, there can be little doubt that some interrelation with calcium deposition exists. Part of its action may be on the synthesis of fibrous proteins described in the "Phosphatase" chapter. (cf. Davidson, 1949; Chapter IX).

VI. The Role of Glycogenolysis

The deposition of bone salt in endochondral calcification is preceded by definite morphological changes in the cartilage cells, which swell to become hypertrophic, and by corresponding alterations in the nearby matrix which at first shows increased basophilia and metachromasia

(Rubin and Howard, 1950) later losing these properties. There is also an increase in alkaline phosphatase and other enzymes. Bone salt is first deposited in the so-called "provisional calcification zone" of the cartilage, beyond the disintegrating cartilage cells. This layer is not reached by the capillary loops of the spongiosa and is thus bathed only in intercellular fluid and is not subjected to contact with the high content of organic phosphate esters in the red blood cell. Transition of cartilage to the hypertrophic state has been known for a long time to be parallel with the accumulation in the cytoplasm of large amounts of glycogen, which disappear either just before or simultaneously with the appearance of bone salt in the adjacent cartilage. In markedly rachitic cartilage Follis (1949) has demonstrated that glycogen is present only in a narrow zone of hypertrophic cartilage cells of recent origin and absent from the older cartilage cell area. Intraperitoneal injection of inorganic phosphate into such rachitic animals leads to calcification only in the glycogen rich cartilage areas. Cobb (1953) has shown that phosphorylase is present in those cells which are known to contain glycogen at some stage of their development. Harris (1932) postulated a connection between glycogen and calcification, suggesting that perhaps hexose phosphate from glycogen breakdown might be the substrate for the alkaline phosphatase in the original Robison theory. Mainly from the work of Gutman using *in vitro* calcification techniques, a large amount of evidence has come forward indicating that phosphorylative glycogenolysis is indeed needed for cartilage calcification. He has used selective inhibitors of enzymes known to be active in the process of anaerobic phosphorylative glycogenolysis in muscle, for example, phloridzin, iodoacetate, and fluoride known for their blocking action at the stages: glycogen → glucose-1-phosphate, 1:3-diphosphoglyceraldehyde → 1:3-diphosphoglyceric acid and 2-phosphoglyceric acid → phosphopyruvic acid respectively. The inhibitors of muscle glycolysis also block *in vitro* calcification of cartilage. Phloridzin, for example, at a concentration of $10^{-2} M$ inhibits cartilage phosphorylase (Gutman and Gutman, 1941) and in its presence calcification *in vitro* fails when phosphorus is supplied as inorganic phosphate and only succeeds when supplied as glucose-1-phosphate, glucose-6-phosphate, fructose-1:6-diphosphate or 3-phosphoglycerate, all of which phosphate esters are formed in the glycolysis cycle subsequent to the phloridzin block. This work has been confirmed by Marks and Shorr (1950) who removed glycogen from the hypertrophic cartilage cells with ptyalin, and found that calcification with inorganic phosphate no longer occurred. Adding glucose-1-phosphate restored the ability to calcify even in the presence of phloridzin. Calcification is also prevented by iodoacetate and fluoride (Robison and Rosenheim, 1934). Iodoacetate is now

known to inhibit the 1:3-diphosphoglyceric aldehyde dehydrogenase. In the presence of iodoacetate, therefore, calcification does not take place if the above glucose or fructose phosphates are added but succeeds if phosphoglycerate is present (Gutman, Warrick, and Gutman, 1942; Gutman and Yu, 1949, and Gutman and Gutman, 1941). Fluoride acts by inhibiting enolase, which catalyses the formation of phosphopyruvate from 2-phosphoglycerate, a still later stage in the glycogenolytic cycle. The effect of fluoride is complicated by its interaction with magnesium, since Goldenberg and Sobel (1951) have shown that in the absence of magnesium, 10^{-3} M fluoride enhances, rather than depresses, the degree of *in vitro* calcification. All the other enzymes of the glycogenolytic cycle except those following enolase have been demonstrated to be present in calcifying cartilage, see Table II). It is likely that pyruvate under the low oxygen tension prevailing in calcifying cartilage is converted to lactic acid. This has been shown manometrically to accumulate when glucose is added anaerobically to cartilage homogenates, and has also been shown to be present in sheep embryonic cartilage (Cartier, 1951b).

TABLE II
SUBSTRATES AND ENZYMES OF THE GLYCOCOGENOLYTIC CYCLE WHICH APPEAR TO BE PRESENT IN CALCIFYING CARTILAGE (Gutman, 1951)

Substrates	Enzymes
Glucose	Hexokinase
Glycogen	Phosphorylase
Glucose-1-phosphate	Phosphoglucomutase
Glucose-6-phosphate	Isomerase
Fructose-6-phosphate	Phosphohexokinase
Fructose 1:6-diphosphate	Aldolase
Dihydroxy acetone phosphate }	Triose isomerase
Glyceraldehyde-3-phosphate }	
1:3-Diphosphoglyceraldehyde	1:3-Diphosphoglyceraldehyde dehydrogenase
3-Phospho glyceric acid	Triose mutase
2-Phospho glyceric acid	Enolase

The presence of a reasonable amount of citrogenase, by which the acetyl group of acetyl coenzyme A (formed from pyruvate or acetate) is condensed with oxalacetate, has been shown in epiphyseal cartilage by Dixon and Perkins (1952) but not a comparable amount of the isocitric dehydrogenase which is the next stage in the citric acid cycle. These authors suggest the lack of the latter enzyme as a reason for the accumulation of citrate, the subsequent incorporation of citrate in the bone mineral taking place by co-precipitation with calcium phosphate.

The demonstration of these enzyme systems leaves little doubt that phosphorylative glycogenolysis must play a part in calcification, and

Gutman suggests that it may be a device for building up the local concentration of phosphate, not necessarily inorganic but perhaps of an as yet unidentified phosphate ester which is then used for bone salt formation. In view of the high content of 5-nucleotidase in cartilage and its relatively greater activity at pH 7 in this tissue than alkaline phosphatase, Reis (1950) suggested that this system might be an important one for bone salt formation particularly as adenosine monophosphate forms a good substrate for the *in vitro* calcification technique. However, Dixon and Purdom (1954) were unable to demonstrate any increased serum nucleotidase activity in various bone diseases although big increases were obtained in liver disease. This still does not rule out the possibility that 5-nucleotidase may play an important part in the calcifying mechanism, the significant feature being the greater activity shown by cartilage to hydrolyze 5-nucleotides such as adenosine-5-phosphate at pH 7 compared to the alkaline phosphatase activity of the cartilage at the same pH. Acid phosphatase is present in bone and plasma in amounts smaller than those of alkaline phosphatase and although the former may not be concerned with bone salt deposition, there is some suggestion that it may be active in the dissolution of bone. For example, Kochakian (1952) has shown an increase in bone acid phosphatase after parathyroid injection. Klendshoj and Koepf (1943) have demonstrated an increase in serum acid, as well as alkaline, phosphatase in hyperparathyroidism with a prompt fall after removal of the parathyroid tumor, and the older observations of Huggins and Hodges (1941) and of Gutman, Sproul, and Gutman (1936) indicated that in patients with prostatic carcinoma, serum acid phosphatase is not greatly raised until the metastases and dissolution of bone occur. In severe Paget's disease the acid as well as the alkaline phosphatase of serum may also be raised. Reference has already been made to the work by Cartier (1950, 1951a), (Cartier and Picard, 1951) on the *in vitro* calcification of normal sheep embryo epiphyses, in which he found limited deposition in the presence of glycerophosphate, glucose-1-phosphate, fructose diphosphate, phosphoglyceric acid or mineral phosphate but very rapid calcification with adenosine triphosphate. With rachitic rat epiphyses Cartier did not find, however, the same degree of calcification produced by adenosine triphosphate, thus agreeing with the investigations of Robison and of Gutman. Cartier concludes that the system using adenosine triphosphate is necessary for calcification of normal cartilage and results in the release of pyrophosphate which precipitates with calcium and is the first bone salt formed. The pyrophosphate may saturate the matrix and become fixed for a relatively short time and then when the local concentration of calcium is sufficient calcium phosphate may precipitate. Cartier has obtained

Ca/P atomic ratios of near unity by direct analyses of the incubated slices, which support the concept of a calcium pyrophosphate compound. A possible explanation of Cartier's findings may be that adenosine triphosphate plays a role in converting the pre-osseous matrix to calcifiable matrix, providing a source of energy. Albaum, Hirshfeld, and Sobel (1952) have also found reasonably high amounts of adenosine triphosphate in epiphyseal cartilage compared with other tissues. Perkins (1954) has found high activities of adenosine triphosphatase in homogenates, particularly in the metaphysis (ratio metaphysis:epiphyseal plate, 2.9:1).

An inhibitor of calcification whose mode of action is not yet fully explained is beryllium. Klemperer, Miller and Hill (1949) and Grier, Hood, and Hoagland (1949) showed that beryllium, which is known to give rise to a type of toxic rickets, inhibits alkaline phosphatase. Gutman and Yu (1951) showed that alkaline phosphatase of a rat epiphyseal cartilage homogenate was inhibited to a maximum of 72% by 10^{-4} M beryllium, whereas glycolysis, observed manometrically, was not inhibited even by 10^{-3} M beryllium. Thus 10^{-4} M beryllium inhibits calcification when phosphorus is supplied as ester phosphate, but not when it is in the form of inorganic phosphate. Hiatt and Shorr (1951) confirmed that 10^{-4} M beryllium inhibited *in vitro* calcification when phosphate ester was the sole source of phosphorus, but found nevertheless that at the pH used (7.4) beryllium made no difference to the amount of inorganic phosphorus liberated from the ester. It seems possible therefore that the effect on calcification is not mediated via phosphatase, but is related to some other part of the deposition mechanism.

VII. Reversible Inhibition of the Calcifying Mechanism

Work carried out by Sobel and coworkers over the last decade (Sobel, 1952) has helped to throw further light on the local factor by studying the reversal and inhibition of the calcifying mechanism. The starting point of these investigations was a comparison of the behavior of rachitic epiphyseal cartilage slices from calcium rickets and strontium rickets. When strontium carbonate replaces calcium carbonate in a rachitogenic diet, rickets develops which is histologically similar to calcium rickets but which does not respond to vitamin D, nor do the strontium rachitic bone slices calcify *in vitro* unless they have been shaken with calcium chloride. Sobel concludes that one of the essential factors is the combination of calcium with something in the matrix with which strontium can compete since it is known that strontium is a mineralizing ion. The inactivation of the calcifying mechanism by incubating slices in salt solutions other than calcium (i.e. Be, Cu, Mg, K, Na) and reactiva-

tion by shaking with calcium again is interpreted to mean that combination of something in the matrix with calcium is a necessary preliminary step to ossification. Conversely to the inactivation experiments, exposure of the bone sections to calcium concentrations much greater than physiological enabled the matrix to become combined with calcium to a much greater extent so that when placed in the calcifying medium a greater deposition of bone salt than ever was obtained. An attempt to show whether chondroitin sulfate or a similar compound is the calcifying factor was made by using inhibitors which have a mucopolysaccharide sulfate as a specific target. Protamine is known to combine readily with chondroitin sulfate and it was found to inhibit the *in vitro* calcification depending on the calcium-protamine ratio in the basal solution. Protamine appears therefore to act by competing with calcium at some point in the process of calcification.

VIII. Conclusion

Attempts to formulate calcification theories in terms of solubility products have been unsuccessful and bone salt deposition is probably not a simple precipitation. Cellular activities may be required to provide enzymes and supply energy as well as to confer calcifiability on the matrix. The wheel has turned full circle and the early theories by Freudenberg and Gyorgy of calcium binding by the ground substance are receiving consideration again. (See also Chapter IX.)

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CHAPTER XI

CITRIC ACID AND BONE

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I. Introduction

Before considering in detail the relationship between citric acid and bone, the importance of citric acid as an intermediate metabolite in tissues generally will be indicated. Citrate was shown by Sherman, Mendel and Smith (1936) to be a product of endogenous metabolism, since it persisted in the urine even during starvation, although as Dickens (1940, 1941) subsequently pointed out, such conclusions are not completely justified without parallel bone analyses. In 1943, however, Class and Smith performed an experiment in which oral administration of sodium bicarbonate or malate to rats led to increased excretion of citrate, without any concomitant change in the bone content.

Citrate occupies a key position in the metabolic pathway of oxidation of carbohydrate, fat and protein in mammalian tissues. Acetyl coenzyme A produced by breakdown of these foodstuffs is condensed enzymatically with oxaloacetate to form citrate. Citrate is then partially oxidized to oxaloacetate, water and carbon dioxide by the series of enzymatic reactions which together comprise the tricarboxylic acid cycle (Krebs and Johnson, 1937). The oxaloacetate so formed is then available to condense with a further molecule of acetyl coenzyme A. Only those steps of the cycle most closely related to citrate will be discussed further. Aconitase is the enzyme responsible for the interconversion of citrate,

cis-aconitate and *iso*-citrate. With pure enzyme, equilibrium is reached when the proportions of the three acids are citric 90%, *cis*-aconitic 4%, *iso*-citric 6% (Krebs and Eggleston, 1943). *Iso*-citrate is oxidized to oxalosuccinate by *iso*-citric dehydrogenase. In the presence of fluoracetate some oxaloacetate condenses with fluoracetate instead of acetyl coenzyme A, forming a fluorocitric acid which inhibits aconitase and so prevents the further metabolism of citrate. This results in a large accumulation of citrate in the tissues, especially those in which citrate formation is normally most active, e.g. kidney. Fluoracetate poisoning, therefore, indicates a large turnover of citrate in the body.

Although by far the greatest proportion of body citrate at any one instant is found in the bones (Dickens, 1941), this comparatively inert reservoir may be affected by happenings in other tissues in which citrate is more labile. In the following sections an attempt is made to assess the relationship between the various aspects of citrate metabolism in the body, and their effect on bone.

II. The Citrate Content of Bone

The presence in bones of a comparatively large amount of citric acid was only recently detected. Most of the earlier analyses of bone were carried out on bone ash, in which the citrate originally present probably appeared as an addition to the carbonate fraction. Early work on tissue citric acid levels was confined mainly to soft tissues and body fluids, the determination being by the enzymic method of Thunberg (1929). This method is not readily applicable to a tissue such as bone, in which citrate occurs in a form only slightly soluble in water. Thunberg, however, stressed the association of citrate with calcium in the body, noting particularly that semen, which contains about five times as much calcium per 100 ml as serum, also has a high citrate concentration (Schersten, 1929, 1936). This association of citrate and calcium was later found to apply to calcium deposited in bone.

Dickens (1940, 1941) estimated citric acid in bone by the chemical method of Pucher, Sherman, and Vickery (1936), and showed that bone contained an amount of citrate large compared with that found in other tissues. In an analysis of a whole mouse the skeleton contained 70% of the total citric acid. Dickens also found that most of the citric acid in bone was located in the calcified regions, the marrow having only a small content. Citrate was not readily extractable by water even from steamed bone meal. (The durability of the citrate bound in bone was shown by Thunberg (1947), who examined medieval and prehistoric bone, and found amounts of citric acid slowly declining with increasing age.) Dickens showed, too, that the compound estimated in bone was

indeed citric acid by isolating the free acid from bone meal. The citrate content of the bones of several species was estimated by Thunberg (1941). His results were similar to those of Dickens but the values observed differed quite widely from one species to another. Thunberg (1948) found high citrate levels in the bones of gulls and values up to 5% of the dry, fat-free bone in the herring. In dry, fat-free human bone a citrate content of 1.6–1.8% was found in femur, clavicle, and rib. The vertebrae contained 0.71% citrate in males and 1.11% in females. In various bones of the rat, Class and Smith (1943) found citrate values of 5–7 mg/g wet tissue. The relation between age and bone citrate in the rabbit was studied by Cartier (1951) who found that during growth from 130 g to 750 g body weight the citrate content of the long bones stayed close to 0.9% of the dry weight, whereas the lactic acid content decreased steadily with increasing age from 0.26% at 130 g body weight to 0.1% at 700 g. This steady diminution of bone lactic acid content with increasing body weight and mineralization of the bones was also observed in rats. He carried out a similar investigation with sheep embryos, determining the citrate content of the epiphyseal cartilage and compact diaphyseal cortex of the long bones. In diaphyseal bone the citrate content was once again unaffected by age but in epiphyseal cartilage it rose from a very low value in the youngest embryos taken to one about one fifth of that found in hard bone in the eldest. These differences very roughly paralleled the observed changes in ash content. In a further experiment on the epiphyses, metaphyses and diaphyses of the long bones of rabbits at various stages of growth (from 0.5 kg to 2 kg) he observed substantially similar citrate contents throughout, with one low value for the epiphyseal region in the youngest rabbit. Some variation of citrate content with bone region was observed in young rabbits (0.6–0.7 kg) by Dixon and Perkins (1952) who found somewhat higher citrate levels in the diaphyseal cortex of long bones than in the metaphyses or epiphyseal line cartilage, but their results were expressed in terms of wet tissue. It seems probable that in normal animals of a given species the ratio of citrate to calcium in calcified parts of the bones is more or less constant. In this connection some results on teeth are also of interest. Zipkin and Piez (1950) examined large numbers of human teeth, and found mean values for citric acid content of 0.89% in dentin and 0.10% in enamel. In contrast to the picture in bone, the more highly calcified enamel contained a far lower proportion of citrate than dentin. It must be remembered however, that the circumstances of calcium deposition in teeth are very different from those obtaining in bone.

One other point related to the occurrence of citrate in bone may be of interest. There is no evidence that bone contains either *iso*-citrate or

aconitate, although aconitase, which catalyses the interconversion of citrate, aconitate and *iso*-citrate, has been demonstrated in bone (Dixon and Perkins, 1952). The methods used for the analysis of bone may not have been sensitive enough to detect these acids, or it may be that these acids, unlike citric acid, are not co-precipitated with bone salt.

III. Complex Formation Between Citrate and Calcium

The fact that citrate forms a soluble complex with calcium which effectively removes Ca^{++} ions from solution is of importance in several aspects of calcium metabolism. Calcium bound to citrate probably forms the bulk of the ultrafilterable, non-dissociated fraction of serum calcium (less than 1 mg/100 ml). This unionized calcium is not available for such purposes as the transmission of nerve impulses. The reabsorption by the kidney tubules of the calcium-citrate complex may be different from that of either of its components. Also, by binding calcium in this way, citrate can promote the solution of bone salt even at comparatively high pH values, and consequently can prevent the precipitation of calcium phosphate from solution by reducing the Ca^{++} ion concentration.

The first direct evidence for complex formation between calcium and citrate was obtained by Shear and Kramer (1928), who titrated trisodium citrate solution against sodium chloride and calcium chloride solutions, and measured the conductivity changes. In the presence of calcium an abnormal decrease of conductivity was observed, the minimal value being reached when the molecular ratio of calcium chloride added to sodium citrate was 1.35:1. Using the frog-heart as a means of measuring Ca^{++} concentration Hastings *et al.* (1934) obtained a value of 3.22 for the dissociation constant (pK) of a complex anion represented by CaCit^- (pH 7.4 and 22° C). They concluded that tricalcium dicitrate was almost completely dissociated as follows:



By an indirect method they showed that magnesium behaved similarly to calcium in respect of complex formation, since addition of magnesium ion tended to displace calcium from the complex. This interaction of magnesium with the calcium-citrate complex was discussed by Nordbo (1938). Further investigation by a conductivity method was carried out by Hennig, Schmahl and Theopold (1951), who showed that calcium, strontium, and barium formed complexes with citrate of stability increasing in that order, each complex being a monovalent anion containing one metal atom and one citrate residue. As regards the structure of the complex, Hastings *et al.* (1934) originally suggested that the calcium atom was linked to two adjacent carboxyl groups of the citric acid molecule,

thus forming an asymmetric complex, whereas Hennig and Theopold (1951) proposed a symmetrical complex in which calcium was linked to the two terminal carboxyl groups. Some additional evidence about the nature of the complex was obtained by Muus and Lebel (1936) who showed that the complex of calcium with citric acid (β -hydroxytricarballylic acid) was fifty times more stable than that with tricarballylic acid, an observation which Schubert and Lindenbaum (1950) confirmed by ion-exchange methods.

In addition to the soluble complex anion CaCit^- discussed above, Muus and Lebel (1936) suggested that a soluble complex Ca H Cit , ($pK = 2.3$) was formed. This complex would presumably be more readily formed at lower pH values, when more of the citric acid is present as H Cit^+ ions, and would account for the fact that calcium will form a complex with citrate even at pH values as low as 3.5 or 4, such as are employed in histological decalcifications. Whatever the precise form of the complexes, it is evident that they are relatively undissociated and that their influence on the relationship of citrate and calcium in the body must be profound.

IV. Citrate and Calcium in Blood and Urine

Since both calcium and citrate are present in bone salt and since they form a soluble poorly-ionized complex, it is hardly surprising that changes in their blood and urine levels have been found to be to some extent mutually dependent. The picture is complicated by the fact that the concentration of citrate in the blood is dependent not only on the absorption and excretion of preformed citrate and its deposition in bone, but also on the relative rates of its formation and destruction within the tissues. These rates are large and might be expected to be the major factors determining changes in the blood level of citrate.

The normal range of blood citric acid concentration in man was found by Schersten (1931) to be 1.5–4.0 mg/100 ml whole blood, and figures in this range have frequently been confirmed by more modern analytical methods, e.g. Harrison and Harrison (1952a). There is evidence that most of the blood citric acid is located in the plasma rather than in the red blood corpuscles. It has been found that when sodium citrate is added to whole blood only a small proportion of the citrate enters the red cells (Taussky and Shorr, 1947) and Martensson (1940) observed about five times as much citrate in the serum as in the corpuscles.

On the whole changes in blood level of calcium and citrate run parallel, whether caused by nephrectomy, injection of neutral citrate or calcium chloride (Chang and Freeman, 1950), injection of parathyroid

extract (Alwall, 1944) or oral administration of vitamin D (Freeman and Chang, 1950). An exception to this was observed by Shohl and Butler (1939) and Harrison and Harrison (1952b) who found that administration of a large dose of an equimolar mixture of sodium citrate and citric acid to rachitic infants on a vitamin D-deficient diet induced a fall in serum calcium, but no great change in serum citrate.

In the urine the picture is more complicated. Oral administration of large amounts of free citric acid caused no appreciable change in urinary citrate in man (Ostberg, 1931), nor did half-neutralized citric acid (Harrison and Harrison (1952b)). On the other hand, intravenous administration of citric acid neutralized with sodium led to a rise in urinary citrate in man (Shorr *et al.*, 1942) and in the dog (Gomori and Gulyas, 1944; Chang and Freeman, 1950). In the dog but not in man urinary calcium was also increased. It has been shown by Ostberg (1931), Sherman *et al.* (1936), and Krusius (1940) that urinary citrate is dependent on the acid-base balance of the body. Citrate excretion increases with a rise in the pH of the urine, and decreases with a fall (Schreier, 1948; Shorr *et al.*, 1948). The increase in urinary citrate induced by injection of neutralized citric acid was probably not solely due to the alkalinizing effect of the treatment, since Chang and Freeman (1950) showed that injection of an equivalent amount of NaHCO_3 did not produce the same effect. When excess calcium was given orally in man (Ostberg, 1931) or intravenously in the dog, (Chang and Freeman, 1950) a rise in urinary calcium accompanied by a fall in urinary citrate was observed. In a hyperparathyroid subject, however, Shorr *et al.*, (1942) found that an increase in dietary calcium led to a rise in urinary calcium and a parallel rise in urinary citrate. Milne (1953) explained the above observations as follows:

- a. The clearance of citrate by the kidney increases as plasma citrate rises.
- b. Free citric acid is more easily reabsorbed by the kidney tubule than the citrate ion. Therefore citrate clearance will increase with rising pH of the urine, since dissociation of citric acid increases with rising pH, becoming maximal at pH 7.0.
- c. The calcium citrate complex is less well reabsorbed than free citric acid, and hence urine citrate rises when urine calcium rises.

Thus in a normal man Milne found a linear relationship between urinary citrate and calcium, the urines having pH values in the normal range, the excretion of each atom of calcium being accompanied by 0.18 molecules of citric acid.

Since the ratio of calcium to citrate in serum and hence in glomerular filtrate is such that calcium is normally in excess of the amount required

for complex formation with the citrate, a change in citrate excretion with a change in calcium excretion would take place only if a change in serum citrate level had also occurred. In contrast to Milne's observations, Ostberg (1931) and Chang and Freeman (1950) found that a rise in urine calcium might be accompanied by a fall in urine citrate.

The fact that serum citrate tends to rise when serum calcium rises might be explained on the assumption that formation of the calcium citrate complex protects citrate from oxidative removal in the tissues, e.g. kidney. However, Perkins (unpublished experiments) found that calcium, even in amounts equivalent to all of the added citric acid, did not affect the oxidation of citrate by minced kidney *in vitro*.

On the whole it appears that the relationship between urinary calcium and citrate would repay further investigation, as the mechanism of the mutual influence of these substances is by no means clear.

V. Citrate and Calcium Deposition, *in Vitro* and in Bone

As described above citrate and calcium occur in bone salt, the two substances form a soluble poorly ionized complex and changes of calcium and citrate contents of blood and urine may be interdependent. Evidence concerning the co-deposition of calcium, phosphate, and citrate, *in vitro* and in bone will now be considered. Although citrate at any given pH tends to increase the solubility of calcium phosphate precipitates by complexing calcium, Kuyper (1938) discovered that small concentrations of citrate could be completely co-precipitated with calcium phosphate. In 1945 Kuyper extended this work, and examined the co-precipitation of calcium, phosphate, carbonate, and citrate, from solutions with a range of concentrations similar to those occurring in serum. The amount of citrate precipitated under these conditions was about 0.7–1.3 molecules/100 atoms of calcium, which is similar to the amount found in bone (i.e., 0.9 molecules/100 atoms of calcium). It would therefore seem likely that citrate in bone has been derived from citrate in tissue fluid by co-deposition. It must, however, be remembered that the components of bone salt as they occur in serum do not precipitate at all outside the bones, except in pathological conditions. Some special mechanism is required in bone to bring about the deposition of bone salt from serum, and it is likely that this mechanism influences the co-deposition of citrate, too.

The effect of citrate upon the calcification of rachitic rat bone slices *in vitro* was studied by Shipley, Kramer, and Howland (1926), who found that calcification was inhibited if calcium were supplied as calcium citrate. They deduced that the removal of Ca^{++} ions by citrate reduced the availability of calcium for calcification, and Dixon and Perkins (1952)

found that calcification was effectively inhibited when citrate equivalent to only a small part of the available calcium was added to the medium. These results should be compared with those of Harrison and Harrison (1952b) who administered oral doses of half-neutralized citric acid to rachitic infants. The serum citrate and phosphorus remained low, while the total serum calcium actually decreased and serum from the patient produced no calcification of a rachitic rat tibia slice ($\text{Ca} \times \text{P}$ product 20–26, each concentration expressed in mg/100 ml). Nevertheless, under these conditions X-ray signs of clinical healing of the rickets were observed. These authors suggest in explanation of this paradox that citrate may increase the calcifiability of the rachitic cartilage *in vivo*, by restoring its citrate content to normal levels (see Section VI). The observed fall in the blood level of calcium might be due to its deposition in bone at a rate greater than its absorption from the intestine. It seems strange, however, that great changes in the calcifiability of cartilage should be brought about by citrate without any previous rise in the blood citrate level. The *in vitro* experiments mentioned above suggest that increased citrate would tend to decrease rather than increase the calcification of pre-osseous cartilage.

The fact that excess citrate in the surrounding medium inhibits *in vitro* calcium phosphate deposition in rachitic bone slices suggests that local increases in citrate concentration in bone produced by cellular activity might lead to dissolution of bone salt, and thus play a part in the processes of bone modelling.

VI. Citrate, Rickets, and Vitamin D

The influence of citrate on rickets is naturally related to the mode of development of the disease. It is known that rickets may be caused by lack of calcium or phosphorus passing into the blood stream from the intestine, a condition which in the absence of vitamin D is favored by extremes of calcium/phosphorus ratio in the diet. Indeed, experimental rickets can only be produced in rats deprived of vitamin D if extreme calcium/phosphorus ratios are used in the diet. The deficient element is excreted in the feces as insoluble calcium phosphate. The deposition of bone salt is probably also directly influenced by vitamin D, and rickets is characterized by bones calcified only in the parts laid down before development of the condition and by hypertrophic, relatively uncalcified growing regions. Rickets may also be produced by a type of kidney dysfunction, in which failure of the tubules to reabsorb phosphorus leads to hypophosphatemia. There are three main processes by which citrate may influence the course of the disease; by affecting

the absorption of calcium from the intestine, the excretion of calcium in the urine, and the deposition of bone salt.

The effect of citrates on calcium metabolism of human adults not suffering from lack of vitamin D was studied by Farquharson *et al.* (1931). They found that daily oral administration of 6 or 12 g of sodium citrate produced only a very slight effect on calcium excretion in the urine and no appreciable effect on either the calcium or phosphorus balance. Nevertheless, healing of rickets caused by administration of citrates was observed by Hamilton and Dewar (1937) and Shohl (1937). The former authors found that addition of sodium citrate to a high-calcium low-phosphorus rachitogenic diet could either prevent development of rickets in rats, or cure previously developed rickets. The free acid was effective, too, but less so than the sodium salt. They also found that when administered in the form of the calcium salt, citrate had no antirachitic effect. The difference between calcium and sodium citrate was thought possibly to be due to an effect on intestinal pH. Shohl (1937) related the antirachitic effect of citrate to the ability of the anion to form a complex with calcium, and Heinz, Müller, and Rominger (1947, 1948) suggested that formation of a soluble complex in the intestine facilitates the absorption of calcium and increases the diffusible calcium available for deposition in bone. An improvement in calcium absorption following oral administration of citric acid was also observed in a case of Fanconi syndrome (Schreier and Wolf, 1950). Shohl and Butler (1939) administered mixtures of 2 parts citric acid and 3 parts sodium citrate to rachitic infants and found X-ray evidence of healing, even though the $\text{Ca} \times \text{P}$ product in the serum was still below 30 (each concentration expressed in mg/100 ml), i.e. considerably less than the value normally considered necessary for calcification to occur. In the work of Harrison and Harrison (1952a, b) mentioned earlier rachitic infants were given a citrate supplement similar to that used by Shohl and Butler. No changes in serum citrate, phosphorus, or phosphatase levels were found but the treatment led to an initial fall of serum calcium by about 2 mg/100 ml. After some time X-ray evidence of healing of rickets was observed, although the $\text{Ca} \times \text{P}$ product remained low. It seems possible that apparent X-ray evidence of healing in such cases may be due to a redistribution of calcium already present in the bones, facilitated by the citrates administered. Glanzmann, Meier, and Walthard (1946) recommended the use of citrate therapy in cases of so-called calcium-poor rickets, characterized by marked osteoporosis or osteomalacia, in cases of vitamin D resistant rickets and in cases of ordinary rickets with considerable lowering of the serum phosphorus. They found that five daily doses of 5 ml of 5 to 10% citric acid were suffi-

cient to cause rapid improvement in craniotabes although sometimes treatment was not complete without further administration of vitamin D. This clinical trial was based on animal experiments in which rachitic rats maintained on a high-calcium low-phosphorus diet were healed by the incorporation of 0.4 mol sodium citrate into each kilogram of diet. Similar quantities of lactate had only a very slight effect. In experiments with Ca⁴⁵ Gordonoff and Minder (1951) found that the lowered rate of calcium exchange observed in bones and teeth of rachitic rats as compared with normals was not raised by the inclusion of citrate in the diet, even though such treatment prevented the development of clinical rickets. The same authors (1952) showed that treatment of rickets with vitamin D, on the other hand, not only caused clinical healing but also brought the calcium exchange back to normal. The mechanism of the healing influence of citrate upon rickets still remains an unsolved problem.

Apart from its direct effect upon the progress of rickets, metabolism of citrate has been shown to be affected by vitamin D. Thus Freeman and Chang (1950) showed that supplying vitamin D orally to normal and to parathyroidectomized dogs led to a rise of both calcium and citrate in the serum. Harrison and Harrison (1952a) found that vitamin D caused a rise of both serum and urinary citrate in rachitic infants. Bellin and Steenbock (1952), working with normal or low-phosphorus rachitic rats, found a rise of urinary citrate and calcium after administration of vitamin D, irrespective of changes in urinary pH induced by inclusion of NaHCO₃ or NH₄Cl in the diet. These results, which show parallelism in the urinary excretion of citrate and calcium, should be contrasted with the findings discussed in Section IV, in which increased calcium ingestion led to increased excretion of calcium and decreased citrate excretion.

The concentration of citrate is altered in rachitic bones. It was observed by Dickens (1941) that the citric acid content of dry fat-free bone from a rachitic kitten maintained on a low-calcium diet, was only about 50% of that of a normal animal, although the calcium, phosphorus, and carbonate contents were not greatly different in the two. Nicolaysen and Nordbo (1943) also found that in animals maintained on a vitamin D-deficient, high-calcium low-phosphorus diet the bone citric acid contents were only about 25% of those in normal controls. Steenbock and Bellin (1953), however, found only a slight lowering of bone citrate in rachitic rats maintained on a low-calcium diet, and no change with a high-calcium diet. They did, however, find an increase in bone citrate, along with that of many other tissues, when physiological doses of vitamin D were added to the rachitogenic diets.

A rachitogenic diet may lead to a lowered serum citrate (Harrison

and Harrison, 1952a) but the difference is not great or invariable (Harrison and Harrison, 1952b) and does not seem to be sufficient to account for large changes in bone citrate.

VII. Citrate Metabolism in Bone

In view of the close connection between calcium metabolism and citrate it is reasonable to suppose that local changes in citrate concentration brought about by enzyme action may affect the deposition and resorption of bone salt. The oxidation of added citrate by bone slices was first demonstrated by Follis and Berthrong (1949), using the methylene blue technique. Dallemande (1951) discussed the significance of citrate in bone and commented on the likelihood of the presence of citric acid cycle enzymes there, but failed to demonstrate citrate accumulation in bone after fluoracetate poisoning. As described above citrate formation from oxaloacetate in kidney homogenate was increased by fluoracetate (Liebecq and Peters, 1948, 1949; Kalnitzky, 1949), the reason being that fluoracetate is condensed with oxaloacetate to form fluorocitric acid, which blocks the tricarboxylic acid cycle. Peters has since demonstrated that the fluorocitrate formed inhibits aconitase and so prevents the conversion of citric acid to *iso*-citrate (Lotspeich *et al.*, 1952; Peters *et al.*, 1953a, b; Morrison and Peters, 1954). Buffa and Peters (1949) injected fluoroacetate into rats, pigeons and guinea pigs, and showed that citrate accumulated in various tissues, the greatest increase being observed in the kidney. The degree of accumulation in any tissue might be taken as a rough indication of the normal activity of the condensing enzyme in that tissue. Following this work Beaulieu and Dallemande (1951) and Lindenbaum, White, and Schubert (1951) determined bone citrate in fluoroacetate poisoning, but found no significant increase. Since, however, Buffa and Peters (1949) found in kidney an increase of only 1 mg citrate/g wet tissue and bone is known to contain about 7–10 mg citrate/g wet tissue, it is evident that even if bone were as active as kidney in this respect, it would still be difficult to demonstrate citrate accumulation by such a technique. In 1952 Dixon and Perkins were able to demonstrate the activities in bone of three enzymes of the tricarboxylic acid cycle, namely condensing enzyme, aconitase, and *iso*-citric dehydrogenase. They found fairly high activities for the first two enzymes in the epiphyseal plate and metaphyses of rabbit bone, and compared the levels with those in kidney and liver from the same animal. The levels of *iso*-citric dehydrogenase activity were relatively much lower in bone than in the other tissues, and it was tentatively suggested that citrate formed enzymically might be co-precipitated with bone salt before it could be further oxidized, thus accounting for part of the citric

acid in bone. Although the activity of condensing enzyme is quite high in the bones of young animals, Perkins and Dixon (1953) showed that in the rat it fell rapidly with age, decreasing fourfold between the 7th and 24th week.

Since it has been shown that active citrate metabolism can occur in bone itself, the possible influences of this must be taken into consideration whenever alterations of bone citrate level are discussed. Citric acid cycle enzymes may have considerable influence on deposition and resorption of bone salt.

VIII. Bone, Citrate and Hormones

The influence on bone of various hormones is discussed in other chapters, but it is pertinent to consider here any possible secondary effects of hormones due to interference with citrate metabolism, assuming that changed citrate levels may affect the excretion of calcium or its deposition in bones.

1. PARATHYROID HORMONE

Dickens (1941) showed that prolonged administration of parathyroid hormone to a puppy led to a value for bone citrate 27% higher than in a normal control. In rats, Perkins and Dixon (1953) could find no appreciable difference in bone citrate levels of normals and animals parathyroidectomized 4 weeks previously. They studied the effect of parathyroidectomy on the citrogenase activity (i.e. formation of citric acid from oxalacetate and acetate) of minced rat bone, and found that 4 weeks after operation the activity was very low compared with that of normal animals of the same age, although they could observe no change within the first 4 days after operation. Massive injections of parathyroid extract into normal or parathyroidectomized animals did not produce any immediate rise in citrogenase activity.

Parathyroid hormone has been shown to affect the serum levels and renal excretion of citrate as well as phosphate and calcium. Shorr *et al.* (1942) found that injection of parathyroid extract to a hypoparathyroid human subject caused parallel increases of urinary calcium and citrate similar to those observed when urinary calcium is raised by other methods (see Section IV). Alwall (1944) showed that injections of parathyroid extract to dogs led to increases in both calcium and citrate of the serum while Freeman and Chang (1950) found that the increase in serum calcium and citrate following nephrectomy in dogs did not occur in animals thyroparathyroidectomized some weeks previously. However, in view of the extensive turnover of citrate in the body as a whole such experiments do not indicate whether or not bone is directly involved in

the changes in citrate levels observed. Alwall's findings, however, of high serum citrate after parathyroid extract injection would accord with the raised citrate content of bone from a similarly treated animal observed by Dickens. The work of Perkins and Dixon (1953) suggests a connection between parathyroid hormone levels and citrate formation in bone, but the effect is far too slow to account for the rapid changes in serum calcium brought about by the hormone.

2. SEX HORMONES

A connection between the menstrual cycle and citric acid excretion was first shown by Shorr, Bernheim, and Taussky (1942) who found maximal excretion at ovulation, with a marked fall premenstrually. Administration of estrogens to subjects with ovarian insufficiency led to a rise in urinary citrate excretion. Progesterone alone was not found to have any effect, but seemed to enhance the effect of estrogen. Androgens, on the other hand, were found to decrease citrate excretion in both men and women. In further studies with a hypogonadal man as subject, Shorr *et al.* (1948) observed that administration of testosterone propionate caused a 50% fall in citric acid excretion, and a simultaneous rise in its reabsorption by the kidney. These observations indicate an enhancement of citrate excretion by female hormones, and a reduction by male hormones. Edwards, Gray, and Wood (1950), although confirming the existence of a regular variation in citrate excretion during the menstrual cycle, found no correlation between urinary citrate and urinary estrone-estradiol. Administration of estradiolbenzoate to a post-menopausal woman produced no significant change in urinary citrate and its administration to a girl with ovarian agenesis caused only a slow rise in citrate excretion. Although morphological changes have been induced in bones by sex hormones (e.g., Day and Follis, 1941), no information is available on any concomitant alterations in bone citrate.

IX. Summary

The evidence for a connection between the metabolism of calcium and citrate has been reviewed. The soluble complex formed between these two substances can be co-precipitated with bone salt. It seems likely that the incorporation of citrate into calcified bone may have the effect of making bone salt more easily soluble and therefore potentially more mobile. Evidence of the precise stage at which citrate is involved in the deposition of bone salt is lacking, since citrate cannot readily be demonstrated histologically. It is also difficult to prove that citrate plays any part in causing local dissolution of bone salt. Assuming, however, that resorption takes place without appreciable change of pH, the

equilibria governing the stability of precipitated bone salt must be affected by the presence of a substance such as citrate which will remove calcium in the form of a soluble relatively nondissociated compound.

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CHAPTER XII

AUTORADIOGRAPHIC STUDIES OF BONE FORMATION AND GROWTH

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I. Introduction

Bone consists of crystals of calcium phosphate held within an organic matrix of collagenous fibers and carbohydrate material. For bone to form and grow, there must be an addition of both minerals and matrix. The purpose of the present chapter is to examine information related to this dual type of growth obtained by use of the autoradiographic technique (also called radioautographic technique).

The pioneer isotopic investigations of mineral acquisition by bone were carried out by Lomholz (1930), Behrens and Baumann (1933), and Copp, Axelrod, and Hamilton (1947) using lead isotopes (radium D, thorium B). Pecher (1942) employed radiostrontium for the same purpose. Today, the ready availability of radiophosphorus (P^{32}) and radio-calcium (Ca^{45}) has rendered obsolete the use of less physiological "bone-seeking" elements, and recent comprehensive works on the subject have been carried out either with P^{32} (Leblond, Wilkinson, Bélanger, and Robichon, 1950) or Ca^{45} (Comar, Lotz, and Boyd, 1952; Tomlin, Henry, and Kon, 1953). Calcium-45 is now generally preferred to P^{32} , since it

produces a greater resolution of the autoradiographic image (as the lower energy of distintegration of Ca^{45} insures a greater space rate of energy loss near the point of emission and, as a result, a more restricted image; Gross, Bogoroch, Nadler, and Leblond, 1951) (See Plate I).

For many years, studies of bone centered on the metabolism of the mineral compartment. Recently, chance discoveries revealed the incorporation within bone matrix of C^{14} -bicarbonate (Greulich and Leblond, 1953; Greulich, 1953a) and S^{35} -sulfate (Bélanger, 1954). By means of these isotopes, autoradiographic data on the formation of matrix became available.

In autoradiography, the minute silver bromide particles of a photographic emulsion act as microdetectors of the radiation emitted by the isotope present in an adjacent preparation of bone tissue. This was first done by the classical technique of juxtaposing a ground section of bone and a photographic plate or film—the “contact method”—which even recently has yielded useful results (Lotz, Gallimore, and Boyd, 1952 (Figs. 1-3); Tomlin *et al.*, 1953 (Figs. 5 and 6). The introduction of an “integrated” method of autoradiography by which an intimate contact between section and emulsion is provided (Bélanger and Leblond, 1946) improved both the resolution and ease of handling of autoradiographs. Only two of the integrated methods have been frequently used in bone studies, namely, the “inverted” technique by Bélanger (1950) (Figs. 7 and 8) and the “coating” technique by the authors (see Fig. 4 and Gross *et al.*, 1951).

The present review will be preceded by general consideration of the acquisition of bone matrix as visualized with C^{14} -bicarbonate and S^{35} -sulfate, and of the deposition of minerals as seen with Ca^{45} or P^{32} -phosphate. The information garnered from these two aspects of the problem will then be used to re-examine the various steps in the ossification of the two types of bone (membranous and endochondral). Brief comments on bone growth will conclude the chapter.

II. General Features of Bone Formation

Little is known concerning the relation between bone matrix and the formation (or maintenance) of the minerals. It will be shown below, however, that the state of growth is associated with the acquisition of both matrix and minerals. Thus, these two components of bone must be examined separately.

1. ACQUISITION OF MATRIX

Most of the autoradiographic evidence dealing with the development and metabolic activity of organic bone matrix has been obtained from

studies of the behavior of labelled carbon (C^{14}) administered as bicarbonate and of labelled sulfur (S^{35}) administered as sulfate. While it is true that other isotopic ions, principally those of a fissionable and/or rare-earth type, also have an affinity for organic matrix (see Bloom, 1948), the limited experimental data presently available (see review of Gross *et al.*, 1951) do not indicate that they can be utilized as indicators of the normal physiological pattern of matrix metabolism. Hence, this section will be concerned only with the experimental findings derived from studies of C^{14} -bicarbonate and S^{35} -sulfate.

The initial investigations utilizing autoradiographic and radiochemical techniques with C^{14} -labelled carbonate or bicarbonate were made on adult animals. In such animals, it was possible to demonstrate the incorporation of C^{14} into the mineral compartment of the bones and teeth, presumably in the form of labelled carbonate salt (Bloom, Curtis, and McLean, 1947; Armstrong, Schubert, and Lindenbaum, 1948; Skipper, Nolan, and Simpson, 1951). A slight labelling of bone proteins was also present (Schubert and Armstrong, 1949).

Autoradiographs of bone sections from newborn rats given small subcutaneous doses of C^{14} bicarbonate revealed a widespread deposition of the labelled carbon (Greulich and Leblond, 1953), but treatment of the sections in 5% nitric acid, a process by which the mineral is removed (compare Figs. 9 and 10), had little effect on the pattern of deposition or the intensity of reaction (compare Figs. 11 and 12). Therefore, most of the radiocarbon taken up in bone of very young animals was present in the organic matrix. The labelled bicarbonate must have been incorporated into the proteins and/or carbohydrates of this matrix (Greulich, 1953a).

When the uptake of C^{14} -bicarbonate in bone matrix was compared by Geiger counter measurements of samples from animals weighing 30, 110, and 260 g, there was a decrease of radioactivity uptake with age (Greulich, 1953b). Autoradiographs revealed that reactive and non-reactive areas were present within bone tissue at all ages, but the intensity of the reactive areas decreased as the animals became older.

For instance, the mid-diaphysis of the tibia of rats injected with C^{14} -bicarbonate when 3 days old was autoradiographed at various time intervals after injection using demineralized preparations. At the early intervals, the diaphysis displayed an irregular, poorly limited band of radioactivity along its periosteal surface (Fig. 22), while the rest of the diaphysis was unreactive. The band was attributed to the appearance of labelled matrix components, presumably due to the activity of the periosteal osteoblasts.

At later intervals, the reactive zone (Figs. 23 and 24, arrow) was

farther and farther from the periosteal surface. This apparent displacement was attributed to the continued apposition of new layers of unlabelled organic matrix: unlabelled, since the rapid fall in the circulating level of injected labelled bicarbonate (Skipper, White, and Bryan, 1949) should result in a rapid decrease and eventual disappearance of labelling in the matrical components being formed.

The same sequence of events was observed in C^{14} -autoradiographs of bones of older animals. However, as the animals became older, the band of reaction became less intense and was more sharply limited to the subperiosteal region soon after injection. With time, the band receded from the periosteum more slowly. It was concluded that the older the animal, the slower was the rate of formation of matrix.

Comparable use of S^{35} -sulfate in very young rats (Bélanger, 1954) revealed similar subperiosteal deposition of radioactive material and later presence deep within the diaphysis.

Thus, the use of either C^{14} or S^{35} demonstrates the same accretional formation of matrix in the subperiosteal area of diaphyseal bone.

The autoradiographic reactions observed with these two isotopes in other types of bone also coincided; and, therefore, both may be used to find out where matrix formation takes place.

With regard to the nature of the labelled compounds detected in the matrix by autoradiography, it may be pointed out that the S^{35} material was soluble in hyaluronidase but the C^{14} material was not. The sulfate component of matrix is, therefore, believed to be a chondroitin sulfate (Bélanger, 1954). The carbon-containing component is a different substance. It may be the collagen and/or the non-acidic carbohydrates known to be present in bone (Glegg and Eidinger, 1954).

2. ACQUISITION OF MINERALS

As bone matrix is deposited, the mineral ions, calcium and phosphate, also appear (in the form of apatite-like crystals). Classical theory held that the mineral crystals arose from a mere physical chemical precipitation. To explain that the crystals appear only where there is matrix, it is generally assumed that the matrix plays a role in the crystal retention *in situ* (see Leblond *et al.*, 1950, p. 323, footnote 1). The minerals may be first deposited as irregular crystalline masses which undergo successive dissolutions and recrystallizations until they reach a form stable under the local conditions of collagenous fiber orientation, mechanical factors, etc. An alternative theory proposed by Neuman and others is that the matrix provides a seeding mechanism which would evoke crystal formation. For instance, if the matrix were to contain an organic substance possessing phosphate groups so arranged that their periodicity

would be the same as that of the lattice of apatite, calcium ions might become associated with them and thus initiate crystal formation. (The regularity of the arrangement of definitive apatite crystals on certain zones of collagenous fibers shown by Robinson and Watson, 1952, is compatible with either theory).

The growth of crystals may be considered as an addition of calcium and phosphate ions—or isomorphic substitutes—onto the crystal nuclei. However, the process of growth is complicated by associated phenomena, particularly *exchange*: exchange in a crystal is generally defined by the fact that ions leave the crystal surface, while an equal number of similar ions are deposited from the medium onto this surface. Thus, whenever P^{32} -phosphate or calcium-45 ions enter the circulation, they may exchange with non-radioactive ions of the crystal surface and by this process, be incorporated into bone tissue even in the absence of growth. The rate at which this incorporation takes place is dependent upon the accessibility of the crystal surface to the circulating ions. Accordingly, since areas of bone growth are known to be highly vascular, the exchange process may be expected to proceed at a rapid rate in these areas.

According to Patterson (unpublished), crystal growth is not a mere addition of ions but the result of complex processes which may be summarized as follows. Crystal faces, particularly in growth areas, are not uniform, but include partially completed layers of ions forming "islands," and even isolated adsorbed ions. Frenkel (1945) showed that the adsorbed ions and those at the edges of the islands are readily disturbed by thermal vibrations and may thus be shaken loose. The less numerous the points of contact between any given ion and the other ions of the surface, the greater will be its chance to be lost to the solution. In fact, ions are continuously leaving the crystal faces. However, similar ions from the medium are added to the edges of the islands or merely deposited on the surface. With a knowledge of these facts, *exchange* may be redefined as the condition under which the withdrawals and additions of ions are statistically equal so that the weight of the crystalline material remains unchanged. *Growth*, on the other hand, is the condition under which there is a predominance of addition over withdrawal of ions, resulting in a net increase in size and/or number of crystals.

The problem of mineral growth may be approached by the autoradiographic technique using P^{32} or Ca^{45} . For instance, when the periosteal region of the mid-diaphysis of long bones was examined in young adult animals at early time intervals after Ca^{45} administration, a band of reaction was observed along the periosteal surface (Fig. 1). Under high power, the radioelement was seen to be deposited over the

area of matrix close to the periosteal osteoblasts, that is to say, approximately that area where C^{14} autoradiographs revealed the deposition of new matrix components. However, while the matrical (C^{14}) reaction band was sharply limited in young adults, the mineral reaction band was prolonged by a weak, diffuse reaction extending through the thickness of the diaphysis.

Mineral reaction bands occurred where new matrix was forming and, therefore, were attributed to the mineralization of this matrix. According to the definition given above, this "mineral growth" would consist of an active interchange of ions in which addition predominates over withdrawal. On the other hand, the weak and diffuse reaction present over the rest of the diaphysis may be attributed to "exchange" processes.

With time after injection, the initially subperiosteal mineral reaction band was found farther and farther from the periosteal surface (Figs. 1-3), although the intervening area between band and periosteum displayed a low level of radioactivity. Whereas the mineral band itself is due to mineral growth at the time of Ca^{45} administration, the area of low reactivity between the band and the osteoblasts is due to mineral growth occurring at a later time, when there has been a gradual drop in the level of circulating Ca^{45} , and a concomitant decrease in the deposition of labelled salts. These observations testify to the accretional mode of deposition of minerals, as the bands demonstrate that successive layers of material blend into one another to form the shaft of the bone.

The mineral reaction bands observed with Ca^{45} (and the similar ones observed with P^{32}) seem to persist indefinitely with little change, at least in animals whose growth is well advanced (Figs. 1-3). Decrease in the intensity of the diffuse "exchange" reactions has been reported and attributed to reverse exchange (Leblond *et al.*, 1950). However, density measurements executed by a semi-quantitative technique suggested that the diffuse reactions did not decrease in intensity with time (Tomlin *et al.*, 1953). The situation was clarified by the *in vitro* experiments of Zemel and Kumamoto (unpublished) which indicated that loss of part of the labelled atoms by reverse exchange did occur, but that only a small portion of them were so affected under the experimental conditions used.

The measurements of the density of diffuse reactions carried out by Tomlin *et al.* (1953) may be used to estimate the extent to which exchange takes place in non-growing areas (assuming that the amount of reverse exchange taking place in these areas under physiological conditions can be neglected). Their data indicated that 24% of the salts in bone were exchangeable and that this exchange was fairly uniform throughout. These results cast suspicion on previous chemical work with

P^{32} (Neuman and Riley, 1947) or Ca^{45} (Harrison, 1950), in which such high figures had been obtained for exchange that the tracer method was felt to be unsuitable for studies of bone growth. On the other hand, the more recent *in vitro* work of Falkenheim, Underwood, and Hodge (1951) indicated that about 20% of the calcium and phosphate ions of bone were involved in a fairly rapid exchange. This figure is in agreement with that of Tomlin *et al.* (1953).

The conclusions on mineral uptake obtained so far applied to adult and young adult rats. Application of the autoradiographic technique to *very young* animals, e.g. newborn rats or cats, revealed the existence of intense autoradiographic reactions diffuse throughout bone spicules and trabeculae (Fig. 4). However, these diffuse reactions were generally more intense in the areas close to the osteoblasts, that is, where young matrix was being deposited. It is suggested that these diffuse reactions are due in part to exchange, since this process is more intense in younger animals (Leblond *et al.*, 1950; Tomlin *et al.*, 1953) but mainly to the interstitial addition of new crystalline material throughout the spicules and trabeculae. Such interstitial addition of mineral salts would account for the progressive hardening of the bone trabeculae during the first few weeks of life in the rat—a phenomenon familiar to all who have dissected bones in such young animals or have attempted to section them for histological study.

III. Intramembranous Bone Formation

The appearance of all membrane bone of the embryo is preceded by the local differentiation of connective tissue cells into osteoblasts. These cells gather in an epithelioid layer, along which a spicule of bone develops. The *formation of matrix* in such a structure was investigated in C^{14} -injected newborn rats by means of autoradiography of demineralized tissues. Soon after injection, a reaction was observed over the edge of the spicules in contact with the osteoblasts. Later, the reaction was seen to be within the spicule.

In some membrane bones (e.g., lateral portion of the mandible of the rat), new spicules and trabeculae are built upon the outer surface, while osteoclastic resorption takes place on the inner surface. Eventually, the spicules which are not resorbed gradually join with one another to form trabeculae and these thicken and fuse to yield a solid type of bone tissue.

In other areas of intramembranous bone formation, e.g., calvaria of the rat, osteoblastic activity may be seen on both surfaces of the initial bony layer (Figs. 19–21), in which case the bone formed is solid from the outset. At one day after injection of C^{14} -bicarbonate, the autoradio-

graphic band of reaction overlies an area of matrix adjacent to the osteoblasts on either surface of the bone (Fig. 19, arrows). At 3 days (Fig. 20) and particularly at 12 days (Fig. 21), the two bands are located at greater distances from the osteoblasts (Fig. 20), although the distance between the bands themselves remains approximately the same. Thus, in such areas of bone at this stage of growth, there is a rapid thickening with no signs of resorption.

Examination of *mineral deposition* with P^{32} revealed that in newborn and very young animals the autoradiographic reaction was present throughout membrane bones with only little predominance in the areas in contact with the osteoblasts. Thus in the mandible (Fig. 4), the bone trabeculae showed a fairly even P^{32} deposition throughout. In older animals, the reactions of bone were limited to superficial areas in contact with osteoblasts.

Thus, in the early stages of development, the mineral growth was to a large extent interstitial, that is, by deposition of salts throughout the matrix. Later, this growth became accretional since minerals were then deposited only in the new matrix present next to the osteoblastic layers.

IV. Endochondral Bone Formation

The bones of the limbs as well as those of the base of the skull, vertebral column and pelvis are termed *cartilaginous bones*, since they first appear in the form of crude models of hyaline cartilage which are later changed into bone by the so-called *endochondral* ossification. Ossification proceeds in two steps: the first (or primary ossification) includes transformation of cartilage into trabecular bone and is associated with formation of periosteal and later endosteal bone; the second (or secondary ossification) includes formation of Haversian systems.

1. PRIMARY OSSIFICATION

Since the process of endochondral ossification requires a cartilage precursor, the features in the formation of hyaline cartilage observable by autoradiography following administration either of C^{14} as labelled bicarbonate or of S^{35} as labelled sulfate will be described (see Plates II and IV).

a. *Cartilage Formation*

The observations made in the hyaline cartilage of bone models were identical to those made in locales which are normally unossified (hyaline cartilage of trachea, and also elastic and fibrous cartilage). Preparations of these types of cartilage examined 1 or 4 hours after administration of

C^{14} -bicarbonate showed a concentration of the label in the chondrocytes (Figs. 13 and 14). Similarly, within 2 hours after administration of S^{35} -sulfate, there was a comparable intracellular accumulation of radioactivity in the various types of cartilage (Fig. 7; Bélanger, 1954). Recently, the S^{35} results were confirmed by Glücksmann and Pelc (unpublished). With either C^{14} or S^{35} at these early intervals, the cartilage matrix was almost completely devoid of the labelled materials.

At later intervals, the labelled carbon or sulfur moved out into the matrix from the cells until, after 3 or more days following injection, the cells were essentially unreactive, while the matrix was more or less uniformly labelled (Fig. 17).

While the morphological behavior of both sulfur and carbon labelled cartilage was the same, there seemed to be no similarity in the chemical nature of the labelled components. Bélanger (1954) observed that treatment of sections of sulfur-labelled cartilage with hyaluronidase removed the labelled material and concluded that the sulfur incorporation occurred in the synthesis of chondroitin sulfate, in agreement with Dziewialkowski (1951, 1952). This substance presumably would be elaborated by the cell prior to its deposition outside as matrix. (An alternate possibility would be that a precursor of this substance, also hyaluronidase sensitive, would be made up in the cell and later synthesized into the completed substance as it is secreted into the matrix.)

However, hyaluronidase treatment did not appreciably diminish the C^{14} reactions or alter their location. Neither did the removal of glycogen by treatment with saliva or amylase preparations have any visible effect upon the reactions. It was concluded that the C^{14} had been incorporated into the cartilage collagen and/or into the carbohydrate-protein complex described in the cartilage matrix by Glegg, Eidinger, and Leblond (1954).

In summary, several of the components of matrix or their precursors are elaborated in cartilage cells and then secreted into the matrix. The use of S^{35} suggests that this is the case with chondroitin sulfate, while the unidentified C^{14} -labelled component might well be collagen.

b. Transformation of Cartilage Into Trabecular Bone

The first indication that the young cartilage model is to transform into bone is the appearance of hypertrophic alterations within the cartilage cells in the center of the diaphysis. At about the same time a blood vessel invades the region of the transformed cartilage cells and soon a vascular connective tissue space (primary center of ossification) is seen within the diaphysis. The edges of this space recede toward each end of the cartilage model. Later, two similar spaces arise within the two extremities of the model (secondary centers of ossification). The

cartilage layer separating the diaphyseal vascular space from that at each extremity is known as the epiphyseal plate.

The space which appears in an ossification center is surrounded with spicules, typical examples of which are seen on the surface of the epiphyseal plate. The changes leading to the formation of these bone spicules and their junction into trabeculae can be subdivided into two main steps: the formation of calcified cartilage, and the addition of bone to the calcified cartilage remnants. Both processes were investigated by the autoradiographic technique.

The transformation of *hyaline into calcified cartilage* is associated with changes in the activity of the cells, which can be visualized using C^{14} or S^{35} autoradiography, with the same results in both cases. The intensity of the intracellular autoradiographic reactions which is weak in hyaline cartilage (Fig. 12, H), is moderately increased in the cells of the zone of proliferation (Fig. 12, Pr) and much more so in those of the zone of cell hypertrophy (Fig. 12, Hyp.), whereas those of the zone of calcified cartilage are completely unreactive (Figs. 11 and 12, arrow). At later time intervals (24 or 72 hours) after injection, the radioactive material migrates from the various types of cartilage cells into the matrix (Figs. 15-18). (However, the enlarged cells of the hypertrophic zone may occasionally have lost the ability to release their material to the matrix; Fig. 16, arrow.)

Another phenomenon observed at the later intervals was that the calcified cartilage was unreactive soon after injection (Figs. 11, arrow, and 13) but later became gradually reactive (Figs. 15 and 17). Presumably, the reactive matrix seen initially above the calcified cartilage remains labelled when it is itself transformed into calcified cartilage.

The interpretation of the observations made in the various zones is that cartilage cells become hyperactive as they proliferate and enlarge, but eventually they become inactive and die, at which time the matrix is described as calcified cartilage.

Examination of the various zones on P^{32} or Ca^{45} autoradiographs revealed that these ions were not taken up in the zones of proliferation and hypertrophy, but they accumulated in the zones of calcified cartilage. The presence of radiophosphorus may be distinguished as a dark band on the lower surface of the epiphyseal plate in Fig. 29 and especially Fig. 32 (see also Fig. 35). The same phenomenon may also be seen with Ca^{45} (Fig. 28). Thus, the minerals are deposited in the very region where there is no uptake of C^{14} or S^{35} , that is, in a region where the activities characteristic of cartilage no longer take place and, in fact, the cells have died. Perhaps their death stopped the production of a factor inhibiting the deposition of calcium phosphate within the matrix.

While blood vessels enter the lacunae containing the dying cartilage cells, the intervening strands of matrix persist as isolated remnants or spicules of calcified cartilage.

The second phase or *trabecular bone formation* results from the appearance of osteoblasts, which coat the cartilage remnants with layers of bone. The formation of the matrix of these bone layers was shown by an autoradiographic reaction around the borders of the spicules after injection of C^{14} -bicarbonate, whereas the cartilaginous core remained free of reaction (Fig. 27) (see Plate VI). The accumulation of mineral salts in this matrix was depicted by the autoradiographic reaction of the spicules after P^{32} (Figs. 29 and 32) or Ca^{45} injection (Figs. 1, 5, and 6)—a phenomenon represented in the left-hand diagram of Fig. 35.

The fate of the bone-coated spicules varies according to their location. However, they always exhibit some degree of osteoclastic resorption and some reorganization of their shape. In the epiphyses, many of the spicules persist and become linked into trabeculae. The resulting trabecular network forms the spongy bone of the mature epiphyses. In the metaphysis of long bones (that is, the spicule-containing region on the shaft side of the epiphyseal plate), the resorption of the spicules is extensive. Thus, at late intervals after Ca^{45} or P^{32} administration, the radioactive spicules located near the axis of the bone are completely resorbed, as may be seen by comparing the autoradiographic reactions of the spicules at 5 minutes (Figs. 29 and 32), 2 days (Figs. 30 and 33) and 8 days (Figs. 31 and 34) after P^{32} injection. The same figures reveal that, with time, the lateral spicules are incorporated within the shaft and thus support the epiphyseal plate as well as whatever weight has to be borne along the axis of the bone (see Plate VII). The complex pattern of spicule replacement in the course of bone growth has been described in detail (Leblond *et al.*, 1950).

Eventually the portion of the shaft comprising the supporting spicules (Figs. 31 and 34) will be resorbed from the outside by osteoclastic activity (Lacroix, 1945; Leblond *et al.*, 1950), so that all autoradiographic reactions of the spicules may then disappear (Fig. 3).

c. *Periosteal Bone Formation*

Soon after the appearance of a primary ossification center, and in some cases prior to its appearance, osteoblasts line up along the surface of the mid-portion of the cartilage model and elaborate matrix. The formation of matrix may be shown by the deposition of either S^{35} (Bélanger, 1954) or C^{14} on the surface of the bone already present (Fig. 22). At 3 days (Fig. 23) and 12 days (Fig. 24) the irregular band of reaction is seen at a progressively greater distance from the osteoblasts as new layers of unlabelled matrix are deposited. This pattern of matrix

formation may be seen throughout life in the rat (but it was pointed out above that in young adult and adult rats the area of matrix formation is more precisely restricted to the subperiosteal surface of the bone than in newborn rats).

The deposition of minerals differs strikingly in very young and in older animals. Thus, in newborn rats soon after administration of P^{32} or Ca^{45} , the reaction will be diffuse throughout the bone with only a slight predominance at the subperiosteal surface, whereas in adult animals, a sharp band of reaction is restricted to the subperiosteal surface (Fig. 1). The inclusion of this band within the compact bone of the cylindrical portion of the shaft as new layers are added on the outer surface and resorption takes place on the inner surface is beautifully illustrated in Figs. 1-3.

Instead of using a single injection prior to autoradiography, Tomlin *et al.* (1953) treated young adult rats chronically with radioactive calcium. The effect of two such treatments with low and high specific activity Ca^{45} , respectively, is shown in Fig. 5, in which the recently administered high-activity calcium may be detected on the outer surface of the shaft while the previously administered one is seen as a band of lesser intensity within the bone tissue itself. These results again demonstrate the accretional formation of periosteal bone in young adult rats.

d. Endosteal Bone Formation

In very young animals, the shaft is regularly cylindrical and an osteogenic periosteum extends along the whole shaft up to and beyond the zone of calcified cartilage bordering the epiphysis. (In Fig. 9, a bone spicule is shown by the von Kossa stain to be present on the left-hand side of the calcified cartilage zone.)

With age, the neck of the epiphysis widens faster than the diaphysis. As a result, the subepiphyseal region takes on a funnel-like shape, as may be seen in the femur (Fig. 1), metatarsal (Fig. 28), tibia (Fig. 29) and humerus (Fig. 32). This region of bone, hereafter referred to as "funnel," includes a spicule-containing portion (the metaphysis) and a spicule-free portion (the narrower portion of the funnel).

The funnel is regularly conical in some bones, e.g. tibia; but may be only partially so in others, e.g. humerus, in which only one side flares out. Histological examination of the walls of regular funnels reveals the presence of osteoblasts at the endosteal surface and osteoclasts at the "periosteal" surface (Lacroix, 1945; Leblond *et al.*, 1950) (see Plate VIII). In the case of partial funnels, osteoblasts are seen on the endosteal surface of only the bent side. Autoradiographic data reveal that matrix formation is shown by the deposition of C^{14} wherever osteoblasts are present. Thus, a reaction is seen throughout the endosteal surface of

the tibia funnel and of the flared portion of the humerus (Fig. 25). With time, the reactive material is located at an increasing distance from the osteoblasts, as may be seen from the small but definite outward displacement of the band in Fig. 26.

Mineral deposition occurs in the same locations in young adult rats. Autoradiographic visualization of calcium deposition on the endosteal surface of a regular funnel is clearly illustrated in Fig. 28, and is also recognizable in Fig. 1 (left) and Fig. 5 (bottom). The displacement of the band of reaction outward with time is schematically represented in Fig. 35, in which it may also be seen that the resorption at the outer surface occurs quite rapidly. Thus, following injection of P^{32} -phosphate to a 50-g rat (see Plate IX), the band of reaction seen in the funnel of the proximal epiphysis of the tibia already reaches the outer surface as early as 8 days later (right-hand diagram, Fig. 35).

2. SECONDARY OSSIFICATION

Secondary ossification, which begins after the walls of the bone have reached a certain degree of thickness, is the sequence of events leading to the formation of Haversian systems within bony tissue. This will be described in the dog using the cylindrical portion of the shaft or cylinder (as opposed to the funnel) as an example.

According to classical histological descriptions, osteoclasts associated with blood vessels erode the endosteal surface and thus enter the bony tissue from the marrow cavity. A hollow channel results which soon becomes orientated along the axis of the bone. Later, the walls of this channel are coated with osteoblasts, which deposit matrix. Mineralization begins as soon as the matrix appears, but is not completed until some time thereafter. The Haversian systems in which the mineralization is not complete can be recognized by their lesser opacity to X-rays (Fig. 38, 1). That such Haversian systems are actually being mineralized can be shown by autoradiography after Ca^{45} injection, when they show a ring-shaped reaction (Fig. 39, 1; Engfeldt, Engström, and Zetterström, 1952). (Even *in vitro*, Amprino, who subjected bone sections to solutions of Ca^{45} or P^{32} , found that the isotope was incorporated in these Haversian systems undergoing mineralization, 1952a, b) (see Plate X).

In the same manner, the formation of Haversian systems continues throughout life. Even after the first or primary ones have appeared, they themselves can be eroded to give rise to secondary systems. Later tertiary, and even more often replaced, systems are formed, all of which behave in a manner identical to that of the earliest or primary Haversian systems.

3. GROWTH OF LONG BONES

Inherent in the normal growth of a long bone is the harmonious development of its various parts: the cylinder, the epiphysis (or epiphyses), and the funnel(s). Their growth proceeds rapidly during the period of primary ossification, but slows down as secondary ossification gets under way.

The *cylinder* expands in width as well as in length. The mechanism of its increase in diameter has been known for centuries. It consists of addition of layers of bone on the periosteal surface, while corresponding resorption of the endosteal surface increases the size of the marrow cavity. These phenomena are illustrated diagrammatically in Figs. 35 and 36, which are based on P^{32} autoradiographs prepared in the laboratory of the senior author. Somewhat similar results were reported by Tomlin *et al.* (1953) using Ca^{45} . Thus, the stages in the growth of the femur illustrated schematically in Fig. 37 confirm that the cylindrical portion of the shaft grows by periosteal addition. In this bone there was also some slight endosteal growth at the anterior wall in young animals, but this soon ceased. Such temporary endosteal growth may be an extension of the endosteal activity of the funnel into part of the diaphysis. Perhaps, this phenomenon is due to a temporary deficiency of calcium since the funnel effect extended farther into the diaphysis in rats fed on a low calcium diet (Fig. 6) than it did in similar animals fed on a high calcium diet (Fig. 5). (A similar extension of the funnel may also be seen in the metatarsus of the calf in Fig. 28.) Bearing such exceptions in mind, it may still be safely concluded that the thickening of the cylinder walls is normally due to periosteal activity.

Increase in length of the cylinder is due to a gradual transformation of the narrow end of the funnel(s) into the cylinder—a phenomenon which will be discussed more fully after describing the changes taking place in the epiphysis and funnel.

An *epiphysis* initially appears with the development of a secondary ossification center at the end of the original cartilage model. To examine the evolution of these centers, a rounded epiphysis (humerus, metacarpal) will be taken as an example. Its shape may be conveniently described as a hollow cartilaginous hemisphere, the flat surface of which is the epiphyseal plate. The outer aspect of its rounded surface would then constitute the articular face. The cavity of the hemisphere is partially filled with trabeculae of endochondral bone, which have arisen from the inner aspect of both round and flat surfaces.

Expansion of the epiphysis involves a series of processes including: (1) addition to the cartilage at the outer aspect of the rounded surface by transformation of fibrocytes into chondrocytes; (2) endochondral

bone formation along the entire inner aspect of the hemisphere (being more marked on the rounded than on the flat surface), resulting in the production and maintenance of a network of trabeculae in the central cavity. In this manner, as the rounded portion of the hemisphere expands, trabeculae are left behind to form the supporting framework of the epiphysis; (3) enlargement of the hemisphere which is of necessity associated with an increase in the diameter of the epiphyseal plate and again results from chondrogenic activity at its periphery (that is, at the junction of the flat and rounded surfaces of the hemisphere).

The *funnel* displays an accumulation of new bone on its inner or endosteal surface as well as bone resorption on its outer surface (Leblond *et al.*, 1950). One may well wonder if the continuous addition of endosteal material should not decrease the diameter of the marrow cavity. Examination of funnels from tibiae of rats of various ages reveals that this is not the case. In fact the inner diameter of the narrow end of the funnel increases in harmony with the inner diameter of the cylinder. Therefore, whatever endosteal bone is added at or near the narrow end of the funnel does not accumulate there indefinitely, but must be eventually subjected to osteoclastic resorption like the inner surface of the cylinder close by. Thus, in the region of the funnel approximating the cylinder there would be a replacement of osteoblasts by osteoclasts—a fact which would automatically insure that this portion of the funnel transforms into cylinder. The occurrence of this phenomenon may be shown in autoradiographs of animals sacrificed at various time intervals after P^{32} injection, by the respective position of the funnel reaction band and the junction between funnel and cylinder (compare the position of the arrows indicating the funnel-cylinder junction at the two time intervals in Fig. 35).

To realize the extent of the addition of material on the endosteal surface of the funnel (and of the simultaneous resorption on the outer surface), it is useful to compare the position of the funnel walls in reference to a fixed point, namely, the labelled calcified cartilage band deposited following P^{32} injection. The funnel wall which is far from this labelled band at first (Fig. 35, left), soon appears to be close to it (Fig. 30), and finally incorporates its remnants (Figs. 31 and 35, right). Thus, there is an "ascension" of the funnel—the end result of the continuous addition of more and more material onto the endosteal surface (Figs. 36 and 37).

Examination of Figs. 29–31 and the schematic diagram in Fig. 36 reveals that the ascension of the funnel is not associated with a decrease in the distance between funnel and plate. Accordingly, the rate at which the lower end of the spicules is either incorporated in the funnel wall or

resorbed is equal to the rate of the growth of their upper or epiphyseal end by endochondral bone formation from the epiphyseal plate. Thus, the length of the various spicules does not decrease with time. As a consequence the distance between funnel and epiphyseal plate does not decrease. In fact, the ascension of the funnel is associated with a rise of the whole epiphysis (Fig. 36).

It has already been pointed out that the diameter of the epiphyseal plate increases as the bone enlarges. As a result, new subepiphyseal spicules appear on the outside (e.g., J on the left of Fig. 35) and the funnel expands correspondingly by lengthening of its wider end.

Thus, growth of the various parts of a long bone may be shown on autoradiographs to proceed in a harmonious manner. Analysis of individual bones remains to be done in detail, although the work of Tomlin *et al.* (1953) represents an excellent beginning along these lines. Autoradiographs of either matrical or mineral elements may be used for such detailed analysis, since the formation of the matrix is immediately followed by a deposition of mineral salts. As a result, the patterns of matrix and mineral deposition are nearly identical, particularly in young adult animals. However, it seems that a greater degree of precision will be obtained by studies of matrix formation, using C^{14} , S^{35} , or some weakly radioactive matrix seeker of physiological importance. Newly-formed matrix reactions are not obscured by reactions which occur extraneously in mineral studies, i.e., those due to exchange, recrystallization, etc.

The ultimate problem associated with bone growth, namely how the matrix holds the mineral crystals, remains unsolved. While it is unlikely that a solution will be arrived at by autoradiographic studies alone, perhaps studies of this type in support of precise biochemical and biophysical investigations will lead to a final answer.

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PLATE I

FIGS. 1, 2, and 3. Contact autoradiographs of the femora of young pigs sacrificed 3 days (Fig. 1), 30 days (Fig. 2) and 60 days (Fig. 3) after intra-arterial administration of 265 μ c Ca⁴⁵ as calcium chloride. (From Comar *et al.*, 1952). The distance between the tips of each pair of arrows corresponds to the thickness of the diaphysis.

At 3 days (Fig. 1), there is an intense accumulation of the isotope in the region of the epiphyseal plates and in some of the subjacent trabecular bone of the metaphyses. Note the sharp image in the subperiosteal regions, and the discontinuity emphasizing the "funnel effect" near the distal end.

By 30 days (Fig. 2), there has been an obvious decrease in the reactivity of trabecular bone, indicating a loss by resorption of labelled mineral in these regions. The periosteal reactions have been displaced inward by means of both the apposition of new unlabelled subperiosteal bone and the resorption of bone on the inner surface. By this mechanism, circumferential growth of the bone shaft occurs. The "funnel effect" is still apparent, particularly in the distal end (left). The areas of low reactivity below the epiphyseal plates (apparent as zones of minimal blackening) represent the endochondral growth in length occurring in the 30 days since administration of the isotope.

At 60 days (Fig. 3), practically all of the radioactivity has disappeared from trabecular bone. The Ca⁴⁵ originally deposited under the periosteum is now in the endosteal region and is about to be removed by the process of resorption.

PLATE I

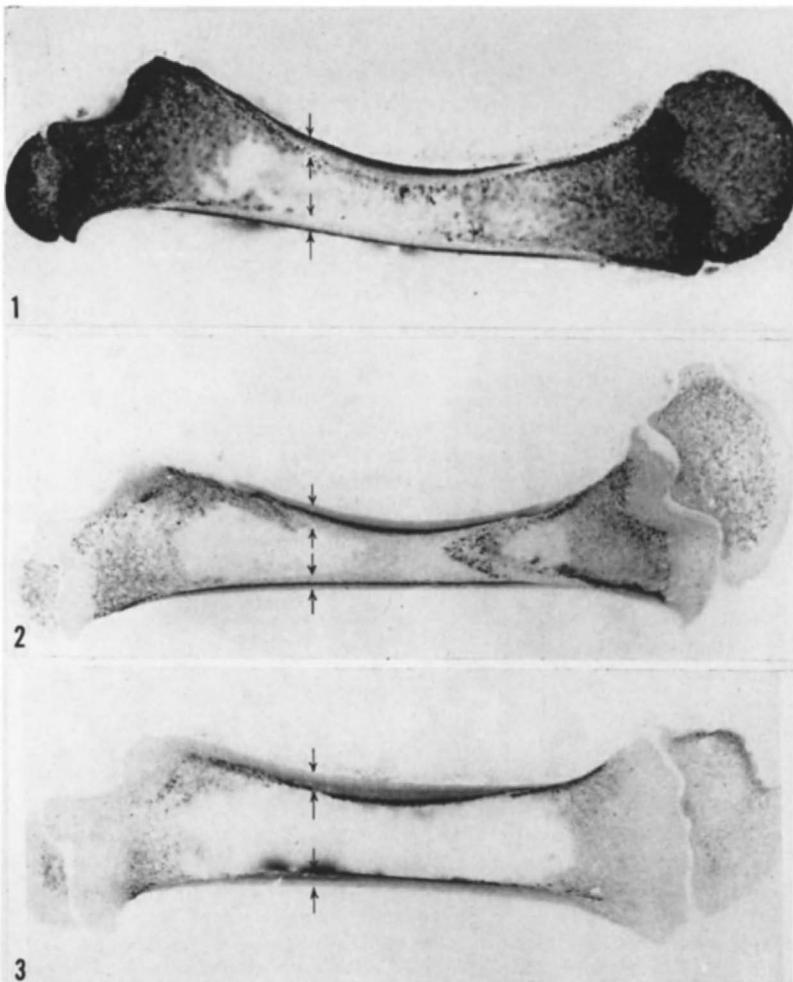


PLATE II

FIG. 4. Coated autoradiograph of a portion of mandible removed from a newborn kitten sacrificed one hour after an injection of 2 mc P³² as phosphoric acid (from Leblond *et al.*, 1950) \times 11.

The reactivity of the alveolar bone (which is membranous in origin) is intense and fairly evenly distributed. This type of diffuse, intense reaction is typical of newly formed or very young bone shortly after isotope administration.

Figs. 5 and 6. Autoradiographs of approximately mid-sagittal ground sections of femora removed from rats given 0.5 μ c per day of Ca⁴⁵ as CaCO₃ in the diet from the 27th to the 107th day of life and again from the 218th day to the 301st day of life, at which time they were sacrificed. The femur in Fig. 5 was taken from an animal maintained on a high calcium diet (Ca = 0.83%; Ca : P = 1 : 1), while that in Fig. 6 was taken from an animal maintained on a low calcium intake (Ca = 0.15%; Ca : P = 1 : 2.5) (from Tomlin *et al.*, 1953).

In Fig. 5, the separate periods of isotope utilization are clearly distinguishable. The reactions resulting from the first feeding of Ca⁴⁵ are less intense (due to the lower specific activity of the radiocalcium used) and more extensive (due to a longer period of administration) than the reactions resulting from the second feeding. Thus, the diaphysis on the left side of Fig. 5 showed from inside outwards, a wide, weak band due to the first feeding, a non-reactive area corresponding to the period when no Ca⁴⁵ was administered, and an intense sharp band due to the second feeding.

Isotope uptake is considerably increased by a low calcium intake (Fig. 6), and this fact somewhat obscures the relationships of the autoradiographic reactions arising from the separate feeding intervals.

Figs. 7 and 8. Inverted unstained autoradiographs of the proximal epiphysis of the humerus of a 9-day old rat, injected 24 hours previously with 5 μ c/g S³⁵ as sodium sulfate (from Bélanger, 1954). \times 24.

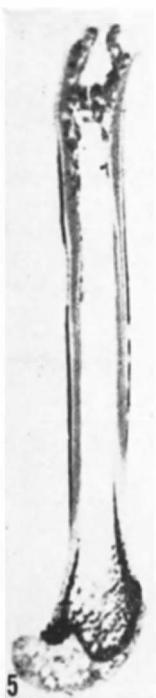
In Fig. 7, the section has been incubated for 6 hours at 37° C in a boiled and therefore, inactivated solution of hyaluronidase, buffered at pH 5.6. The resultant autoradiograph is quite comparable to that of an untreated slide. Note the dense autoradiographic reaction in the most hypertrophic zone of the epiphyseal cartilage. Part of the isotope is extracellularly located in the cartilage matrix, while some still remains within the chondrocyte lacunae, presumably as intracellular material. The latter phenomenon is well illustrated by areas in the vicinity of the developing epiphyseal center of ossification wherein focal reactions correspond to individual chondrocytic lacunae.

Figure 8 is a section from the same tissue, but has been treated for 3 hours in a solution of 0.1% hyaluronidase at 37° C. The greatest part of the sulfur-labelled material has been removed by hyaluronidase treatment. It is, therefore, concluded that most of the labelled sulfur was present as chondroitin sulfate in the cartilage.

PLATE II



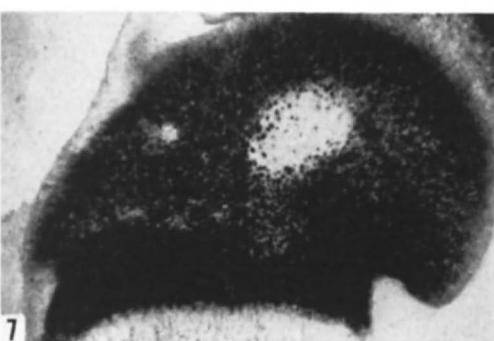
4



5



6



7



8

PLATE III

Figs. 9-12. Semi-serial sections of the proximal end of a tibia removed from a rat given a single subcutaneous administration of $40 \mu\text{c}$ C^{14} as sodium bicarbonate at birth and sacrificed 4 hours later (from Greulich, 1954). \times about 35.

Figure 9 illustrates a non-demineralized section stained by the von Kossa technique for bone salts. Areas of blackening indicate the location of mineral deposits in the region of calcified epiphyseal cartilage, in periosteal diaphyseal bone, and in subepiphyseal and medullary bone trabeculae.

Figure 10 illustrates a similar section decalcified by immersion in 5% nitric acid and subsequently stained by the von Kossa method. No von Kossa positive material remains following acid demineralization. The substage condenser of the photomicrographic apparatus was lowered to resolve the outlines of the unstained bone.

Figures 11 and 12 illustrate comparably prepared unstained coated autoradiographs of the mineralized (Fig. 11) and demineralized (Fig. 12) sections. There is little alteration in either the intensity or the distribution of the radioactive material. Note the area of calcified cartilage (indicated by the arrows) which is autoradiographically negative in both the mineralized and demineralized sections.

From comparison of these preparations, it is concluded that most of the C^{14} taken up by the bone of newborn animals is incorporated in the organic matrix.

The presence of C^{14} in cartilage cells at early time intervals after injection of C^{14} -bicarbonate may be seen in Figs. 11 and 12. For instance, Fig. 12 shows the reaction in the chondrocytes of the hyaline cartilage zone (H), proliferation zone (Pr), and zone of cellular hypertrophy (Hyp). In contrast, the area of calcified cartilage (arrow) is autoradiographically negative in both the mineralized (Fig. 11) and the demineralized section (Fig. 12).

PLATE III

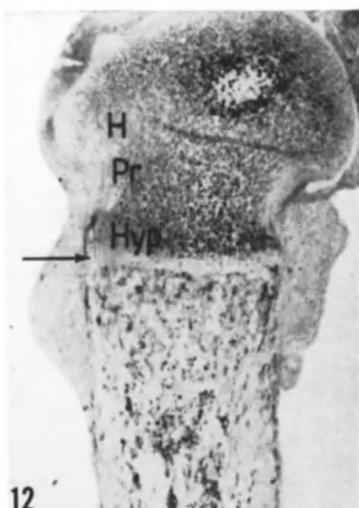


PLATE IV

Figs. 13, 15, and 17. Unstained coated autoradiographs of proximal tibial epiphyses removed from rats sacrificed at 4 hours (Fig. 13), 24 hours (Fig. 15) and 72 hours (Fig. 17) following the administration at birth of 40 μc C^{14} as sodium bicarbonate. $\times 30$.

Figs. 14, 16, and 18. Hematoxylin-eosin stained coated autoradiographs of hypertrophic epiphyseal cartilage from bones respectively comparable to those shown immediately to the left, but at a higher magnification. $\times 290$.

At 4 hours (Figs. 13 and 14), note the presence of strongly reactive chondrocytes, particularly in the zone of cellular hypertrophy (center of Fig. 13, and Fig. 14). In comparison, the cells of resting articular cartilage (top of Fig. 13) show only slight reactivity. At this time, note the contrast between the heavy accumulations of black silver granules over the chondrocytes in the hypertrophic epiphyseal cartilage and the relatively unreactive matrix (Fig. 14).

By 24 hours (Figs. 15 and 16), a good deal of the reaction of cartilage is in the matrix, but a weak reaction is still visible in the chondrocytes. In some regions, a few chondrocytes maintain a cellular reaction similar to that seen at 4 hours (arrow in Fig. 16).

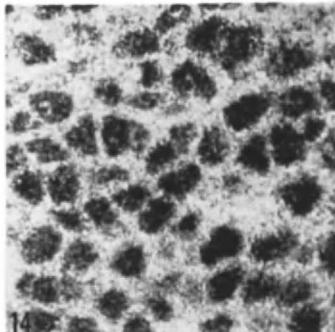
The cartilage at 72 hours (Figs. 17 and 18) shows only a greyish cast due to a diffuse reaction of the matrix. The cells are no longer reactive. The black bodies apparent in Fig. 18 are the stained nuclei of the chondrocytes, which themselves contain little or no C^{14} .

It is concluded that C^{14} derived from sodium carbonate is utilized in the synthesis by chondrocytes of an organic component which is later deposited into the cartilage matrix.

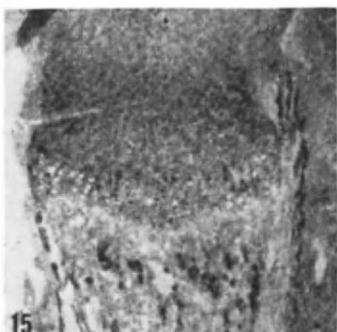
PLATE IV



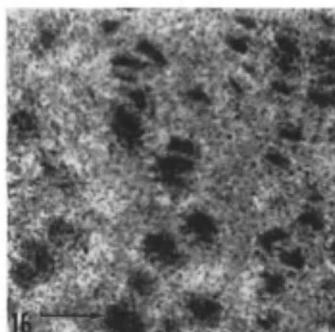
13



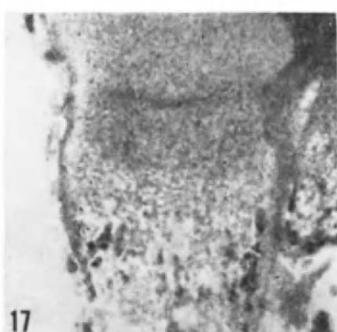
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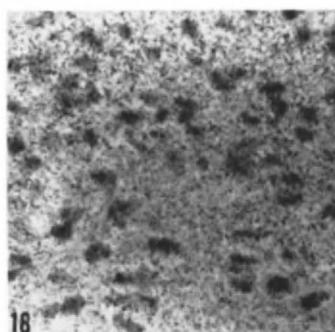
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16



17



18

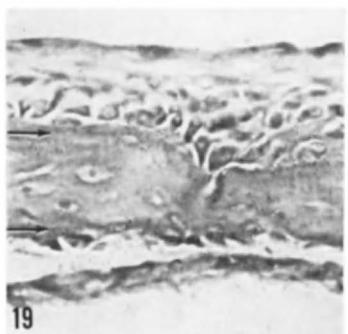
PLATE V

FIGS. 19-24. Coated autoradiographs of portions of membrane bone (calvarium; Figs. 19, 20, and 21) and of periosteal bone (tibial diaphysis; Figs. 22, 23, and 24) removed from rats sacrificed one day (Figs. 19 and 22), 3 days (Figs. 20 and 23), and 12 days (Figs. 21 and 24) following administration of approximately $30 \mu\text{c}$ C^{14} sodium bicarbonate at the age of 3 days. Sections decalcified in formate-citrate, stained with hematoxylin-eosin, and photographed by dark contrast phase microscopy. $\times 300$.

In both membrane and diaphyseal bone at 24 hours (Figs. 19 and 22), it is possible to visualize the incorporation of C^{14} into a component(s) of the organic matrix immediately beneath the bone surface. Each arrow indicates the position of a band of autoradiographic reaction. In the membrane bone (Fig. 19) there seems to be periosteal activity at both inner (lower arrow) and outer (upper arrow) surfaces. However, the synthetic activity at the inner surface is somewhat less than that of the outer surface. In the pictures of tibial diaphysis (Figs. 22-24), the letter "P" indicates the periosteal surface.

The layers of labelled organic matrix may be visualized at later intervals as being displaced inward from the surfaces as new non-labelled matrix is formed at these surfaces and the bone increases in width. Compare relative positions of arrows and the relative thickness of bone at 1 day (Figs. 19 and 22), 3 days (Figs. 20 and 23) and 12 days (Figs. 21 and 24).

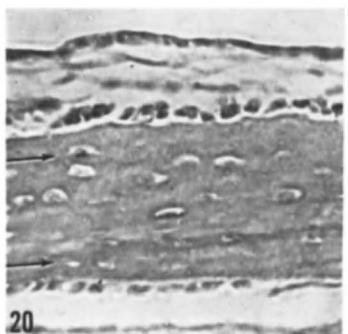
PLATE V



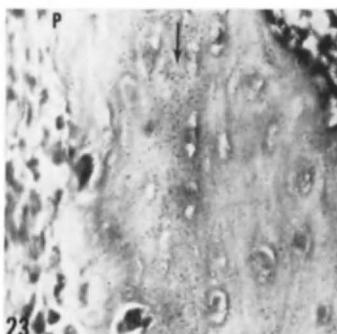
19



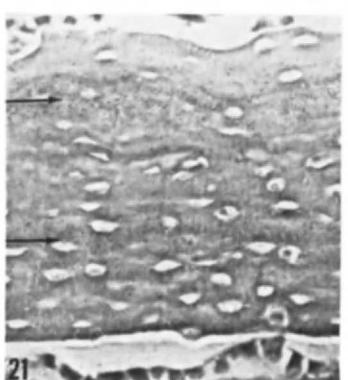
22



20



23



21



24

PLATE VI

FIGS. 25 and 26. Unstained coated autoradiographs of longitudinal sections of humeri removed from 30-g rats following administration of $80 \mu\text{c}$ C^{14} as sodium bicarbonate. Sections decalcified in tetra sodium ethylene diamine tetraacetate and photographed by dark contrast phase microscopy. $\times 140$.

The sections illustrated are taken from the wall of the proximal funnel and indicate the endosteal (E) deposition of labelled matrix at 24 hours (Fig. 25) and at 72 hours (Fig. 26) following administration of the labelled bicarbonate. It may be seen that there is a small, but definite outward displacement of the layer of labelled matrix (arrows) toward the periosteal surface with time after injection. This phenomenon is also reflected in the behavior of mineral-seeking isotopes under comparable experimental conditions (see text).

FIG. 27. Unstained coated autoradiograph of an area in the secondary epiphyseal ossification center of a humerus removed from a 30-g rat 24 hours following administration of $80 \mu\text{c}$ C^{14} as sodium bicarbonate. Sections decalcified in tetra sodium ethylene diamine tetraacetate and photographed by dark contrast phase microscopy.

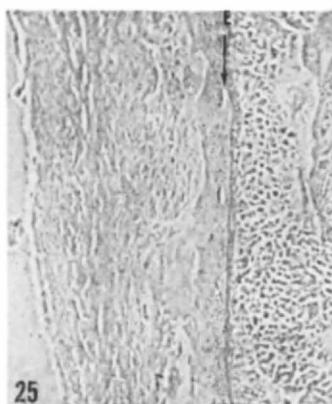
The field illustrates a cross-shaped cartilaginous spicule which is undergoing ossification. The organic bone matrix appears as a slightly more refractile peripheral coating about the limits of the spicule. Examination reveals that the autoradiographic reaction is confined to the new bone matrix, while the cartilage matrix of the central core is essentially unreactive.

FIG. 28. Autoradiograph of a metatarsus of a calf given 0.5 mc of Ca^{45} orally (from Lotz *et al.*, 1952).

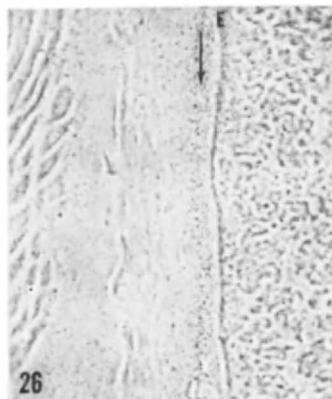
Narrow black lines on the outer surface of the shaft represent the location of the Ca^{45} in the subperiosteal bone of the "cylinder." The low energy beta-particles emitted by the isotope allow a high resolution.

The endosteal deposition in the funnels is clearly visible as a dotted line (since the reactive subendosteal bone is associated with many non-reactive trabeculae). In this case, the intense reaction of the upper funnel is prolonged by a weak reaction along the endosteal surface of the cylinder.

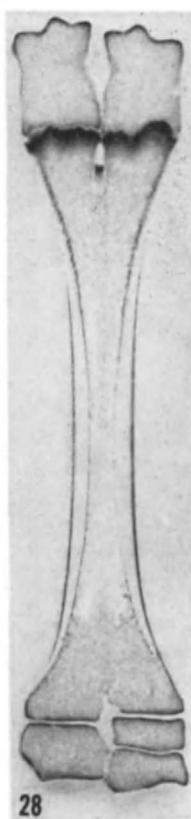
PLATE VI



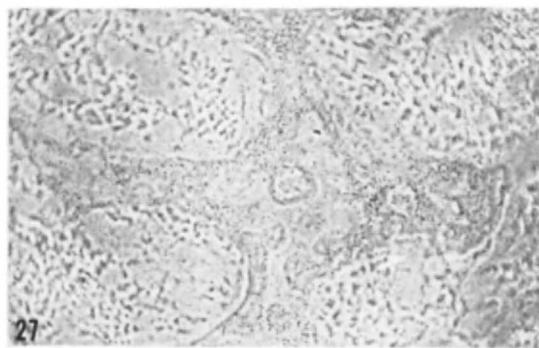
25



26

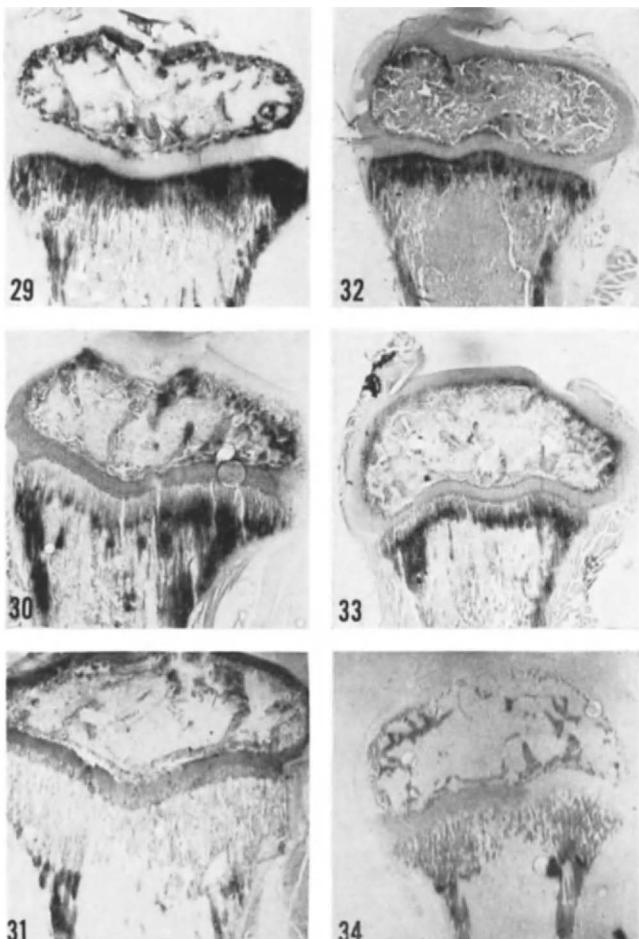


28



27

PLATE VII



Figs. 29-34. Coated autoradiographs of heads of tibia (on the left side of the page) and humerus (on the right side of the page) of 50-g rats sacrificed at various time intervals after an injection of $350 \mu\text{c}$ P^{32} as phosphoric acid. Safranin stain (from Leblond *et al.*, 1950). $\times 11$.

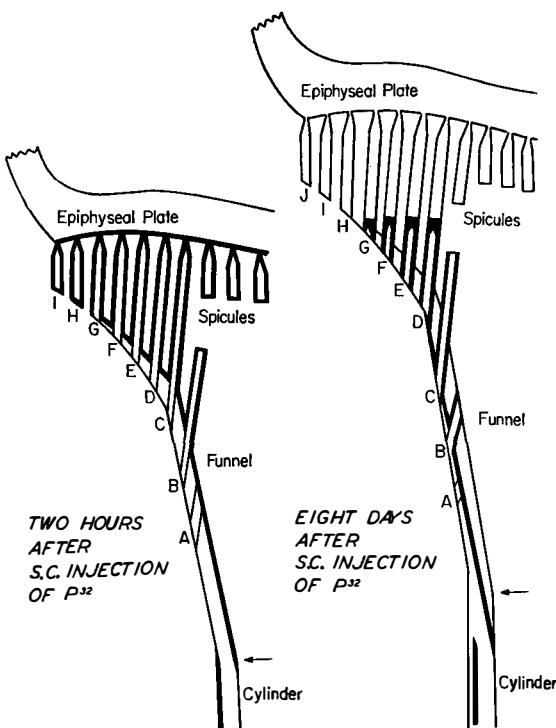
FIG. 29. Head of tibia 5 minutes after injection. The lower limit of the cartilage of the epiphyseal plate shows an intensely dark band (calcified cartilage) beneath which there is a moderate reaction of the spicules (due to their bone coating).

FIG. 30. Head of tibia 2 days after injection. Only the lowest part of the spicules shows a reaction.

FIG. 31. Head of tibia 8 days after injection. The apparent downward motion of the dark line may be seen only in those spicules which are close to the wall of the diaphysis, as the more central and more peripheral spicules have been resorbed.

FIGS. 32-34. Head of humerus 5 minutes (Fig. 32), 2 days (Fig. 33), and 8 days (Fig. 34) after P^{32} injection. The apparent downward displacement of the band arising in calcified cartilage can be seen at the later time intervals.

PLATE VIII



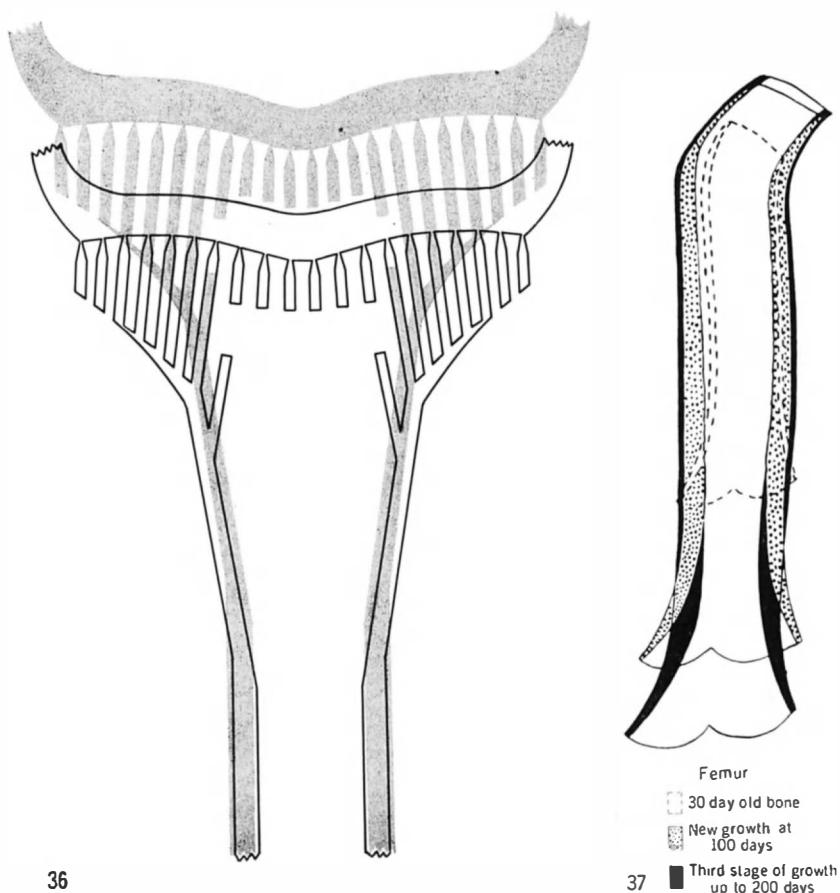
35

FIG. 35. On the left, a diagram represents part of the head of the tibia of a 50-g rat 2 hours after a subcutaneous injection of radiophosphorus. The heavy lines indicate the surfaces where an elective deposition of radiophosphorus takes place.

On the right, a diagram represents the corresponding region of the tibia 8 days later. The reactive areas have been drawn as heavy lines in a position corresponding to that in the left diagram.

The solid reactive band bordering the epiphyseal plate soon after injection—which is due to P³² accumulation in the zone of calcified cartilage (left)—persists as a broken line across some of the spicules (right); the rest of the line has been eroded. The reactive coating on the spicules soon after injection (left) persists on the lower parts of some of the spicules (right); by tracing the individual spicules from one diagram to the other, their role in making up the wider end of the funnel becomes apparent. The reactive line found on the endosteum of the funnel soon after injection (left) becomes deeply embedded in the bone along most of the length of the funnel and is even resorbed at the wider end (right). The reactive line found on the periosteal surface of the cylinder soon after injection (left) becomes embedded in the bone (right) (Leblond *et al.*, 1950).

PLATE IX



36

37

FIG. 36. Increase in size of the tibia of the 50-g rat over an 8-day period. The 50-g rat tibia (drawn in white with dark outlines) is placed next to a diagram of the same bone as it appears 8 days later (drawn in grey), in such a way that the bony structures that are retained over the 8-day period are superimposed.

The bone parts of the grey diagram that are not covered by the white diagram correspond to new bone formed during the 8 days, while the bone parts of the white diagram that do not cover the grey diagram correspond to the bone resorbed during the 8 days.

The rise of the funnel and epiphyseal plate, and the lengthening of funnel and cylinder may be seen (Leblond *et al.*, 1950).

FIG. 37. Diagrammatic representation (not to scale) of three stages in the growth of the femur in the rat (Tomlin *et al.*, 1953).

The major funnel is seen at the lower extremity where its position at the three intervals studied may be recognized.

PLATE X

Figs. 38 and 39. An X-ray microradiograph (Fig. 38) and an autoradiograph (Fig. 39) of the same section of the compacta of femur of a dog, given 10 mc P³² as disodium phosphate and sacrificed 3 days later (from Engfeldt *et al.*, 1952). \times about 30.

The Haversian system at 1 appears darker than most of the bone in the microradiograph (Fig. 38)—a fact indicating a lesser absorption of X-ray and, therefore a low content of mineral salts. This Haversian system shows a high uptake of labelled phosphate (Fig. 39, 1). In contrast, the Haversian system at 2 has the same high content of mineral salts as most of the bone and shows no significant uptake of radioactivity. Bone surrounding the resorption cavity at 3 also has a high content of mineral salts and shows no activity. The old parts of the bone, lying between the Haversian systems can be seen microradiographically to be heavily mineralized and take up relatively small amounts of radioactive phosphate.

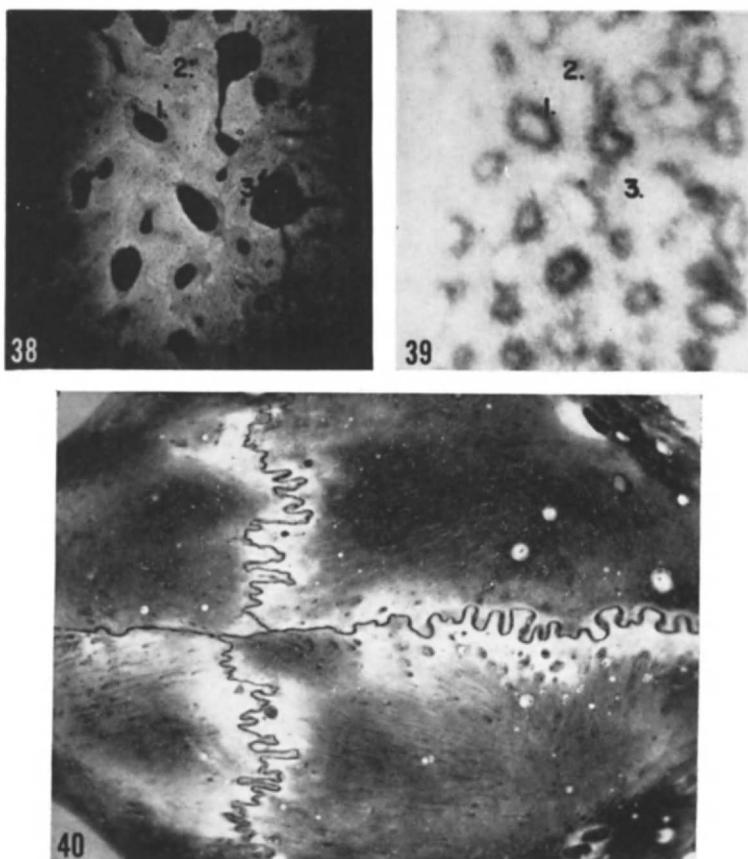
FIG. 40. Surface view of the calvaria of a rabbit injected at the age of 3 months with Ca⁴⁵ and sacrificed 3 months later. The bone was cleaned and then coated with photographic emulsion. After exposure in the dark the bone-emulsion complex was photographically developed and fixed. (Preparation supplied through the courtesy of Prof. P. Lacroix.)

As in the previous figures, the areas of isotope uptake are indicated by a smooth blackening of the photographic emulsion. (The suture lines are seen because of their refractiveness and not because of a radioautographic reaction).

The pale areas in the vicinity of the suture lines represent zones of minimal isotope uptake. The greatest uptake appears to have occurred in the central regions of the individual frontal and occipital plates.

This technique appears useful in determining growth patterns in complex bony structures of membranous origin.

PLATE X



For explanation, see p. 357.

CHAPTER XIII

OSTEOGENESIS IN THE HUMAN EMBRYO AND FETUS

ERNEST GARDNER

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I. Introduction

Osteogenesis involves a number of related aspects, as Brash (1934) has clearly indicated. These include the nature of ossification and of the absorption of bone, and the chemical and mechanical influences on or controls of these processes, as well as the general mechanisms by means of which characteristic external form and internal structure are deter-

mined, organized, and maintained as integrated elements within the skeletal framework. Many details are considered in other chapters, particularly the role of the osteoblast and osteoclast, the mechanism of calcification, the structure of ground substance and fibers, and various other features. It seems more pertinent, therefore, to discuss here in some detail the prenatal development of bones in man, and to deal particularly with the initial formation of bones, the environment in which ossification begins, the relation of ossification to general growth patterns, shape and structure of bones during prenatal growth, and the sequence of ossification. After a general discussion of osteogenesis, certain bones have been chosen to illustrate some of these points. They have been selected as representative and are carried throughout the embryonic and fetal periods, with particular regard to the sequence of specific growth patterns. It will be apparent that for many aspects of osteogenesis, only purely descriptive or morphological data exist, particularly for the human skeleton.

II. Ossification and Growth

If the term osteogenesis is used in its all-inclusive sense, then ossification is but one phase of osteogenesis and is a process that is fundamentally similar wherever it occurs. All bones are indicated first as condensations of the mesenchyme which appear early in the embryonic period, before ossification begins. Some condensations are predominantly fibrous or membranous; ossification in them forms membrane or dermal bones. Most bones, however, begin as cellular condensations which go through a stage of chondrification leading to the formation of cartilage. The bones which then form are the so-called cartilage or replacing bones. Thus, it is the environment in which ossification occurs and not the nature of the process of ossification which differentiates membrane from cartilage bones. Objections are often raised to the use of these terms on the grounds that it is not clear that they refer merely to the substratum in which ossification occurs, and the terms intramembranous and endochondral ossification are often used instead. The criticisms are valid but the latter terms are no better in this regard. Endochondral ossification, for example, refers only to one phase of growth in a bone which has been preformed in cartilage and is an incomplete term so far as the total growth pattern of the bone is concerned.

1. MEMBRANE BONES—INTRAMEMBRANOUS OSSIFICATION

Membrane bones include most bones of the brain case or vault of the skull, many around the sense organs and in the facial skeleton, and, in part, the clavicle and mandible.

Intramembranous ossification is preceded by a fibrocellular condensation which forms during the embryonic period. The primary center (or centers) of each bone is indicated by an increase in cells and fibers. The enzyme phosphatase is synthesized by these differentiated cells, now called osteoblasts, and coincident with this is the formation of a calcified organic matrix which cements the fibers together. The precise manner in which the matrix is formed is unknown, although it seems likely that connective tissue cells are directly concerned and probably form or secrete the matrix. This matrix or ground substance contains complex compounds, including mucopolysaccharides, in varying degrees of polymerization (Gersh and Catchpole, 1949). The recent work of Heller-Steinberg (1951) indicates that the matrix of new bone is less highly polymerized than it is later on. But the exact metabolism, and, of course, the initiating factors, are unknown.

The first bit of bone is a spicule or very small trabecula surrounded by osteoblasts. Some of the osteoblasts become enclosed in matrix being formed around them and thereby become osteocytes. Others divide and continue to add bone to the trabecula. Similar changes begin in the immediate vicinity so that new trabeculae are formed and the center of ossification expands. Some trabeculae radiate from the center. These primary ones soon become connected by secondary trabeculae. The primary ones increase in length by accretion on their free ends. All trabeculae increase in thickness as new bone is added to their surfaces.

The study of undecalcified rat bones (Bloom and Bloom, 1940; McLean and Bloom, 1940) has shown that ground substance is calcifiable as soon as it is formed. The term osteoid refers to bone matrix which has not been calcified. According to Bloom and Bloom (1940), osteoid is present at the end of a trabecula, and sometimes on the sides, when such a trabecula is first formed, but not during subsequent growth. Generally then, formation and calcification of bone matrix are simultaneous, at least in rats.

The pattern of growth and orientation of bony trabeculae depends upon the bone being considered. In the case of bones such as the parietal or frontal, the first or primary trabeculae soon form a radiating network, mainly parallel to the surface of the skull. Some are directed at right angles and thus add to the thickness of the center. The primary trabeculae become interconnected by secondary trabeculae. The spaces thus enclosed contain vascular connective tissue, the forerunner of hematopoietic tissue. The spaces become smaller as trabeculae increase in thickness, so that there is an increasing density of the bone in the area where ossification first began, and the radiating pattern becomes less apparent. The bone continues to expand by the formation of new

trabeculae at the periphery, together with free islands and nodules of bony tissue. This combination of a denser central plate, with a more peripherally situated trabeculated region of advancing ossification, together with free islands and nodules at the peripheral margins, constitutes *open reticular bordered plate bone* (Noback, 1943). Throughout the period of ossification, with the expansion of the central plate, there is a gradual reduction in amount of open reticulum and marginal zones. When the bones have grown to occupy more or less their definitive regions and come into closer relationships with other bones, a bony border begins to form around peripheral edges as the trabeculae become interconnected. Subsequently, increments of growth decrease sharply and reorganization becomes prominent.

Membrane bones pose certain problems, some of which are common to all bones, whereas others are peculiar to the vault region. These have to do with the factors controlling early formation or differentiation, the relation of skull bones to the meninges and the brain, the mechanism of absorption and reconstruction in changing radii of curvature, the significance of sutural positions and the problem of sutural growth, the formation of diploe, and relationships between bones. Of all the bones in the body, those of the vault seem most subjected to mechanical influences during the prenatal period because of pressures of growing and expanding intracranial contents. Such influences may well be important in trabecular orientation. It has been suggested that they are also responsible for the onset of ossification, but this hardly seems likely. The vault bones can form in the absence of the brain, as in cases of anencephaly, although they are small and abnormal in shape.

This chapter is not concerned with the osteoclast, the exact function of which is still enigmatic (Hancox, 1949), or the precise mechanisms of bone absorption. Osteoclasts are, however, commonly found where bone is being removed, and there seems little doubt that they are involved in absorption processes. Information derived from plotting the location and numbers of osteoclasts (Barnicot, 1947), combined with that obtained from madder feeding and other experimental work (Brash, 1934), shows that reconstruction of vault bones begins very soon after ossification starts. Once they have reached their definitive size and relation to other bones, the vault bones accommodate the growth of the brain primarily by a change in curvature. This is brought about chiefly by deposition of bone on the external surface, progressively increasing toward the periphery, with absorption of bone from the inner surface, although to a lesser degree since the bone increases in thickness. The problem of sutural growth hardly concerns the prenatal period, nor does the possible absorption of bone within diploic spaces rather than on surfaces or at sutures, since formation of diploe is a postnatal event.

The nature of the bone formed by intramembranous ossification is predominantly fibrous, that which Weidenreich (1930) describes as plexus-like or coarse-fibered. The collagenous fibers in each trabecula are gathered in coarse bundles which are arranged in an irregular plexus-like manner with lacunae of variable shape. Reconstruction and formation of lamellar systems, and the resorptive mechanisms forming diploe, occur after birth. During growth, the connective tissue adjacent to each surface of the bone as a whole gradually forms a fibrous periosteum, that on the inner surface becoming dura mater. At birth, and for some time after, the vault bones consist only of a single plate, a type of compacta, with mixtures of fine- and coarse-fibered bone, and containing spaces filled with loose tissue and vessels.

In other membrane bones, such as maxilla and mandible, ossification is similar but the pattern of growth and orientation of trabeculae are different. Central plates develop more rapidly, borders are smooth and there are few if any bony islands or nodules. The trabeculae extending from the central plate are short and the general appearance of the bones during proliferation led Noback (1943) to term these *smooth bordered plate bones*. The general trabecular pattern is more complex than that of the parietal or frontal bones, and constructive growth is likewise more complex in view of necessity of providing for teeth and their migration, and for keeping teeth in the maxilla and mandible in proper relation to each other.

a. *Secondary Cartilage*

This is a special type of cartilage associated with certain membrane bones, particularly the mandible and clavicle. It forms after ossification begins and consists of large, vesicular cells with relatively little intercellular matrix. It is not part of the cartilaginous primordium but nevertheless takes part in growth processes, just as does cartilage which antedates ossification in other bones.

2. CARTILAGE BONES—PERIOSTEAL AND ENDOCHONDRAL OSSIFICATION

During the period of early formation or differentiation, bones of this type are indicated first by mesenchymal condensations in which cells with oval or round nuclei are packed together; their cytoplasm appears to have a syncytial arrangement. This condensation or blastema appears early in the embryonic period. An intercellular matrix or ground substance which contains compounds characteristic of cartilage matrix is then formed, appearing first in the center of the mesenchymal condensation. The cells become separated and the region presents a lighter staining appearance. This process represents the beginning of chondrification and constitutes a stage often called precartilage. The deposition of

ground surface spreads peripherally until it reaches the margin of the original condensation. Here the mesenchymal cells become so oriented as to form a perichondrium, the cells of which contribute to subsequent growth. With the formation of a perichondrium, each skeletal part is clearly outlined, and before the embryonic period is over resembles the adult in form and arrangement. The cartilage is then gradually replaced by bone.

a. *Long or Tubular Bones*

The cartilaginous models of bones such as the humerus are sharply defined, with well-developed perichondria. They grow by apposition from perichondrium and from tissue forming the interzones of joints, and through multiplication of cartilage cells already formed, together with increase in intercellular matrix. Streeter (1951) has described the growth of cartilage in some detail for the human humerus. The youngest cells are at the ends where most growth occurs. These cells are small, lack a specific arrangement and constitute a phase which Streeter (1951)

PLATE I

All illustrations in this plate and Plates II and III are unretouched photomicrographs of human embryonic and fetal material. Magnifications are approximate.

FIG. 4. Section of upper humerus, not including head, from a 28-mm embryo, illustrating phases of cartilage growth. The zone of hypertrophied cartilage cells occupies the lower part of the section. There is a very thin layer of periosteal bone next to this zone. Specimen No. 1465, $\times 22$.

FIG. 5. Sagittal section of upper humerus from 30-mm fetus. Note cartilage phases, periosteal bone, and invasion of shaft. Specimen No. 906, $\times 15$.

FIG. 6. Section of humerus at 198 mm. Many cartilage canals in head of humerus. The growth zone, even though close to the head, is still somewhat irregular. Specimen No. 1301, $\times 2\frac{1}{2}$.

FIG. 7. In this frontal section of a humerus at term (348 mm), note curved endochondral growth zone, containing a sharply defined basophilic zone. Specimen No. 1299, $\times 2\frac{1}{2}$.

FIG. 8. A sagittal section of lower humerus from a 37-mm fetus, containing one of several points of erosion of periosteal bone. The invasion is not as advanced as that shown in Fig. 5. The cartilage is characteristically narrow in the region of periosteal bone formation. Specimen No. 907, $\times 15$.

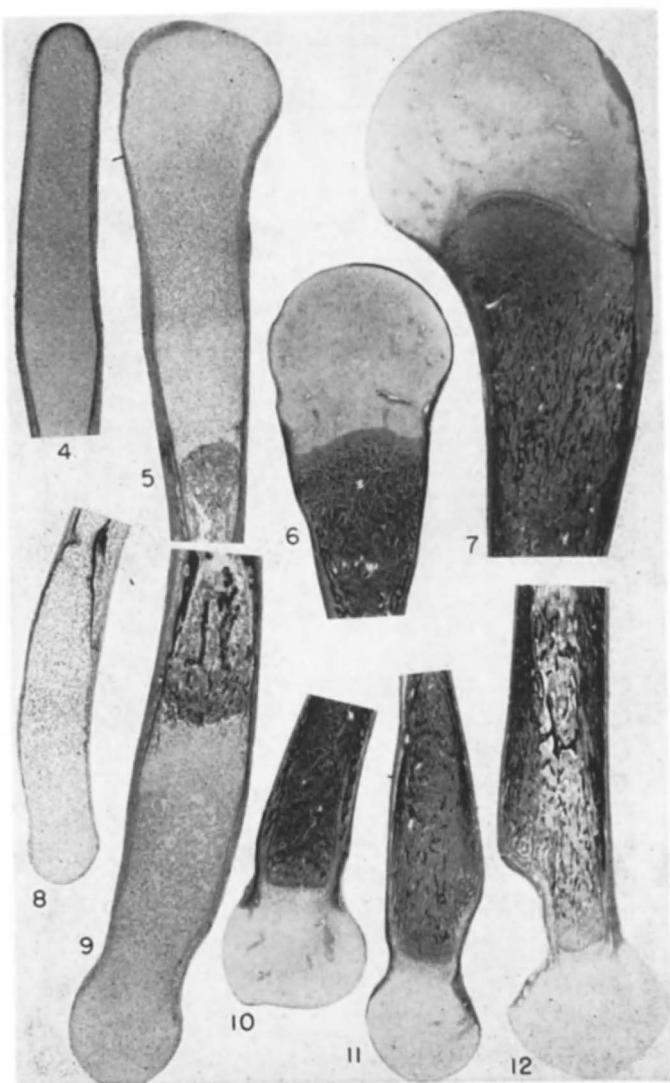
FIG. 9. A sagittal section of lower humerus at 60 mm showing periosteal bone extending toward lower end, ahead of endochondral ossification and at same level as the zone of hypertrophied cartilage. Most endochondral trabeculae are removed since only a few relatively large ones are seen in the marrow space. Specimen No. 1024, $\times 15$.

FIG. 10. Lower humerus at 253 mm. Endochondral growth zone is sharply defined. Specimen No. 1292, $\times 2\frac{1}{2}$.

FIG. 11. Sagittal section of lower humerus at 285 mm. Periosteal bone is especially prominent on the posterior surface (right side). In this plane and region, the growth zone occupies the narrowest part of the shaft. Specimen No. 1211, $\times 2\frac{1}{2}$.

FIG. 12. Section of lower humerus at term, 348 mm. Same specimen as Fig. 7 but cut in sagittal plane. Periosteal bone formation very prominent posteriorly (left side). $\times 2\frac{1}{2}$.

PLATE I



designated phase 1. These cells multiply and form rows of flattened, closely packed cells, Streeter's phase 2. The intercellular matrix then increases in amount, the cells enlarge and their cytoplasm becomes vesicular (phase 3). In phase 4, cartilage cells reach their maximum size and undergo extreme vacuolization. In the last growth stage, phase 5, the cells begin to disintegrate or liquefy and some of them disappear. Fell (1925) has described cartilage phases for the chick, including epiphyseal, flattened (comparable to Streeter's phase 2) and hypertrophied (phases 3 and 4).

The oldest cartilage cells, those of phases 4 and 5, are found in the center or midsection of the shaft, where they form a lighter staining region. The shaft is narrower here than anywhere else (Figs. 4, 8). This may be due in part to the relatively small number of cells originally present, and in part to the fact that cartilage grows more by addition of new cells at the ends than by multiplication after being formed. The perichondrium around the shaft contributes very little to the growth in width.

(i) *Periosteal and endochondral ossification.* Ossification begins at the middle of the cartilaginous model. A thin layer of calcified bone matrix is laid down between perichondrium and that portion of the shaft containing hypertrophied cartilage cells and by extending around the shaft forms a ring or collar. This *periosteal bone* (primary bone collar) is directly in contact with cartilage (Fig. 4). The inner cells of what is now termed periosteum differentiate into osteoblasts and alkaline phosphatase can be demonstrated in them. These cells line up in relation to bundles of collagen fibers and trabeculae begin to form, just as in the case of intramembranous ossification described previously. The primary bone collar thus gradually becomes multilayered.

In the cartilage itself, alkaline phosphatase appears in the hypertrophied cartilage cells and then in the matrix of this zone (Greep, 1948). Sometimes concurrently with the formation of the primary bone collar, but more often a little later, the matrix of this zone becomes calcified.

The time between the formation of the primary bone collar and calcification of cartilage matrix on the one hand, and the next phase, vascular invasion of the cartilage, is extremely variable, depending on the bone. In the case of the humerus and other large, long bones, there is very little delay. Once the primary collar is well established, it is penetrated at several points by vascular tissue, and osteoclasts, derived from the periosteum (Fig. 8). But in the case of other bones such as metacarpals or phalanges, days or weeks may intervene between the formation of the primary collar and vascular invasion.

The tissue which penetrates the bone and invades cartilage is mainly

cellular. It contains few if any fully formed blood vessels. The cartilage is rapidly replaced by this proliferating tissue. Blood vessels and a circulation are then established. Some of the cells differentiate into osteoblasts while others are forerunners of blood forming cells. This invasive, proliferating process extends rapidly toward the ends of the bone. At a variable time after invasion, the osteoblasts form bone around the bits of cartilage which are left, both in the region of the original entry and at the advancing zone of cartilage removal. This formation of bone constitutes *endochondral ossification*. According to Bloom *et al.* (1940), osteoid is not present as a preliminary stage in the formation of endochondral trabeculae of rat bones.

The rapid advance toward the ends of the bone leaves behind a loose network of endochondral trabeculae which fuses with the multilayered periosteal shell. This network contains in its meshes a vascular tissue, the forerunner of hematopoietic tissue. All cartilage growth phases are still present, but the zone of calcified cartilage matrix becomes quite thin. In some regions, the advance of ossification may be so rapid that preliminary calcification of cartilage matrix may not have time to occur.

Periosteal ossification likewise extends toward the ends of the bones, preceding the endochondral and maintaining a level with the zone of hypertrophied cartilage until the epiphyseal regions are reached (Figs. 4-12).* The advance consists of a continuing deposition of bone matrix adjacent to the hypertrophied cartilage. New trabeculae are then formed so that the shaft increases in thickness (Fig. 22). These trabeculae form at right or acute angles to bone already present, in such a manner as to surround vessels running longitudinally in the periosteum.

Many of the endochondral trabeculae are removed almost as soon as they are formed, so that a short marrow cavity with but a few trabeculae is formed. This removal of endochondral trabeculae continues as the zone extends toward epiphyses, so that relatively few remain. Those which do become progressively larger as more and more bone is laid down around them. Many fuse so that a loose network is formed.

When epiphyses are reached, the growth zone becomes much more orderly in appearance (Figs. 7, 21) and the rate of ossification is naturally slower. Hypertrophied cartilage cells are arranged in longitudinal columns with thin strips of calcified matrix between the cell columns. Osteogenic tissue and blood vessels invade the cells, leaving matrix around which bone is later deposited. Many of the endochondral trabeculae thus formed are removed almost immediately. Those which remain increase in thickness and form an irregular network in the interior of this part of the shaft.

* Fig. 4-12 in Plate I (see p. 365).

This growth zone is responsible for increase in length of the bone. The reconstruction which is necessary to maintain form as length increases is now clearly evident. Part of the shaft is uniform in diameter but near the ends it widens as it continues into epiphyses. These wider regions must be reduced as the bone lengthens to prevent club-shaped ends from being formed. This is brought about in a manner illustrated

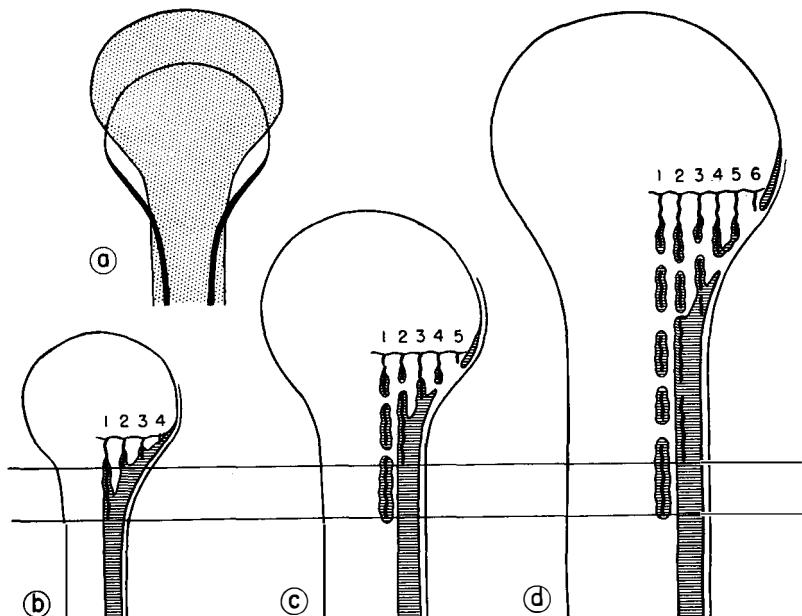


FIG. 1. A schematic representation of certain features of bone growth. In *a*, two stages in growth are superimposed so as to illustrate as simply as possible how bone must be added to the surface of the shaft below but removed from the surface where shaft increases in diameter. *b*, *c*, and *d* illustrate some of the specific changes. In *b*, four endochondral trabeculae connect growth zone and shaft. Calcified cartilage matrix is shown in solid black. Trabecula number 1 joins that portion of the shaft which is uniform in diameter. Since bone is removed from the inside of this portion of the shaft, trabecula number 1 is separate from the shaft in *c*. Moreover, this trabecula is itself eroded in several areas by osteoclastic activity. Trabecula number 2 in *b* joins that portion of the shaft which increases in diameter on approaching the epiphysis. Bone is removed from the surface of this region, as shown in *c*. It is deposited internally or endosteally and by this process incorporates adjacent trabeculae, such as number 2. This explains why remnants of calcified cartilage are temporarily found in the primitive compacta of the shaft. In *d*, another trabecula, number 3 has become incorporated in compacta. *c* and *d* also illustrate that surface removal of periosteal bone may isolate that which is adjacent to the growth zone. If this occurs all the way around the shaft, the isolated portion is then a ring encircling the growth zone. The changes described have recently been clearly demonstrated by autoradiographic studies in the rat (Leblond, *et al.*, 1950).

in Fig. 1, which shows that bone is removed from the external surface and laid down internally (endosteally). Those endochondral trabeculae which fuse with the internal surface of the primitive periosteal compacta become incorporated in this compacta as bone is formed endosteally (Fig. 1).

The removal of bone from the external surface is most prominent in

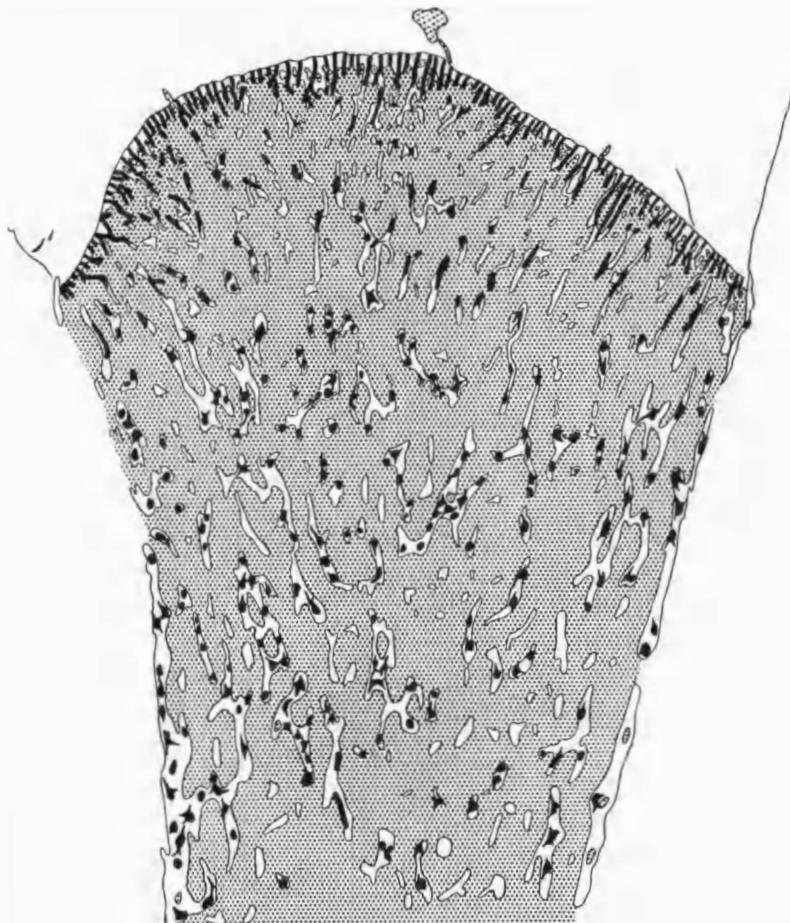


FIG. 2. This is a frontal section of the upper humerus, exclusive of the cartilaginous epiphysis, from a 302-mm human fetus, about 34 weeks. Specimen number 1274, $\times 10$. The section was projected on to bromide paper and a tracing made from the print. The bars of cartilage matrix (solid black) extending down from the growth zone are shown larger than they actually are. The drawing illustrates the complicated trabecular pattern and the widespread distribution of cartilage remnants in endochondral trabeculae and compacta. Marrow spaces and vessels are stippled, including communicating channels from cartilage canals, while bone is clear.

the region just below the level of the growth zone, to the extent that periosteal bone may be completely removed, exposing endochondral trabeculae (Fig. 1). The periosteal bone above this level remains as a ring encircling the growth zone, the periosteal or perichondral ring or "l'encoche de Ranzier." A complete ring, however, is not common in human bones. Figures 2 and 3 are drawings of the humerus showing this process, especially on one side.

Osteoclasts are numerous where bone is being removed, as Kölliker (1873) pointed out in some detail. Figures 3a and b, both drawings of humeri, give the distribution of osteoclasts in those regions where remodelling is prominent.

The portion of shaft which is uniform in width, including regions which were previously wider, increases in diameter by surface accretion and endosteal removal. By this process those endochondral trabeculae which had become incorporated in compacta are eventually removed.

The reduction of width described above does not take place at each end of every bone, nor does it necessarily occur all the way around the end of a bone. The distal end of the humerus for example, narrows in the antero-posterior direction but widens from epicondyle to epicondyle. Therefore, on anterior and posterior surfaces, periosteal bone thickens above the growth zone, and removal of bone from the surface occurs only in medial and lateral epicondylar regions. Furthermore, local processes related to development of ridges, grooves and torsion may also modify general processes.

The epiphysis grows in width primarily by addition of cartilage cells from perichondrium above the bony ring. This ring itself grows upward by addition of new bone to its upper edge, but remains about the same length because bone at its lower edge is removed.

The shaft at birth is composed of a thick compacta, in which trabeculae are directed mainly longitudinally, and a short marrow cavity. The inner surface of the primitive compacta is irregular and trabeculated. Haversian systems (osteones) with lamellar arrangements characteristic

FIG. 3. *a* is a drawing of part of a cross section through the upper humerus from a 180-mm human fetus, about 22 weeks. Specimen number 1208, $\times 20$. The plane and level of section are approximately indicated by the arrow pointing to the humerus of the older fetus in *b*. This drawing shows the distribution of osteoclasts (solid black) which are represented larger than they actually are. Note how the thin layer of periosteal bone has been removed in the region where osteoclasts are especially numerous. *b* is a drawing of the same specimen as Fig. 2 to show distribution of osteoclasts (solid black). Even in this one section there are many such cells, especially at the growth zone. Part of the periosteal compacta has been completely removed, especially on the left. Marrow spaces and vessels are stippled, while bone is clear, $\times 10$.

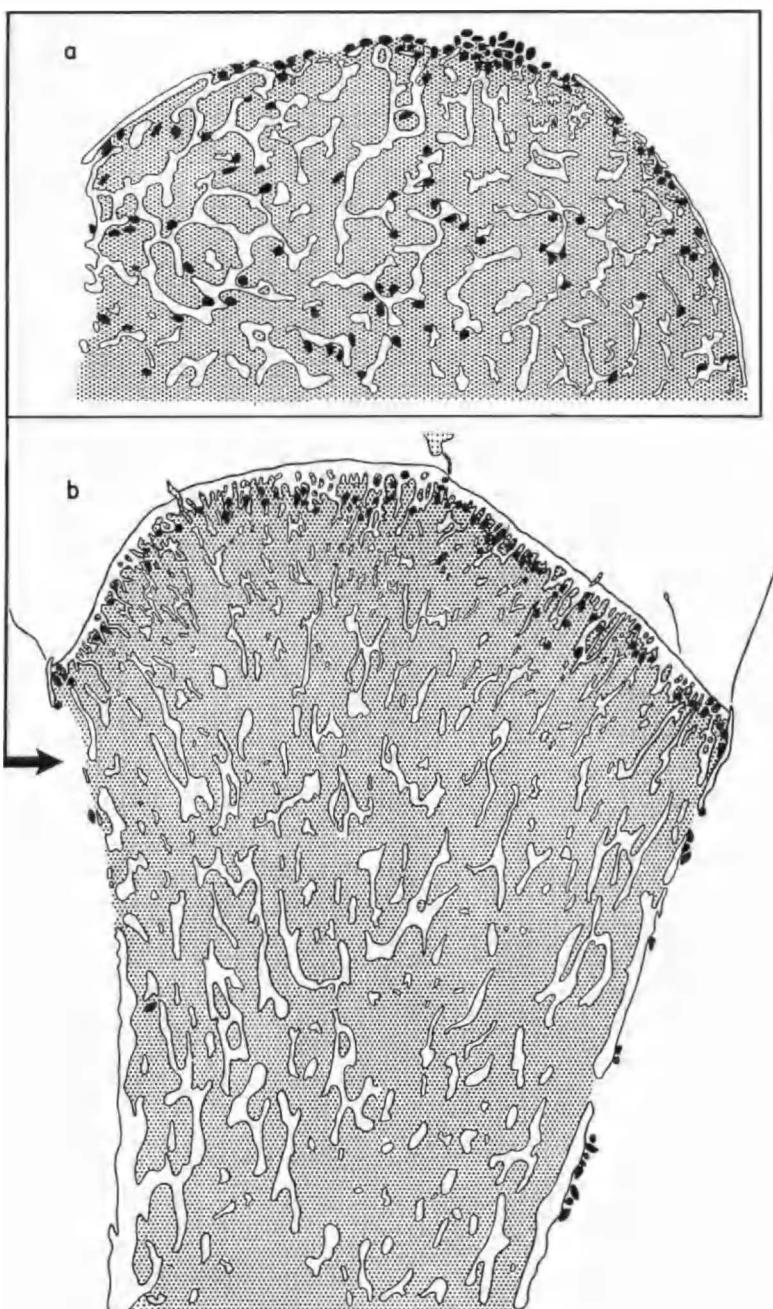


FIGURE 3

of adult compacts are usually not present at birth. Postnatal changes in this regard are clearly described by Amprino and Bairati (1936).

The periosteum, which is anchored to each end of the bone, increases in length primarily by interstitial expansion. Periosteal growth is important, particularly in the remodelling associated with muscular and ligamentous attachments. Space does not permit discussion here and reference should be made to the treatment of this subject by Lacroix (1951).

The blood supply of bone becomes well established during the prenatal period. The initial vascularization of shaft and marrow is multiple and a well formed or single nutrient artery is not present in the early stages (Pinard, 1952).

(ii) *Epiphyseal ossification.* Although the general appearance of the growth zone for the diaphysis is similar to that seen after an epiphyseal plate is well established, a discussion of the epiphyseal ossification which leads to the formation of this plate is not within the scope of this chapter. Yet there are certain phases of this growth which are first indicated in prenatal life.

Early in the fetal period epiphyseal cartilages are invaded by blood

PLATE II

FIG. 13. Clavicle from horizontal section of 18-mm embryo. There are two centers, a smaller lateral one on the left merging into a cellular mass, and a larger medial one on the right continuous with the condensation directed toward the ventral midline. Specimen No. 1502, $\times 27$.

FIG. 14. Horizontal section of shoulder region in 28-mm embryo. The cartilaginous head of the humerus is upper left, with the coracoid process to the right. Below is the acromion process and to its right is the cartilaginous acromial end of the clavicle merging with vascularized membrane bone. Specimen No. 1465, $\times 20$.

FIG. 15. Frontal section of humeral head at term, 348 mm. There are many cartilage canals and an epiphyseal center is just beginning. Note the sharply defined endochondral growth zone. Specimen No. 1299, $\times 2$.

FIG. 16. Horizontal section through sternoclavicular region of same embryo as Fig. 14, showing cartilaginous end of clavicle, lower right. Sternum, upper left. $\times 20$.

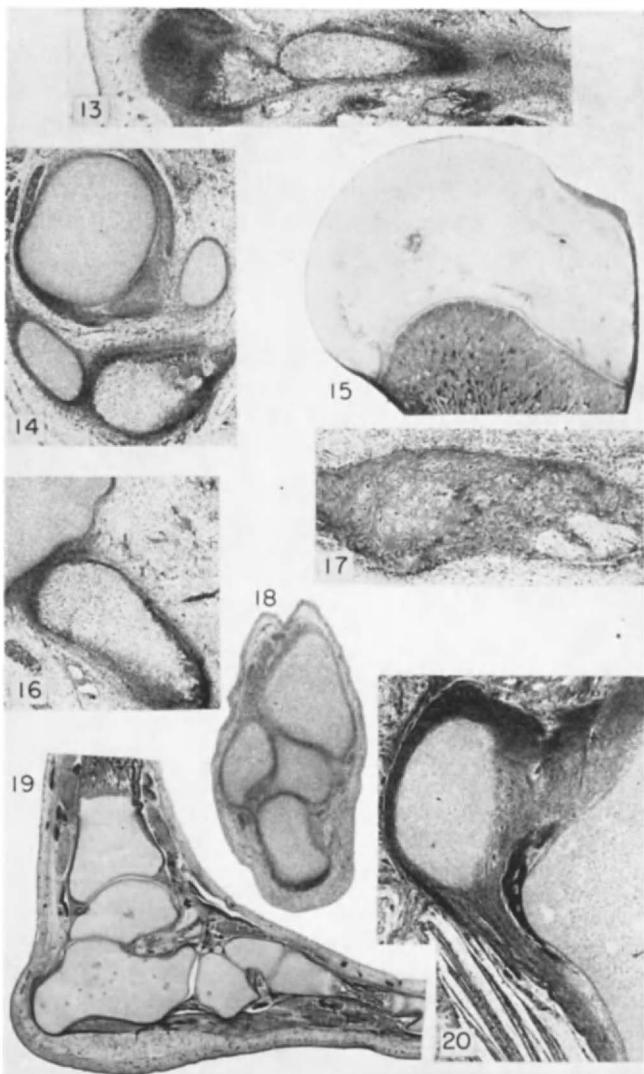
FIG. 17. Shaft of middle of clavicle. Same embryo as Figs. 14 and 16. Clavicle here composed of membrane bone in lower right part of which are two points of vascular invasion. $\times 40$.

FIG. 18. Frontal section through ankle and tarsal region of a 27-mm embryo. Cartilaginous tibia above, talus immediately below it, fibula to its left, and calcaneus below talus. Specimen No. 1439, $\times 20$.

FIG. 19. Sagittal section of foot and ankle of 107-mm fetus. Tibia above. Note cartilage canals in talus and calcaneus. There is a sharply defined multilayered periosteal shell on one of the metatarsals (right side). Specimen No. 1098, $\times 3\frac{1}{2}$.

FIG. 20. Section through calcaneus of a 132-mm fetus. Both apparently separate cartilages are part of calcaneus. Note periosteal bone in center of photograph. Specimen No. 1235, $\times 13\frac{1}{2}$.

PLATE II



vessels which form cartilage canals (Schaffer, 1930; Haines, 1933; Gray and Gardner, 1950), preceding ossification by many months. These cartilage canals are filled with loose connective tissue, containing one or more arterioles, venules, capillaries and a few nerve fibers accompanying the blood vessels.

Ossification in the head of the humerus is usually indicated at or before term (Fig. 15). Cartilage cells in the region of the future center enlarge and become vesicular. Intercellular matrix calcifies, following which vascular buds from adjacent cartilage canals invade this area, erode the matrix and deposit bone around cartilage remnants. Endochondral ossification then spreads rapidly in all directions. In most long bones, however, epiphyseal ossification, including the distal end of the humerus, begins postnatally, even though canals appear early in the prenatal period. Generally, those centers which appear first, as in the head of the humerus, distal end of femur and proximal end of tibia are in those ends from which most growth in length takes place. After an epiphyseal center is well established, its growth zone is in the deep part of the articular cartilage.

(iii) *Clavicle*. The clavicle is a long bone which is preceded by a membranous rather than a cartilaginous stage, but shortly after ossification begins, cartilage forms secondarily and thereafter takes part in the growth in length.

b. *Short, Flat, and Irregular Bones*

Bones of this type are preformed in cartilage (Fig. 18) but have patterns of ossification which differ somewhat from those of the tubular bones. They are usually formed in cartilage before the embryonic period is over, but the onset of ossification is extremely variable. In some bones, such as the scapula, ossification begins by the end of the embryonic period or very early in the fetal period and is characterized by cartilage calcification and periosteal bone formation before vascular invasion occurs and endochondral ossification starts. In other bones, such as vertebrae, the pattern varies for different parts. In the neural arches, cartilage calcification and periosteal bone appear first, but in vertebral bodies calcified cartilage matrix is invaded by blood vessels and endochondral ossification begins. Only later, as a rule, does periosteal bone form. Of the tarsal and carpal bones, only the talus and calcaneus as a rule begin to ossify before birth. In all of the tarsal and carpal bones, however, vascular invasion and formation of cartilage canals (Fig. 19) begin in fetal life (Watt, 1928). When ossification does begin, it is indicated first by hypertrophy of cartilage cells, and calcification of matrix in an avascular area of cartilage. Vessels then invade from adjacent cartilage canals and endochondral ossification starts. The gen-

eral process is therefore much like that in epiphyses. Ossification spreads in all directions until it reaches the periphery of the cartilage. At this time, sometimes before, periosteal bone forms so that continued growth by surface accretion is possible. Occasionally periosteal bone forms on the surface of the calcaneus sometime before the primary center begins (Fig. 20). Whether or not epiphyses occur varies with the bone. In some, such as the scapula and vertebrae, epiphyses are present, but in others, for example, most carpal and tarsal bones, there are none.

The pattern of trabeculation in these bones is characteristic of them. After ossification is established, thick peripheral bands of bone with thin, inner trabeculated borders develop. These are *peripheral band bones*. Remodelling is complex in these irregularly shaped bones and no general rule can be established. Whereas it is commonly present within the bone as growth proceeds, yet changes in shape, development of torsions and other shifts, are for the most part brought about by external absorption in specific sites.

The establishment of cartilage canals before ossification begins is characteristic of the bones of larger animals and is thought to be related to cartilage size in that they nourish cartilage which grows beyond a certain volume. There is still controversy as to whether the entrance of vessels is an active invasion (Hurrell, 1934), or whether they are merely included as cartilage grows (Haines, 1933, 1937). The histological appearance of the invading tissue is similar to that seen in the initial invasion of the shaft. The canals increase in size and number and branch within cartilage. If the mechanism is passive, it is a rather remarkable response to variable mechanical factors and is at variance with the active vascular invasion of the shaft and of epiphyseal and other centers of ossification.

III. Development of Specific Bones

The specific features of bones and their development can best be treated by selecting certain bones and carrying them throughout their prenatal development. In the interests of space, major attention is given to the humerus. Accurate determinations of age are foremost in such a treatment.

1. EMBRYONIC AGE

In any discussion of development, some standard of age or degree of development must be available by which different embryos can be compared. Single criteria, such as crown-rump length, have generally been used as an index of age, but any one criterion is subject to considerable individual variation. Discrepancies between reports of different investi-

gators can in part be attributed to the use of single criteria. Furthermore, lengths may change because of shrinkage and distortion, and clinical histories are often incomplete or unreliable.

Staging of embryos has been a standard procedure for lower forms, especially amphibians. Hamburger and Hamilton (1951) have recently provided a method of staging chick embryos according to a number of external and morphological criteria. The importance of their work lies in the fact that it is now possible to discuss and compare differentiation and growth in terms which are not confused by variations inherent in stages using time since fertilization or but a single morphological criterion such as length.

Streeter (1951), in an important series of studies of human embryos, divided the embryonic period of development into 23 stages, each stage being identified by a number of external and internal characteristics. Each age group or "horizon" is characterized by a certain degree of differentiation and organization of various tissues and organs, as well as by general shape and size. Furthermore, by comparing embryos in each age group to macaque embryos of known age, in which rate and degree of development during the embryonic period are quite similar, an accurate index of ovulation age has been obtained. It is therefore possible for the early development of bone to be outlined in terms of age groups. Discrepancies in the literature regarding time and sequence of chondrification and ossification may be partially resolved, since some are due to the fact that while two embryos may have different crown-rump lengths they may nevertheless have reached the same degree of development and belong to the same age group. At present, relating specific

TABLE I
AVERAGE LENGTHS AND OVULATION AGES OF STREETER'S
AGE GROUPS 12-23

Age group	Usual length in mm	Ovulation days
12	3- 4	26±1
13	4- 5	28±1
14	6- 7	28-30
15	7- 8	31-32
16	9-10	33±1
17	11-13	35±1
18	14-16	37±1
19	17-20	39±1
20	21-23	41±1
21	22-24	43±1
22	25-27	45±1
23	28-30	47±1

features of bone development to specific age groups of the embryo is based mainly on Streeter's studies of material at the Carnegie Institute of Embryology. Some of this material was studied previously by Bardeen (1905), Lewis (1902) and Bardeen and Lewis (1901). These studies have been supplemented in the present chapter by personal observations of sectioned limbs and other regions of embryos, as well as of a fairly extensive series of fetal limbs. Most bones, however, ossify during the fetal period for which no staging has been devised. Weekly increments in weight and crown-rump length or some other external characteristic are perhaps the best index of fetal development during later development.

The embryonic period occupies about the first 7 weeks (since ovulation) of development. At the end of this time differentiation is practically completed. Streeter found that vascular invasion of the shaft of the humerus begins at about the end of this period and arbitrarily designated any specimen in which marrow was forming in the humerus as a fetus, beyond the 23rd stage. This begins in specimens which are usually 28–30 mm crown-rump length. Table I gives certain characteristics of those age groups which are pertinent to this discussion.

2. THE HUMERUS—A LONG OR TUBULAR BONE

a. *Embryonic Period*

The first indication of an upper limb is found in human embryos belonging to Streeter's age group 12. It is only in the older members of this group, however, that an early condensation for the upper limb is visible. The numerous cells within this area exhibit no differentiation. They arise from the unsegmented body wall, the somatopleure, opposite the 8th to 10th somites (5th to 7th cervical). In age group 13, the buds of both upper and lower limbs are visible. In age group 14, the arm buds curve forward and inward, and in age group 15, a hand segment is present distally. During this time, from age group 12 through age group 15, circulation is established and large nerve trunks are growing into the limbs. Cellular proliferation has resulted in a central, longitudinally arranged skeleto-muscular condensation. By age group 16, the condensation is defined by large nerve trunks entering differentiating muscle groups. At this stage, the hand region of the bud shows a central carpal part and a digital plate.

Differentiation proceeds so that by age group 17 finger rays appear and muscle groups are distinguished as well as major branches of the brachial plexus. The more central part of the skeleto-muscular condensation is now evident as the avascular, skeletal blastema, and chondrification is under way in a proximo-distal sequence. By age group

18, individual muscles can be recognized. The hands have distinct finger rays, and in the older embryos, interdigital notches at the rim. An elbow region is present and the cartilages now have the general shapes of the future bones.

As the cartilages form, the regions of future joints become indicated by homogeneous blastemal interzones which are continuous with the perichondria of the cartilages. The cartilages grow by apposition from perichondrium and from chondrogenic zones of the blastemal interzone and by interstitial growth. The sequence of growth leads to a characteristic arrangement of cartilage cells (Figs. 4, 5, 8, 9). Streeter designated the youngest cells as belonging to phase 1. In the humerus, these cells are located at the ends of the cartilage, adjacent to the interzones, and intercellular material is just beginning to form between them. The cells have no specific arrangement, but as they divide the resulting cells become somewhat flattened and elongated and arranged in rows transverse to the long axis of the bone. This constitutes phase 2 and is of course closer to the center of the shaft. Then the cells enlarge and become cuboidal in shape. Their cytoplasm begins to vacuolate and intercellular substance is increased in amount. This is phase 3 cartilage. In phase 4, cartilage cells are maximal in size and have undergone extreme vacuolization, accompanied by a thinning out of intercellular material. Calcification of this matrix may begin in phase 4. In phase 5, according to Streeter, disintegration or liquefaction of cartilage cells is advanced.

In embryos of age group 19, the first three phases of cartilage growth are present. By age group 20, the arms are bent at the elbow. A clearing area is visible in the center of the shaft of the humerus. In age group 21, the distal phalanges are slightly swollen and touch pads are beginning to form. The hands are slightly flexed at the wrist and nearly come together over the heart eminence. Four phases of cartilage growth are present and the perichondrium is beginning to specialize around the middle of the shaft. By this time the general form of the humerus and associated structures resembles that of the adult. A humeral neck is present, as well as greater and lesser tubercles. The intertubercular sulcus contains the tendon of the long head of the biceps which had differentiated by age group 18. The characteristic form of the distal end of the humerus is also established. Interzones in elbow and shoulder joints begin to show a three-layered arrangement, preliminary to cavitation. Capillaries are evident in the perichondrium and at the periphery of the joints.

In embryos of age group 22, outer fibrous and deeper cellular layers can be distinguished in the perichondrium. A very thin layer of bone

tissue has now been deposited directly adjacent to the cartilage matrix. This deposition may begin in age group 21. The primary bony shell or collar is like a cuff or ring around the center of the bone. Osteoblasts line up external to it, and phase 4 cartilage, the matrix of which may be in the process of calcification, is directly internal to it. In this group, cavitation has usually started in the shoulder joint. It may also begin in the elbow joint, but ordinarily not until stage 23.

In embryos of stage 23, phase 5 cartilage is present. Cells are extremely vacuolated, cytoplasm is liquefied and many appear to be disintegrating. The matrix is now being calcified, if it had not already begun to do so in the preceding stage. The general shape and relationships of the humerus are clearly similar to those of the adult. Cavitation is under way in the shoulder and elbow joints. All ligaments are present, certain bursae are forming, and the joints are supplied by vessels and nerves which have an adult type pattern of distribution. As cavitation proceeds, an early synovial tissue is formed, containing many capillaries. Beginning now and especially noticeable throughout the fetal period is a striking vascularity in regions where fat pads will form. The primary bony shell of the humerus is a single layer without openings or channels. Then, at several sites, a vascular, proliferating cellular bud erodes the shell and comes into contact with cartilage. Matrix is removed and proliferating vessels extend toward both ends. This invasion marks the end of the embryonic period and has been noted in specimens of 28 to 30 mm crown-rump length. This is the last stage of the embryonic period.

b. *Fetal Period*

During this period, removal of cartilage takes place and extends rapidly toward both ends. Adjacent to the advancing zone, phase 5 cartilage with calcified matrix persists as a much thinner layer. Coincident with the central processes, trabeculae form in the periosteal bone so that a multilayered arrangement is evident. By the 10th week, endochondral trabeculae begin to form in the marrow. These grow and unite and the resulting network blends with the outer shell. By the 12th week endochondral ossification occupies more than half of the shaft, and periosteal bone extends to the region where the shaft widens on becoming continuous with epiphyses. Vascular invasion of the head of the humerus may begin by 37 mm and is well under way by the 10th week. Similar invasion of the distal end does not begin until about 73 mm, however (about 13 weeks). In the proximal epiphysis, the first vessels enter mainly from the bicipital sulcus but soon thereafter from areas of tendinous insertions and capsular attachments. The pattern of blood supply to the humerus is thus established, including nutrient, periosteal and epiphyseal arteries.

From the time that cartilage invasion begins, osteoclasts are present, particularly in the advancing ossification zones. Here they may be seen next to cartilage itself and at the free ends of newly formed endochondral trabeculae. Many of these trabeculae are removed as soon as they are formed. By the end of the 3rd month, growth zones are nearing the lines of capsular attachments. Toward the end of the 4th month, osteoclasts can be found on the external surface of the wide or metaphyseal portion of the proximal humeral shaft. Periosteal bone begins to be removed from the external surface in these regions. This is the first visible evidence of the remodelling mechanisms illustrated in Fig. 1. In the early part of the 5th month, many areas in the wide portion of shaft show removal of periosteal bone (Figs. 1-3). Once this remodelling is evident, and especially from the early part of the 6th month on, remains of calcified cartilage matrix can be found in the outer shell or compacta (Figs. 1 and 2). This process is much less prominent in the distal part of the humerus, which contributes less to growth in length than does the proximal end. The growth zone distally is at the narrowest part of the shaft in the antero-posterior plane. Figures 11-12 clearly show that the shaft proximal to this zone thickens by heavy periosteal bone formation. A separate periosteal ring is rarely seen in this plane.

Even at the proximal end of the humerus, the periosteal ring is not completely isolated. Although this general region is being narrowed, there are local sites where bone forms externally, particularly on the edges of the bicipital groove.

Figures 7 and 15 illustrate the humerus at term and an early epiphyseal center. The zone of ossification is characteristically curved, although in sagittal planes it is much straighter. The cartilaginous head and tuberosities consist mainly of cells with no specific arrangement. At the articular surface there are one or two rows of flat cells. More deeply, cells are thin, with round or oval nuclei and cytoplasmic processes. Toward the growth zone, cells occupy more definite spaces or lacunae and tend to lose cytoplasmic processes. Near the growth zone, there is a transition to groups of two to four flat cells, and then clumps of such groups (Fig. 21). There is then a sudden arrangement into columns of hypertrophied cells, with a thin line of basophilic matrix running through the columns. The ends of the columns are invaded by blood vessels.

With regard to associated structures, synovial tissue in shoulder and elbow joints develops early. Synovial villi appear by the 3rd month. Bursae associated with the insertions of pectoralis major, latissimus dorsi, and teres major muscles also develop during the 3rd month. Pacinian corpuscles are found in association with periosteum during later fetal months, and elastic fibers in periosteum, ligaments, and tendons are also present.

3. THE TALUS AND CALCANEUS—SHORT, IRREGULAR BONES

Many bones preformed in cartilage do not ossify until the fetal or postnatal period. Some of these, such as the carpal and tarsal bones, usually have only the so-called endochondral phase, that is, periosteal bone is not initially deposited and no secondary or epiphyseal centers ordinarily form. Of the carpal and tarsal elements, only the calcaneus and talus commonly begin to ossify prenatally.

a. *Embryonic Period*

The initial appearance of the lower limb lags slightly behind the upper limb, appearing usually in embryos of age group 13. In age group 16, one can clearly see a lateral cephalic part to which are distributed the femoral and obturator nerves, a medial caudal part for the distribution of the peroneal and tibial nerves, and a foot region. As in the upper limb, an axial condensation forms in which, with the ingrowth of nerves, skeletal and muscular parts can be distinguished. In age group 17, the leg bud has a rounded digital plate and at age group 18, some feet have toe rays. Chondrification of the blastema has begun and the femur seems to be slightly ahead of the coxal cartilages, all three of which are present. By this time also the tibia and fibula are chondrified. In embryos of age group 19, all of the tarsal cartilages appear to be present. Although toe rays are prominent, interdigital notches are not present, and the distal parts of the digits are still blastemal. In age group 20, interdigital notches are present and in group 21, the toes are well formed and chondrification has practically reached the distal phalanges. A primary bone collar is often present in the femur and tibia and sometimes in the fibula. Just as in the arm, the form and arrangement of the various structures resemble those of the adult. The tarsal cartilages are in their characteristic relations (Fig. 18), most ligaments are present and joints are indicated. Cartilage growth proceeds by both interstitial multiplication and perichondral deposition. No outstanding changes are noted during the remainder of the embryonic period (age groups 22 and 23).

b. *Fetal Period*

The calcaneus and talus maintain their form and relations while increasing in size. At the end of the 3rd month or early part of the 4th (85 mm specimens and larger), blood vessels begin to invade these and other tarsal cartilages, establishing cartilage canals (Fig. 19). No other changes are apparent until ossification begins late in the fetal period when, in the region of the primary center, cartilage cells hypertrophy, matrix becomes calcified and vessels grow in from adjacent cartilage canals. This generally takes place during the latter part of the 6th or early part of the 7th month (Table IV).

Periosteal bone occasionally forms on the anterolateral surface of the

calcaneus by the end of the 4th month (Fig. 20), thus preceding the endochondral center. The calcanean center reported by Noback and Robertson (1951) at 120 mm was undoubtedly periosteal. This early center occurred in about one quarter of the specimens examined by Trolle (1948). It fuses with the main center after the end of the 6th month.

Ossification spreads from the primary center until it reaches the surface, after which periosteal bone is laid down. The calcanean epiphysis is postnatal in onset. The talus occasionally has two primary centers of ossification, and two have likewise been found in the calcaneus.

There is no specific time which characterizes the beginning of reconstruction. Early in the embryonic period, the fibula for a time articulates with the calcaneus. But more than that, the talus is quite low, especially by the 4th fetal month (Straus, 1927). One of the main changes after this time is an increase in width and height, relative to length, so that the talus becomes broad and high. The change in height is especially marked, and it is interesting that this change begins before ossification starts and approaches the adult ratio by term. Likewise, the neck of the talus points acutely toward the medial side of the foot. The angle

PLATE III

FIG. 21. Growth zone in humerus of term fetus, 348 mm. From above down, note changing picture of cartilage cell arrangement, culminating in columns of hypertrophied cartilage cells. Specimen No. 1299, $\times 21$.

FIG. 22. Cross section through middle of shaft of humerus from 150-mm fetus. The trabeculated compacta of periosteal bone is thick and the marrow cavity is relatively narrow. Specimen No. 1561, $\times 15$.

FIG. 23. Section through both Meckel's cartilages from a 22-mm embryo. Early bone of mandible beside each cartilage. Specimen No. 911, $\times 22$.

FIG. 24. Same specimen as Fig. 23, but section is more posterior. Two trabeculae here, and mental nerve occupies middle of light staining region. $\times 22$.

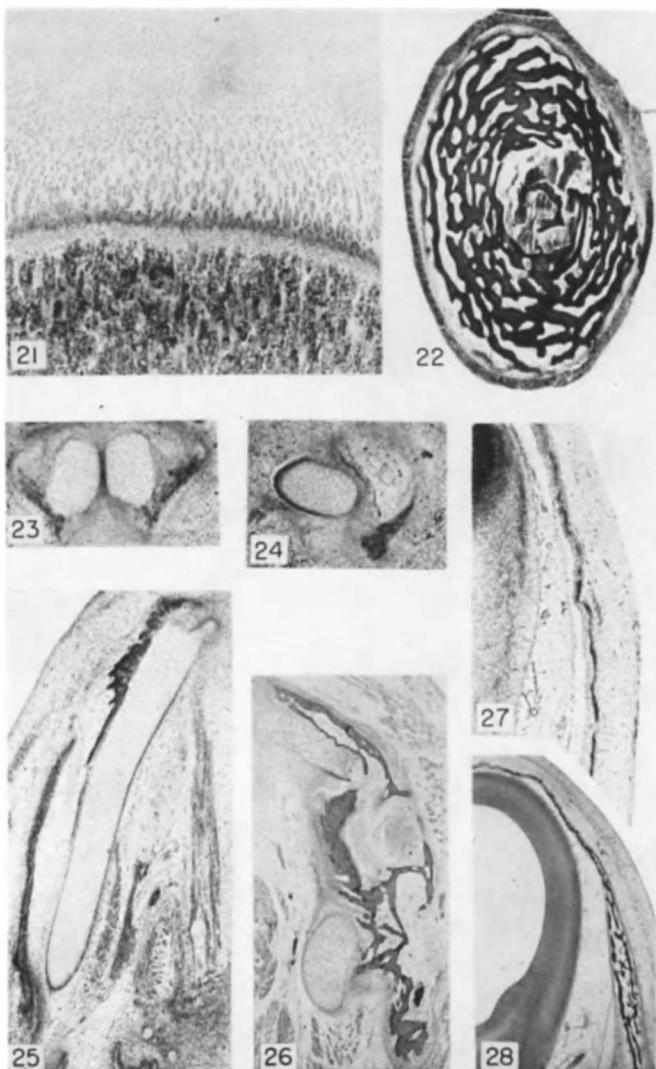
FIG. 25. Section through a considerable extent of Meckel's cartilage in a 28-mm embryo. Note bone closely applied to outer (left) surface of cartilage anteriorly, while the bone formation posteriorly is separated from the cartilage. Mental nerve emerging through foramen. Specimen No. 1465, $\times 22$.

FIG. 26. Mandible from a 58-mm fetus. Trabecular arrangement now quite complicated. Bone is closely applied to Meckel's cartilage anteriorly (upper) and part of it (on right side) is being removed. It is only this region of the cartilage which can be said to undergo endochondral ossification. Part of Meckel's cartilage is also visible in lower part of photograph. Specimen No. 1500, $\times 15$.

FIG. 27. Horizontal section through head of 28-mm embryo. Brain to the left. In a condensation to the right, dark staining early frontal bone is evident as thin spicules. Specimen No. 1465, $\times 22$.

FIG. 28. Horizontal section through head of 58-mm fetus. Brain on left. Frontal bone on right shows increasing thickness and trabeculated pattern. Specimen No. 1500, $\times 7$.

PLATE III



decreases throughout the fetal period from the 4th month on, again before ossification begins, and continues to decrease postnatally. In early fetal months, the body of the calcaneus is short, but increases in length until term. Talo-calcanean relations also change and the two bones do not reach their final position until after term. These two bones undergo greater changes during the fetal period than do the other tarsal bones. Thus, while reconstruction and definitive architecture of bone are undoubtedly influenced by functional use postnatally, there are many characteristics due to changes beginning in the cartilaginous state.

4. THE MANDIBLE (DENTARY)—A MEMBRANE BONE WITH SECONDARY CARTILAGE

This bone is interesting, not only because it is a membrane bone in which cartilage forms secondarily, but because of its close association with Meckel's cartilage.

a. *Embryonic Period*

Jacobson and Fell (1941) have shown that in the embryonic chick there are three types of mesenchymal cells in the early mandibular arch, myogenic, chondrogenic, and osteogenic. These are independent in origin and distinct in time of appearance. The myogenic cells do not arise in the mandible but migrate into it from another source. Other experimental work indicates the possibility that the cartilages of the trabeculae and most visceral arches, including Meckel's cartilage, may come from neural crest cells (de Beer, 1937, 1947). Osteogenic cells are characteristically associated with a thickened patch of mouth epithelium which corresponds to the rudiment of the enamel organ in mammals.

Meckel's cartilages begin to form in human embryos of about age group 17 and rapidly become cartilaginous. They soon become relatively large and by age group 18, the right and left cartilages meet in the ventral midline (but do not actually fuse). Ossification may begin at this time in the membranous condensation which indicates the future mandible. The smallest specimen reported in which it had begun is 15 mm (Mall, 1906; Augier, 1931). According to Noback *et al.* (1951), it is always present after 24 mm (age group 21). Ossification begins on the outer side of the ventral part of the cartilage, in the region of the future body of the mandible (Figs. 23–24), in a condensation which extends posteriorly along the lateral side of the cartilage (Low, 1909). By age groups 20–21, this condensation can be traced back to the lateral pterygoid muscle and the ossification has spread from about the symphysis in front to about the level of the auriculotemporal nerve behind. The form of the temporomandibular joint is also indicated at this time (Symons, 1952). By the end of the embryonic period, the pattern of

the mandible is already quite complex and the mental foramen is clearly indicated (Fig. 25).

b. *Fetal Period*

By the end of the embryonic period, the condensation for the entire mandible is clearly defined, and ossification is spreading posteriorly. Early in the fetal period, there may be changes in Meckel's cartilage at a point between lateral incisor and canine tooth germs suggesting incipient ossification. Periosteal bone then forms on the upper and lateral aspects of the cartilage, following which there is vascular invasion and endochondral ossification (Fig. 26).

Intramembranous ossification extends rapidly into condylar and coronoid processes. By about 11 weeks, a vesicular type of cartilage has formed on the articular surface of the condyle (Symons, 1952), but separated from the temporal bone by fibrocellular tissue. Between 11 and 12 weeks, cavitation begins in the temporomandibular joint and an intra-articular disc is indicated. Soon after this, the secondary cartilage calcifies and is then vascularized. Endochondral ossification begins later and is well under way by 18-19 weeks. Vascular channels may pass from the fibrocellular layer through the cartilage into the marrow space. The entire process is quite similar to that which takes place at the ends of the clavicle. Secondary cartilage also appears along the anterior border of the coronoid process at 13-14 weeks. At term the cartilage is reduced to a narrow zone (Symons, 1952).

That part of Meckel's cartilage from the central incisor to the canine tooth germ becomes surrounded by periosteal bone by the 13th to the 14th week. The rest of the cartilage usually disappears. Small cartilaginous masses may form secondarily along both alveolar margins in the region of the incisor teeth and along the lower border of the jaw in front, but these usually disappear completely by term.

Thus, each half of the lower jaw develops as a single skeletal element. Meckel's cartilage contributes but slightly to it and takes no part in the formation of the condylar cartilages.

Throughout the prenatal period the mandible is depressed and not opposed to the maxilla (Noback, 1944). Its trabecular pattern is quite complex. Noback (1943) and especially Wissmer (1927) discuss this pattern in detail, the latter giving measurements of body and ramus as well. Reference should be made to Brash (1934) for details of the pattern of growth, which is largely by accretion in special sites. Increase in width is due to bone formation on lateral surfaces. Forward growth comes from deposition on the anterior surface of the symphysis, and backward growth from accretion on the posterior border from the condyle to the angle. The main site of increase in height appears to be the alveolar

border. The condyle grows up and back and the remodelling mechanisms around the neck are similar to those in long bones. Accretion occurs at the tip of the coronoid process. Posterior deposition and anterior absorption in tooth sockets allow teeth to move forward and make room for molars.

5. CLAVICLE—A LONG BONE FORMED IN MEMBRANE, WITH SECONDARY CARTILAGE

The clavicle is a long bone which begins in membrane and secondarily develops growth cartilages, just as does the mandible.

a. *Embryonic Period*

The first indication of a clavicle is a mesenchymatous condensation stretching from the neighborhood of the scapula, downward, forward, and medially to the midline region (Rutherford, 1914; Hanson, 1920). Intercellular matrix increases in the lateral portion of this condensation, suggesting incipient chondrification, but no definite perichondrium forms at this stage. In embryos of 15–20 mm (age groups 18–19), and always after 24 mm (Noback *et al.*, 1951), intramembranous ossification begins in two adjacent centers, a lateral and a medial (Mall, 1906; Rutherford, 1914; Hanson, 1920). These centers are solid rather than trabeculated (Fig. 13). The matrix first laid down is homogeneous, but that which forms later is much more fibrous. The lateral region or center is smaller and more irregular than the medial (Fig. 13). The connective tissue cells around both are so oriented as to suggest a periosteum. Bone cells are large and irregularly arranged and two or three are sometimes seen in a group. Both centers fuse very early. The lateral part grows toward the acromion from which it remains separated by a fibrocellular mass. The medial part extends toward the sternum and by 22 mm (stage 21 or 22) has just about reached its lateral edge. Here, as at the acromial end, the bone gradually merges into a fibrocellular mass. By age group 20 to 21, the tissue at the ends of the clavicle resembles early cartilage. The cells, however, are larger and there is less intercellular matrix than in other cartilage. At this stage also, blood vessels begin to invade the bone (Fig. 17). By 26 mm (age group 22), cartilage is definitely present at both ends (Figs. 14, 16). At the end of the embryonic period, the bone is vascularized to the extent that a marrow space is present. Growth is now similar to that in other long bones. Surface accretion and internal removal lead to an increase in width. Osteoclasts are found where bone is being removed.

b. *Fetal Period*

Shortly after the fetal period begins, the central invasive process

reaches the cartilage and endochondral ossification begins. A growth zone forms but it rarely shows the orderly arrangement of zones in other long bones. The hypertrophied cartilage cells tend to be arranged irregularly rather than in longitudinal columns. Most endochondral trabeculae are removed almost as soon as they form, so that relatively few are found in the marrow space.

The pattern of growth is similar to that of other long bones in that bone is removed from the surface of that part of the shaft near the growth zone, and endochondral remnants can be found in compacta. The remodeling is not as marked, however, as in other bones because the growth zones themselves are not much wider than the shaft. The bone which is adjacent to growth zones is quite thin and often seems to continue directly into cartilage.

The epiphyseal cartilages become progressively thinner throughout the fetal period and by term are reduced to thin strips. Like the condylar cartilage of the mandible, they are vascularized during the fetal period by vessels at the periphery or from the fibrocellular tissue on their articular surfaces.

Throughout the fetal period the clavicle is markedly S-shaped, with a very thick trabeculated compacta and small marrow space.

6. PARIETAL AND FRONTAL BONES—MEMBRANE BONES

The vault bones form in response to genetic factors, but mechanical factors seem to influence their subsequent course of development. The orientation of trabeculae may well be related to pressures from the growing brain. Such pressures may also be a factor in absorption of bone from inner surfaces. Attempts have been made to relate the initial appearance of ossification to pressure from the growing brain, but this seems unlikely. It is difficult to see why meninges would not also ossify under such conditions, and why the bones may form in the absence of brain.

a. *Parietal Bones*

Each parietal bone develops from a pair of centers, or occasionally from but one, which appear by 31 mm (Mall, 1906) to 37 mm (Augier, 1931), that is, in the early fetal period. The centers are always present after 45 mm (Noback *et al.*, 1951). In the case of the parietal bone, ossification begins in what corresponds to the morphological center of the bone. The two centers fuse and the trabeculae radiate from the growing central plate.

The parietal bone grows rapidly, in constant increments in relation to sitting height, until about the 20th week, at which time it has reached

its definitive form and area (Inman, 1934; Noback, 1944). From this time on until birth, it grows more slowly, but still with constant increments, at about the same rate as the rest of the skull. At 140–160 mm (about the 20th week) the peripheral edges of the bone fuse to form a bony border. The manner in which remodeling occurs to accommodate the expanding intracranial contents was discussed previously.

b. *Frontal Bones*

The two frontal bones which develop prenatally usually fuse after birth. Each bone is first indicated as a membranous condensation early in the embryonic period. Ossification begins in the late embryonic period or early fetal period (29–35 mm), and always after 35 mm. It has been found as early as age group 22 (26 mm) in sectioned material (personal observation) (Fig. 27). Bone first forms in the region of each superciliary arch. Each such center simultaneously gives rise to a portion of both the pars frontalis and pars orbitalis and to the primitive medial angular process. Ossification spreads more rapidly in the pars frontalis where heavy islands of bone are formed. These islands fuse and form primary radiating trabeculae, following which secondary trabeculae are formed (Fig. 28). Ossification in the pars orbitalis is less rapid, the radiating trabeculae are smaller and an expanding meshwork is maintained. Accessory centers of ossification are not usually found. Just as in the case of other calvarial bones, the frontal bones grow rapidly, in constant increments until about 140–160 mm (20th week) when their rate of growth slows down and keeps pace with the rest of the skull (Inman, 1934; Noback, 1944). The initial rapid growth represents the expansion of the bones until they cover their definitive area of the vault. The post frontal element is not a separate center of ossification (Inman, 1934; Inman and Saunders, 1937). It is a region of retarded growth in the posterior inferior angle of the frontal bone and becomes apparent only after the 5th fetal month.

Beginning at about 11 weeks the zygomatic process and most of the orbital margin start to thicken, and at 12 weeks the orbital rim is indicated. By 13–14 weeks most of the orbital plate is completed and it has begun to fuse with the medial angular process. From 140 to 160 mm or from about the 20th week until term is the period of reconstruction.

The formation of frontal sinuses is a postnatal event, accompanied by absorption of bone and ingrowth of lining tissue, but the first indications of the sinus can be found prenatally (Schaeffer, 1920). By the end of the 3rd fetal month or beginning of the 4th part of the middle nasal meatus starts to extend in a ventrocephalic direction. This is the beginning of the recessus frontalis and is really the first step in the formation of the

frontal sinus. Late in fetal life this recess is complicated by the formation of frontal furrows or pits. The various potential rudiments of the sinus are well advanced at term. Occasionally the sinus itself may be present but one cannot usually be certain of its presence until 6-12 months after birth.

7. GENERAL CONSIDERATIONS OF OTHER BONES

Space is too limited to do more than indicate some pertinent features about other bones and to supply references to work dealing with them. For a detailed discussion of the chondrocranium, reference should be made to de Beer (1937), and to his excellent bibliography. Bast and Anson (1949) should likewise be referred to for particular details on the temporal bone. For the skeleton in general, Bardeen's account in 1910 is a classic.

It is of interest that some bones ossify relatively late in the fetal period or else postnatally. Of these, the talus and calcaneus have been discussed. The sternum is another of these. Despite its close association with the ribs, which ossify early, the sternum does not show ossification centers until well along in the fetal period and even then there is considerable variation in the time of appearance and number of centers. Cartilage canals are present by the 4th month, before ossification begins. Morphological studies in man, and experimental studies of birds and mice, show clearly that the sternum originates independently of the ribs (Hanson, 1919; Chen, 1952, 1953). Migration of undifferentiated tissue in the dorsolateral body wall gives rise to two ventrally placed rudiments which later fuse. The development of the sternum is largely due to intrinsic factors and can occur in the absence of ribs.

The early development of vertebrae in man is covered by Sensenig (1949), whose paper contains a valuable bibliography. Certain features of vertebral ossification were mentioned earlier in this chapter. Some recent experimental work in the chick is of considerable interest as regards development of vertebrae, ribs and sternum. Straus and Rawles (1953), by carbon marking in the chick embryo, traced the origin of trunk musculature, ribs and vertebrae. They found that somites alone gave origin to the vertebrae, to the dorsal parts of the ribs and to the muscles of about the dorsal third of the body. The mesoderm of the lateral plate (somatopleure) formed the sternum, the ventral parts of the ribs and the ventral parts of the trunk muscles. In between was a region of body wall to which both somites and lateral plate contributed. At the time of the experiments (27-34 somites), the somatopleure was a mosaic in which topographical organization was already determined.

Other findings of interest have been reported by Rowe and Roche

(1953), on the subject of separate neural arch. From their studies of human fetuses they found no evidence of centers other than one for each half of the neural arch.

Sesamoid bones form in cartilage early in embryonic or fetal life but do not ossify until after birth. Some sesamoids are quite constant and develop early in the fetal period in an intracapsular position. Others, such as fabellae, are inconstant. Gray *et al.* (1950), in their study of the knee joint, reported fabellae in 5 of 45 embryos and fetuses, the earliest being found at 14 weeks (85 mm) in an intratendinous position.

In this discussion of bones, one of their most important functions is only mentioned. Blood formation begins early in fetal life, during the 3rd month. By the end of the 3rd month bone marrow is the most important site for formation of erythrocytes as well as for some leucocytes.

IV. Onset of Ossification

The onset of ossification has always been of practical importance, especially for postnatal periods, and many studies and tables have been published. As a matter of fact, few fields of investigation have such a long and voluminous history. A completely accurate list is still not available, however, especially for the prenatal period. Difficulties result from (1) method used, (2) error in determining age, and (3) individual variation. It is possible that sex differences may exist in the onset of ossification, but this remains to be determined.

Four methods of study may be listed. (1) Sectioned and stained material. With good material and proper staining this method detects ossification slightly earlier than any of the others. The disadvantages are that serial sections must often be used and reconstruction may be necessary so as to identify bones. (2) Staining and clearing whole specimens. Once prepared, these allow rapid study and provide beautiful preparations. The early stages of ossification are easily recognized and there are but slight differences in results between this method and sectioning. (3) Radiological study. Although this is not as accurate a method for onset of ossification as the first two, even relatively recent articles (Flecker, 1942) in radiological journals neglect the fine work which has been done by anatomical methods. It is more difficult to identify individual bones by radiological methods, especially bones of the skull. The tremendous advantages in the postnatal period are obvious. (4) Gross dissection. Useful mainly for studying gross form and making measurements.

Individual variation can only be evaluated by having a series large enough to define such variation. Noback *et al.* (1951) have made such

a study on stained and cleared specimens. As yet, no similar study of the entire skeleton has been made by using sectioned and stained material.

There appears to be a definite sequence in appearance of ossification centers. Based mainly on the work of Noback *et al.* (1951) the sequence is as follows:

In the skull, the sequence is facial and calvarial centers, basicranial centers, and hyoid centers.

In the infracranial axial skeleton, the sequence is costal, primary vertebral, and sternal centers.

In the appendicular skeleton, the sequence for girdle parts is clavicle, scapula, ilium, ischium, and pubis. For the upper extremity it is humerus, radius and ulna, distal phalanges, metacarpals, proximal phalanges, and middle phalanges. For the lower extremity it is femur and tibia, fibula, metatarsals, distal phalanges, proximal phalanges, and middle phalanges.

The sequences listed are general and, of course, do not indicate the extent of variation, overlap or asymmetry. Nevertheless, the entire process is sufficiently regular and precise to indicate a genetic control.

Tables II-IV give times of onset of ossification, based on data from Noback *et al.* (1951). Their article lists similar data published by others, such as Augier (1931) who compiled a table of onset of ossification in the human skull based on a study of 250 cleared and stained specimens. Personal observations indicate that the onset of ossification may be detected slightly earlier in sectioned and stained material than with cleared and stained specimens, at least in the embryonic and early fetal periods. If such a difference is true for the rest of the prenatal period, it is easily masked by the difficulty in determining degree of development on the basis of crown-rump length. The series of Noback *et al.* (1951) is the best one available for the skeleton as a whole.

V. General Considerations of Bone Development

Within the space available, it seems worthwhile to provide a short discussion relating to factors concerned with the early development or embryology of skeletal primordia.

1. DEVELOPMENT OF THE SKELETON

Experimental studies of the early development of bones and joints have been carried out mainly in birds and amphibians, and to a limited extent in mammals. Much of this material is the subject of another chapter. Key references, including original papers and reviews, are Murray (1926, 1936), Murray and Selby (1930), Fell (1939), Fell and Canti (1934), and Gardner (1950). One outstanding conclusion follows from

TABLE II

ONSET OF FACIAL, CALVARIAL, BASICRANIAL, AND HYOID CENTERS IN STAINED CLEARED HUMAN EMBRYOS AND FETUSES DURING THE FIRST 5 PRENATAL MONTHS*

Facial	(1) mm	(2) mm	Men- strual weeks	Basicranial	(1) mm	(2) mm	Men- strual weeks
Mandible	20	24—	8	Anterior process of malleus	34	40—	10
Maxilla	23	28—	8½	Exoccipital	38	45—	10½
Premaxilla	24	35		Basioccipital	52	60—	12
Zygomatic	29	35		Lesser wing	65	71—	13
Vomer	29	35		of sphenoid			
Palatine	29	35		Basisphenoid	68	83—	14
Hamulus	29	35		centers			
Nasal	38	44—	10½	Lingula	86	102—	15
Lacrimal	44	60—	12	Presphenoid, 1st pair	84	133—	17
<u>Calvarial</u>							
Frontal	29	35		Petrosum	94	116	
Squamosal	29	35		Incus	108	120	
Supraoccipital	29	35		Malleus, inferior	108	120	16
Tympanic				center			
annulus	35	40—	10	Malleus, super-	120	139—	18
Interparietal	34	44		rior center			
Parietal	38	45		Presphenoid,	120	175—	21
Greater wing	34	52—	11	2nd pair			
of sphenoid				Stapes	139	148—	19
				Presphenoid	165	235 or	27
				median unpaired		after	
<u>Hyoid centers</u>							
				Greater horn	161	after	after
					235—		27

* The crown-rump lengths of specimens in which ossification was first noted are given in column 1 to the right of the list of bones. The lengths after which ossification is always found are given in column 2. For example, ossification of the mandible was first noted in a 20-mm embryo, and was always found in specimens 24 mm or more. The table therefore gives an index of variation in onset. The column on the right gives approximate ages in menstrual weeks, based on Streeter's (1920) tables, for lengths in column 2.

The data in this and Tables III and IV are from the original article by C. R. Noback and G. G. Robertson, *Am. J. Anat.* **89**, 1 (1951), an article which contains further and more complete tables. Permission to use granted by authors and publishers.

most of the experimental work. It is that bones and joints are self-differentiating structures. This does not mean that they are independent of external influence or environment. It means that the attainment of initial form and arrangement is not due to or dependent on such extrinsic

TABLE III
ONSET OF OSSIFICATION IN VERTEBRAL, STERNAL, AND COSTAL CENTERS*

Vertebral Centra			Vertebral Arches				
	(1)	(2)	Age		(1)	(2)	Age
C1	135	161	20	C1	45	49	11
C2	69	120	16	C2	40	45	10½
C3	69	102	15	C3-T2	38	45	
C4	57	85	14	T3	40	52	11
C5	57	71	13	T4-6	40	60	12
C6	52	71		T7-L2	45	60	
C7	52	68		L3, 4	45	68	13
T1	52	69		L5	60	69	
T2	52	57	12	S1	65	76	13½
T3, 4	48	57		S2	102	127	17
T5-L1	40	52		S3	102	161	20
L2, 3	45	52	11	S4	135	161	
L4	45	54		S5	163	173	21
L5	45	57	12				
S1	52	65	12½				
S2	60	68	13				
S3	60	97	15				
S4	84	143	18	T1	37	40	10
S5	135	after	after	T2-11	29	35	9½
		175	21	T12	38	68	13
<u>Sternum</u>							
Manubrium	136	235 or after ?	—————				
Body 1	143	235 or after ?		27			
Body 2	143	235 or after ?		or			
Body 3	140	235 or after ?		after			
Body 4	235	—————					

* Conditions as for Table II.

factors as movement or pressure of growing parts, but rather that primary form is due to factors intrinsic to the developing part acting in a favorable environment. This self-differentiation is true only of the cartilaginous stage. The form of bone itself is dependent on cartilage form. Furthermore, even after bones are formed their rate of growth with respect to each other and to other parts of the body is so regular that they are obviously influenced by a growth-controlling factor common to the whole skeleton. The role of such extrinsic factors as movement must therefore be minimal even during the fetal and postnatal periods.

The factors which lead to differentiation of a limb and which control its development are for the most part unknown. According to Saunders

(1948), the chick limb differentiates in proximo-distal sequence, under the control of the apical ectoderm. By various experimental methods, he showed that the mesoderm in the apical region of the wing bud progressively forms the cells for future wing parts in a proximo-distal sequence and a definite spatial pattern. The process is suppressed by excision of the apical ectoderm, and paraxial hemimelia can be produced by removal of part of this ectodermal cap. The important role of the ectoderm brings to mind the fact that, in the mandible of the chick, the

TABLE IV
ONSET OF OSSIFICATION IN UPPER AND LOWER LIMBS *

	Upper Limb			Lower Limb			
	(1)	(2)	Age	(1)	(2)	Age	
Clavicle	20	24	8	Ilium	29	38	10
Scapula	29	35	9½	Ischium	105	124	17
Humerus	23	30	9	Pubis	161	161	20
Radius	24	35	9½	Femur	23	35	
Ulna	24	35		Tibia	23	35	9½
Metac. 1	45	56	11½	Fibula	29	35	
" 2	37	44	10½	Metat. 1	49	60	12
" 3	37	45		" 2	38	49	
" 4	38	45		" 3	38	49	11
" 5	38	49		" 4	40	51	
Prox. ph. 1	52	60		" 5	45	60	12
" " 2	50	60	12	Prox. Ph. 1	69	78	
" " 3	50	60		" " 2	69	76	13½
" " 4	52	60		" " 3	88	91	
" " 5	60	60		" " 4	91	91	14
Middle ph. 2	60	72		" " 5	91	115	16
" " 3	60	70	13	Middle Ph. 2	116	147	19
" " 4	60	70		" " 3	140	after	
" " 5	69	102		" " 4	235		
Distal ph. 1-5	29	35	9½	" " 5	" "	27	
				Distal ph. 1	38	49	11
				" " 2	45	56	
				" " 3	45	56	11½
				" " 4	45	68	13
				" " 5	60	147	19
				Calcaneus (periosteal center)	120	? after	16
				(Endochondral center)	235	after 27	
					"	"	

* Conditions as for Table II.

center of ossification is associated with a thickened part of the buccal epithelium (Jacobson and Fell, 1941). The interrelationships of parts of differentiating limbs, the possible role of inhibitory influences, and the complexities of limb abnormalities are discussed and summarized by O'Rahilly (1951).

Chen (1952, 1953) has recently made a series of experimental studies which show that the mouse sternum originates from lateral wall mesoderm, independent of ribs, and has self-differentiating properties. Chondrification and fusion of the sternal bars can take place in the absence of the ribs, but segmentation does not occur. Apparently, the ribs exert some inhibitory influence for, in their presence, cartilage cells of the sternum at the levels of rib attachments do not hypertrophy.

Critical phases in early development have been studied by a variety of methods. Russell (1950), for example, by irradiation of pregnant rats, has shown that skeletal primordia are sensitive at the time of visibly fastest degree of change. The critical period for digital reductions, that is, by loss or fusion, corresponds to the growth of limb buds. Sensitivity, however, is not predictable, since the critical period for overgrowth, that is, excess lengthening or doubling of digits, occurs before limb buds are visible.

Most of the experimental work which has been cited and which deals with self-differentiating qualities of the skeleton, concerns limbs and parts of the trunk. One special and extremely complex field, for which there exists an extensive literature, concerns the vertebrate skull. For information on experimental, morphological, and embryological studies of the skull, reference should be made to de Beer (1937). A few conclusions regarding differentiation and early development can be summarized here.

From the available evidence, it is possible that most of the visceral arches are derived from the neural crest and not from mesoderm. The formation of the cartilages of the neurocranium is dependent on the brain and other neural structures. For example, the morphological differentiation of the auditory capsule is dependent on the presence of the auditory vesicle or otic sac. Once formed, the chondrocranium is particularly susceptible to endocrine changes, as during metamorphosis from tadpole to adult stages. Of particular interest are experiments showing that phosphatase is synthesized in the tissue that contains it, and that it does not exist in cartilages, such as Meckel's cartilage, which do not ossify.

a. *Human Limbs*

Only morphological data are available for man, but these support the experimental evidence and indicate that neither growth pressures nor movement are essential for the primary development of the skeleton

(Hesser, 1926; Haines, 1947; Gardner, 1950). The formation of the limb skeleton serves as an example. Very shortly after limb buds appear, proliferation of cells leads to an axial condensation or blastema. Chondrification begins in the regions of the future bones, but does not spread across the zones of future joints. The blastema in these regions remains as homogeneous interzones which become thinned as chondrification proceeds. According to Fell and Canti (1934), these interzones in the avian knee joint are composed of immature cartilage. The form of the bones and associated joints is rapidly established, and by the end of the embryonic period resembles that of the adult, before concerted muscle activity seems likely. As differentiation is proceeding, an intermediate looser zone appears in the blastemal interzone. This is a transitory stage, preceding the onset of cavitation. Hagen-Torn (1882) was perhaps the first to ascribe the origin of most intra-articular structures to the vascular mesenchyme (synovial mesenchyme) at the periphery of the joint rather than to the original blastemal condensation.

Haines (1947), in a recent general study of joint development, emphasized the validity of this concept, and subsequent studies by other workers (Gray *et al.*, 1950, 1951; Gardner and Gray, 1950, 1953) led to similar conclusions. It seems clear that for most synovial joints the vascular mesenchyme around the joint becomes continuous with the intermediate layer of the blastema. Some of this mesenchyme becomes intra-articular in position and active ingrowth of vessels aids in this process. The joint capsule condenses in this mesenchyme and where this occurs, the intra-articular position of synovial mesenchyme is particularly clear cut. Intra-articular ligaments and synovial tissue then form from this tissue. The avascular blastemal zones adjacent to cartilages, and continuous with their perichondria, persist as chondrogenic zones.

The differentiation of bones and joints proceeds very rapidly from a generalized blastema to structures having a form and arrangement characteristic of the adult, the process requiring no more than a few days. There is no recapitulation in the sense that structures characteristic of adult lower forms appear as an intermediate phase. Furthermore, articular structures such as ligaments develop *in situ* and undergo no migration having phylogenetic significance. Joint cavitation begins after joint form is established. The available evidence indicates that cavitation is not dependent upon mechanical factors and it may well be enzymatic in nature. Once cavitation is under way, the form and arrangement specific for the joint is soon established. Since the cavity must increase in absolute size while maintaining relative size, there must be active proliferation of lining tissue, as well as advance of the cavitating process.

It seems quite unlikely that movement or other mechanical factors could account for this process. Many bursae likewise begin to form and undergo cavitation by the end of the embryonic period or early in fetal life.

There are a number of movable or synovial joints that deviate from this general process. Some, particularly small ones, may show considerable delay between differentiation and cavitation. Some of these, such as the acromioclavicular joint, may never show the usual homogeneous and three-layered interzones. Others, such as sternochondral, have cartilaginous continuity in early stages and may or may not subsequently develop cavities (Hesser, 1926; Gray *et al.*, 1943).

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CHAPTER XIV

SKELETAL DEVELOPMENT IN TISSUE CULTURE

HONOR B. FELL

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I. Introduction

Skeletal tissue was one of the earliest to be cultivated *in vitro* in an organized state (Strangeways and Fell, 1926). In these original experiments the limb buds of 3-day chick embryos were grown on the surface of a clot composed of plasma and embryonic extract at the bottom of a small centrifuge tube. Nodules of cartilage developed in the axial mesoderm of the buds but in shape they bore little resemblance to the normal limb skeleton. Demuth (1928) cultivated entire bone rudiments explanted at a stage when they had already assumed their normal anatomical structure, and found that they enlarged as a whole and became calcified. At about the same time the formation of periosteal bone was first observed in culture (Fell, 1928). Since this rather crude early work, technical improvements have made it possible to obtain much better development, both histological and anatomical, of cartilage and bone *in vitro*.

As experimental material, cultures of skeletal tissue have several advantages. In the first place, changes in the living tissue can be watched and photographed under the microscope. Secondly the explants are very

easily manipulated, so that microsurgical operations can be performed *in vitro*, which would be impossible or extremely difficult *in vivo*. Finally, the direct effects of experimental agents on cartilage and bone can be studied in the absence of the many complicating factors which usually obscure the issue *in vivo*; thus the *in vitro* method becomes a valuable adjunct to experiments on the intact animal.

In the following chapter the main results that have been obtained by means of tissue culture, in the study of the developmental mechanics and physiology of the skeleton are reviewed. The differentiation of cartilage and bone in living tissue cultures is first described; anatomical problems are then considered, such as how a bone rudiment acquires its characteristic shape, where particular primordia originate in the mesoderm, how joints are formed and the nature of certain morphogenetic movements concerned in skeletal development. Lastly an account is given of recent work on the effects of hormones and of a vitamin on skeletal rudiments in culture.

1. METHODS

A detailed description of the techniques employed in the cultivation of skeletal tissue *in vitro* would be out of place in the present work, and is available elsewhere (Fell, 1951; Fell and Mellanby, 1952). For the benefit of readers who are not familiar with tissue culture, however, it may be useful to indicate briefly some of the ways in which bone and cartilage are commonly grown.

To observe the details of ossification and chondrogenesis in living material, the old-fashioned hanging-drop method is the best in the

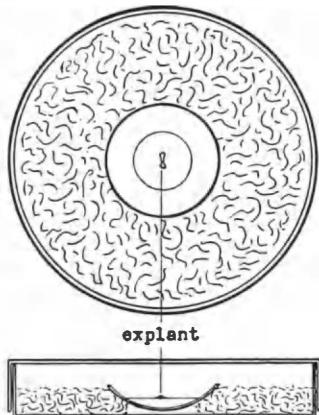


FIG. 1. Diagram of a watch-glass culture. The watch glass is enclosed in a Petri dish carpeted with wet cotton wool; the explant rests on a clot of plasma and embryo extract contained in the watch glass. (After Fell, 1949, by courtesy of the Royal Microscopical Society.)

writer's experience. The tissue is embedded in a clot of plasma and embryo extract on a one-inch-square coverslip which is then inverted over a large hollow-ground slide and sealed with molten paraffin wax.

Every few days the tissue is cut out of the clot and transferred to fresh medium, great care being taken not to damage the interior of the culture where differentiation is proceeding.

Larger rudiments are grown by the watch-glass method (Fell and Robison, 1929) or one of its modifications (Fig. 1). A watch glass is enclosed in a Petri dish carpeted with absorbent cotton-wool; to permit transillumination, a hole is cut in the middle of the cotton-wool which is soaked with sterile distilled water to provide a moist chamber. The medium, usually consisting of a mixture of blood plasma and embryonic tissue juice extracted with physiological saline, is placed in the watch glass and allowed to clot. The explant is then laid on the surface of the clot; at intervals of a few days it is detached from the old medium and transplanted to fresh. Embryological watch glasses sealed with large coverslips have been used satisfactorily (Wolff and Wolff, 1952) instead of ordinary watch glasses in a moist chamber.

II. Morphogenesis

1. THE DIFFERENTIATION *in vitro* OF CARTILAGE AND BONE

Before proceeding to investigations on the developmental mechanics of the skeleton, it seems appropriate to describe shortly the normal differentiation of cartilage and bone *in vitro*.

Chondrogenesis can be seen in considerable detail in large hanging drop cultures of the undifferentiated mesoderm from the limb buds of 3-day chick embryos (Fell, 1928). When first explanted, the tissue appears as a small, compact block, but after 48 hours' incubation it has spread into a thin sheet with a ragged margin of migrating fibroblasts. In places local thickenings of closely packed cells are seen, which represent centers of chondrification. Two days later, areas of so-called "epithelioid" cartilage have appeared in the interior of the centers, and consist of polyhedral cells separated from each other by delicate partitions of refractile, colorless matrix. In places the young cartilage still merges imperceptibly with the surrounding tissue, but elsewhere the latter has formed a perichondrial membrane of elongated cells, which is continuous with, but clearly differentiated from the cartilage.

The nodules of cartilage enlarge fairly rapidly until about the 10th day, after which the growth rate declines; the amount of glistening intercellular material has greatly increased and most of the nodules are completely enveloped by a perichondrium in which fibers can now be distinguished. In cultures of 28 days' growth, the cartilage is very hard and slippery and enclosed by an extremely tough, fibrous capsule; there is no significant change during succeeding weeks. Histological sections of the cultures show typical hyaline cartilage.

If the limb bud is teased into tiny fragments in a fluid medium on

a coverslip, during subsequent incubation the tissue expands into little sheets in which minute nodules of cartilage develop, sometimes containing less than 30 cells.

Osteogenesis is readily observed in hanging drop cultures of the mesodermal primordia of the mandibular membrane bones from 6-day chick embryos (Fell and Robison, 1930). The tissue is rather diffuse when first explanted, but after 24 hours it contracts into a fairly compact mass which then spreads out as a thin sheet with here and there fairly large aggregations of cells, some of which are the future ossification centers. These centers rapidly become more distinct and restricted and by the 3rd day appear as an oval mass of closely packed cells separated from one another by apparently amorphous intercellular partitions which are less refractile than those of early cartilage.

During the next 24 hours, the cellular mass differentiates into two regions: an inner part composed of polyhedral cells and an outer periosteal layer which at the periphery merges with the adjacent tissue. When neutral red is added to the culture medium, the cells of the inner mass are rendered conspicuous by the intensity with which they take up the dye.

A few hours later, delicate intercellular fibers appear in the ossification center and increase in number until they form a fine network between the closely packed cells. The inner region of the center now consists of a mass of young osteocytes embedded in osseous fibers and surrounded by a single layer of osteoblasts, while the outer region has formed a fibrous capsule. The living osteoblasts and young bone cells stain even more deeply than before with neutral red, and under high power, the cytoplasm is seen to be filled with fine, spherical red granules.

As ossification progresses, the identity of the original intercellular fibers is obscured; the delicate criss-cross network of the previous stage becomes condensed into refractile bundles, and under high magnification the osseous matrix is seen as a dense mat. The matrix steadily increases in density and soon begins to calcify; refractile granules appear in the intercellular substance which gradually becomes opaque. In places the original intercellular partitions are resorbed and replaced by small cavities filled with osteoblasts, others remain unaltered, while elsewhere they thicken to form irregular walls and columns. In this way a trabecular structure develops which is well seen by the 9th or 10th day. By this stage calcification is dense and the bone looks nearly black by transmitted light. When examined under high power, the bone trabeculae are found to have acquired a nodular appearance, being thickly studded with brilliant granules of calcium salts. Histological preparations show that typical early membrane bone has been formed.

Rodova (1948) has studied the formation of intercellular fibers in

detail, by growing the embryonic frontal bone in a fluid medium. The osteoblasts form a very thin sheet on the coverslip and the intricate network of fibers and the long, radiating fibrous bundles are beautifully distinct in such cultures. Using Gomori's histochemical method, she demonstrated that the osteoblasts gave a more intense reaction for alkaline phosphatase than did fibroblasts in non-osteogenic cultures.

2. THE DEVELOPMENT *in vitro* OF SKELETAL FORM

In the previous section we have seen how pieces of undifferentiated chondrogenic and osteogenic tissue acquire an almost normal histological structure in culture. When entire skeletal primordia are cultivated *in vitro*, they not only differentiate histologically but also develop to a surprising degree their characteristic shapes, though anatomically they always deviate from the normal in certain respects.

a. *Explanted Blastemata*

Skeletal primordia will develop *in vitro* (Fig. 2) even when the explant consists merely of an apparently undifferentiated condensation of mesoderm, the blastema, in which there is no sign of joints or of the individual bone rudiments.

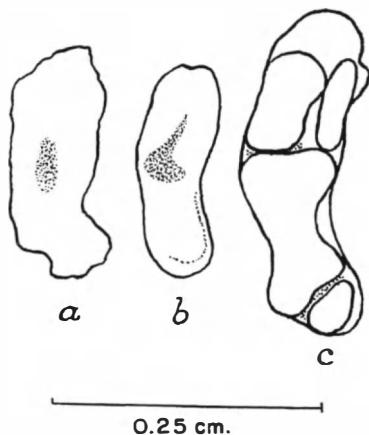


FIG. 2. Camera lucida drawings of a living explanted blastema from the leg bud of a 4-day chick embryo, showing the development of the knee joint. (a) Immediately after explantation; (b) after 24 hours and (c) after 4 days' cultivation. (After Fell and Canti, 1934, by courtesy of the Royal Society.)

The development in culture of the blastema from the leg bud of 4-day chick embryos has been studied in some detail (Fell and Canti, 1934). In the normal bud the skeletal rudiment is a Y-shaped mass of unchondrified mesoderm in which the tail of the Y represents the femur and the two arms the tibia and fibula. This condensation was dissected from the leg bud and grown in a watch-glass culture, where its development was recorded by cinematography. During the first 24 hours, the general outline of the three bone rudiments become vaguely distinguishable and after 2-3 days the future knee joint appears as an opaque, T-shaped region. As *in vivo*, chondrogenesis begins in the middle third

of each rudiment and spreads towards the epiphyses, where in section the cells are seen to be oriented parallel to the future articular surfaces of the three cartilages as *in vivo*; the femur is still directly continuous with the tibia and fibula.

As the three rudiments continue to develop, the cross bar of the T extends completely across the joint, and at the same time the outlines of the femur, tibia, and fibula become increasingly well defined. Originally the diameter of the tibia is only slightly greater than that of the fibula, but as in the normal limb, the tibia widens more rapidly than the fibula as development proceeds. The femur gradually assumes a fairly typical shape with condyles at the distal, and head and trochanter at the proximal end. Part of the pelvis is also usually present and becomes separated from the femur by an opaque line like that of the knee joint.

The articular line of all the joints is most distinct by about the 4th day *in vitro*, and at this stage sections show a clear division between the articular surfaces of the condyles and of the tibia and fibula. In the normal limb a densely cellular layer covers the articular surfaces, but this is less pronounced in the explants; in later development *in vitro* it completely disappears and becomes transformed into hyaline cartilage like that of the rest of the epiphysis. After 6 days in culture, further anatomical development ceases; no joint cavity is formed and the opposed articular surfaces fuse with one another so that the joint is gradually obliterated.

The development in culture of the blastema of the sternum has also been studied (Fell, 1939). In normal development, the sternum arises as two dorso-lateral plates of mesoderm which are in close contact with the ribs, and it has long been a controversial question whether the sternal

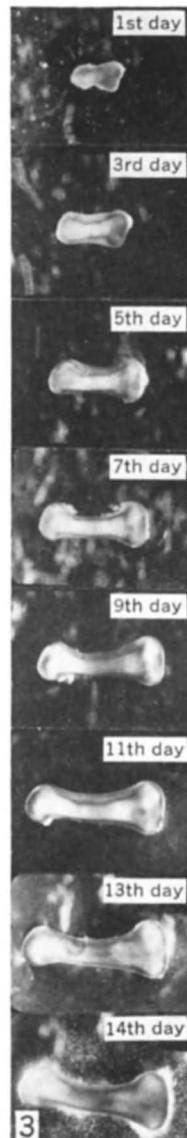
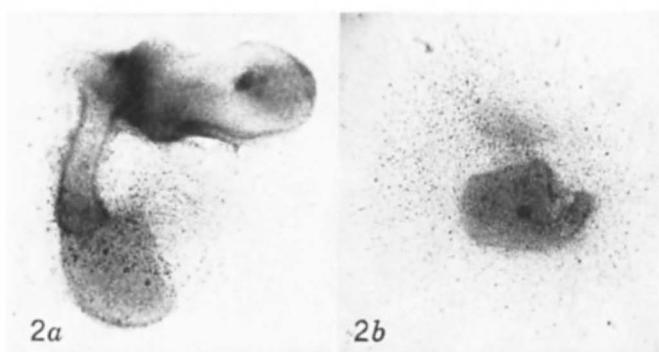
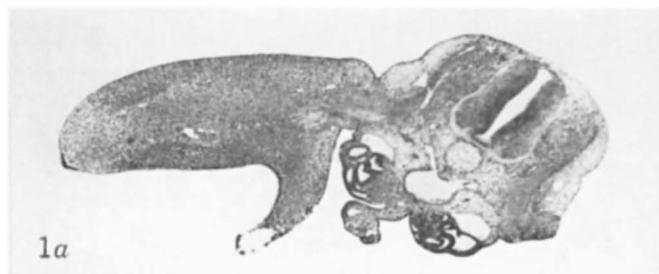
PLATE I

1. *a*. Transverse section through the wing-bud region of a 4-day (control) fowl embryo. One bud and the adjacent lateral body wall have been removed for explantation. *b*. The wing bud and body wall removed from the embryo shown in *a*. Part of the wing skeleton, a fragment of the scapula, the coracoid and the sternal plate have developed during cultivation. Note the complete absence of ribs. Whole mount; same magnification as 1*a* (after Fell 1939, by courtesy of the Royal Society).

2. *a, b*. Photographs of two living explants from the same 4-day chick embryo grown *in vitro* for 7 days. *a*. The explant comprised the wing bud and the lateral body wall from the first intersomitic septum in front of the bud to the first septum behind it; the proximal part of the wing skeleton, part of the scapula, the coracoid and anterior portion of the sternal plate have developed. *b*. A region of the lateral body-wall from the same side as *a*, extending from the 1st to the 4th intersomitic septum behind the wing bud. The posterior part of the sternal plate has differentiated (after Fell, 1939, by courtesy of the Royal Society).

3. A series of photographs from a cinema film by R. G. Canti and H. B. Fell, showing the development of a femur rudiment from a 5½-day chick embryo. Note the elongation of the shaft and the development of the condyles. The middle segment of the shaft has become opaque by Day 9; this is due to periosteal ossification.

PLATE I



plates are formed from the ends of the ribs or as part of the appendicular skeleton; the following experiments were made in the hope of deciding this issue. The axial mesoderm of the wing bud and a piece of the body wall immediately ventral to the bud, were removed before the ribs had appeared and were cultivated as a single fragment (Plate I, 1a and b). Such explants formed the proximal portion of the wing skeleton, i.e. the humerus, part of the radius and ulna, the coracoid and an oval sternal plate, but no ribs.

Experiments were then undertaken to determine the exact site of the presumptive sternal tissue in the early chick embryo. To find the anterior and posterior boundaries of the sternal region, fragments of the body wall were excised from 4-day embryos, the position of the fragments being defined with reference to the intersomitic septa, and were cultivated *in vitro* (Plate I, 2a and b). If part of the sternal tissue were included in a given explant, it developed during cultivation. Similarly, the ventral border of the sternal region was ascertained by explanting strips of the lateral body wall taken at different levels beneath the wing bud. By a series of such fragmentation experiments, the presumptive sternal tissue was found to occupy a small oblong area immediately below, and extending some distance behind the posterior half of the wing bud.

The results of this investigation showed that the sternum was not derived from the ribs, but should be regarded as part of the appendicular skeleton. Chen (1952b) has demonstrated that this is also true of the sternal rudiments of the mouse, which like those of the bird, will develop in culture in the absence of ribs.

When the blastemata of the limb skeleton and sternum were cultivated for longer periods, they continued to enlarge but their gross anatomy not only failed to develop further, but became increasingly abnormal. As stated above the joints disappeared by secondary fusion of the articular surfaces, and the bone primordia lost much of the characteristic shape they had acquired at an earlier stage. Their histological differentiation also, was usually arrested. Although, as we have seen, the mesoderm chondrified readily, the next stage in development, viz., hypertrophy of the diaphysial chondroblasts associated with periosteal ossification, was either greatly retarded or altogether inhibited. It was curious that certain primordia differentiated more often than others; thus the humerus and tibia showed maturation of the cartilage and ossification more frequently than the femur, radius, and ulna, which usually remained small-celled and unossified even after 2 weeks' cultivation. This phenomenon will be considered in more detail in a later section.

Wilde (1948) cultivated the urodele limb bud in a nutrient medium and records some interesting observations on the factors which influence

its development. If the very early bud was explanted by itself, it formed only a blastematous protuberance, though buds from later embryos produced a nearly complete limb skeleton (Wilde, 1950). When, however, the peribrachial flank tissue was left attached to the very early bud, a well formed limb skeleton differentiated. On the other hand, if a regulated amount of gill tissue was explanted with the limb bud, the latter grew much more slowly than controls without gill primordia, and histological examination showed that, while morphogenesis and differentiation were advanced in the gill, the limb remained blastematous (Wilde, 1952). The effect of the gill tissue was cumulative, i.e., the greater the amount included in the explant, the greater was its inhibitory effect on the limb.

b. *The Developmental Mechanics of Joint Formation*

The fact that joints would develop in culture (Fig. 2), provided a new approach to their mode of formation. The results described above, showed that the early stages of joint formation, i.e. the development of the articular surfaces, can proceed in the absence of the muscles and of a nerve and blood supply, although conditions *in vitro* are not adequate for the completion of the process, viz., for the development of the joint capsule. It was decided to investigate the mechanism whereby separate articular surfaces were formed in the originally continuous blastema (Fell and Canti, 1934).

Experiments were first made to find whether the presumptive joint tissue was self-differentiating when isolated from the rest of the skeletal blastema and cultivated *in vitro* (Fig. 3); the results showed that under

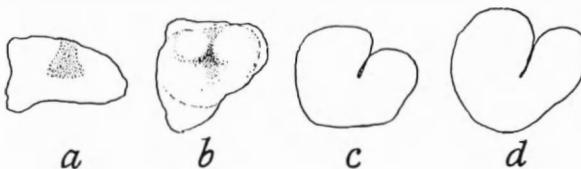


FIG. 3. Camera lucida drawings of the living joint region isolated from the leg bud of a 4-day chick embryo. (a) Immediately after explantation; the lower cut surface is the proximal edge. (b) After 24 hours' cultivation. (c) After 48 hours' cultivation. (d) After 4 days' cultivation; no joint has developed, and the explant has formed a bilobed nodule composed of the femoral condyles and incorporated tibio-fibular tissue. (After Fell and Canti, 1934, by courtesy of the Royal Society.)

these conditions it was not, and the explants merely formed a kidney-shaped nodule of cartilage, the lobes representing the articular ends of the tibia and fibula in direct continuity with the distal extremity of the femur. This suggested that in order to form an articulation, the joint region had to be associated with presumptive diaphyses. To test this, the tibio-fibular region of one blastema was cut transversely in half; the

presumptive joint tissue was excised from the opposite leg bud and interpolated between the two halves of the tibio-fibular region (Fig. 4). Provided the graft fused completely on either side with the tibial shaft

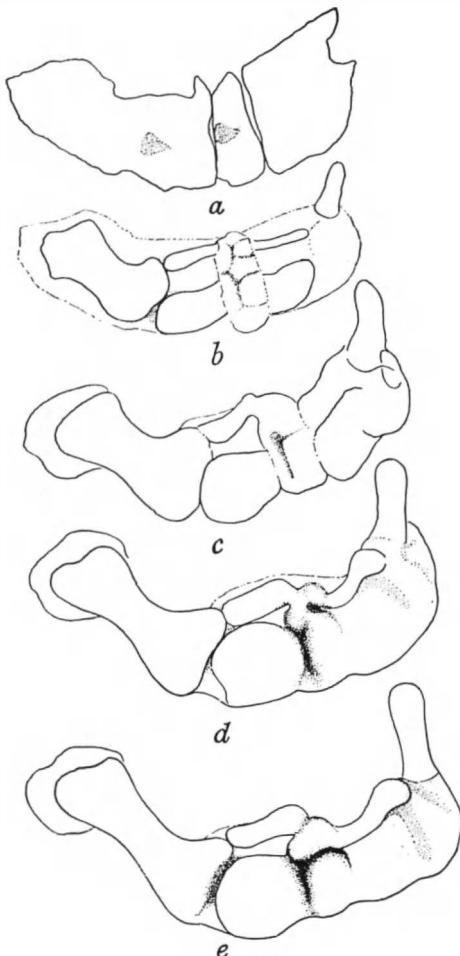


FIG. 4. Camera lucida drawings of a living blastema from the leg bud of a 4-day chick embryo; the isolated knee-joint region from the opposite leg bud of the same embryo has been grafted into the middle of the tibio-fibula region and is oriented in the same direction as that of the host blastema. (a) Immediately after explantation. (b) After 24 hours' cultivation; the graft is already partly fused with the host tibia and fibula and shows indications of an articular line. (c) After 48 hours' cultivation; the relations of the graft with the host fibula have become distorted and the articular line has disappeared in the graft on that side. (d) After 3 days' cultivation. (e) After 4 days' cultivation; note the perfect incorporation of the graft with the host; the graft is longitudinally divided and has also formed a complete transverse joint across the part fused with the tibia but not across that fused with the fibula. (After Fell and Canti, 1934, by courtesy of the Royal Society.)

(the cut fibula usually became distorted), an articulation appeared across the middle of the graft. From this it seemed clear that the shaft played an essential part in the development of the joint.

Attempts were then made to see whether the blastema could form a joint in the absence of the presumptive joint tissue. In one series of experiments the region of the future joint was excised from a completely unchondrified blastema, the two fragments were pushed together on the clot and rapidly fused; a joint subsequently appeared, but the distal part of the femoral condyles was missing. In another series of operations (Plate II, 4a and b), the future joint was removed from slightly older blastemata in which chondrification had already begun in the three long-bone rudiments. Provided only a small part of the articular ends was cut away, so that some unchondrified mesoderm remained, a joint developed; but if a larger portion was removed, so that no unchondrified material was left, the cut surfaces of the tibia and fibula fused directly with that of the femur to form a single cartilage shaped like a tuning fork. These results indicated that the joint was not rigidly localized in the limb mosaic and that an articulation could form in the absence of the presumptive joint region, provided sufficient undifferentiated material remained at the ends of the bone rudiments. On the other hand, the characteristic shape of the articular surfaces appeared to be part of the mosaic.

It was concluded that the separation of the articular surfaces is probably due to the mechanical effects of differential growth. A chondrifying region enlarges more rapidly than the surrounding tissue by the combined effects of the formation of matrix and cell division; consequently a chondrification center orientates the cells of adjacent undifferentiated tissue at right angles to its direction of expansion. In the skeletal blastema of the leg there are three chondrification centers—those of the femur, tibia, and fibula. Chondrogenesis begins in the three shafts and as it spreads into the future epiphyses, the expanding chondrification centers "compete" with one another for the available soft tissue in the undifferentiated joint region; the cells thus become orientated around and incorporated into one or other of the chondrifying epiphyses until no free cells remain. In this way the articular surfaces are automatically produced. We have seen that if all the undifferentiated tissue is removed from the joint region, no joint is formed and there is complete fusion between the femur and the distal segments.

c. *The Development of Isolated Bone Rudiments*

Many observations have been made in the writer's laboratory, on the development *in vitro* of isolated bone rudiments from chick embryos of about 5½ days' incubation; at this stage the joints are usually recognizable but hypertrophy of the diaphysial cartilage cells and ossification

have not yet begun. The primordia that have been most studied are those of the limb skeleton and of the sternum.

When the individual long bones of the wing or leg are cultivated in watch glasses, they sometimes enlarge to four times their original length and usually acquire a fairly normal shape (Plate I, 3). Thus the femur, when first explanted (Fell and Robison, 1929) is a short thick rod with two rounded distal processes representing the condyles, and two smaller, proximal protrusions corresponding to the head and trochanter. After 3 days *in vitro* the condyles have assumed a more definite shape and by the 6th day have acquired their characteristic pulley-like form; the head also is fairly well developed, but the trochanter is less pronounced. *In vivo*, the percentage increase in the diameter of the bones is much higher in the epiphyses than in the middle of the shaft; this regional difference in growth is much exaggerated in the explants, so that in the cultures the epiphyses are larger in relation to the shaft, than they are in the normal bone.

This anatomical abnormality may be due, at least in part, to the histological structure of the explants as compared with that of the normal bone. Unlike the early blastemata described in the last section, the slightly older rudiments reach an advanced stage in differentiation and develop at almost the normal rate, in spite of their greatly sub-normal growth rate *in vitro*. The chondroblasts in the middle segment of the shaft hypertrophy, a periosteum composed of an outer fibro-

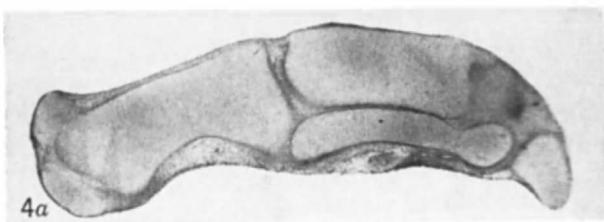
PLATE II

4. *a*. Section of an explanted blastema from the leg bud of a 4-day chick; a narrow strip of tissue had been excised from the joint region, but some of the undifferentiated articular tissue was left and after 4 days' cultivation a joint developed. *b*. The explanted blastema from the opposite leg bud of the same embryo; a relatively large fragment was removed from the joint region, so that no undifferentiated articular tissue remained; after 4 days' cultivation no joint has been formed and the tibia and fibula have developed in continuity with the femur. (After Fell and Canti, 1934, by courtesy of the Royal Society.)

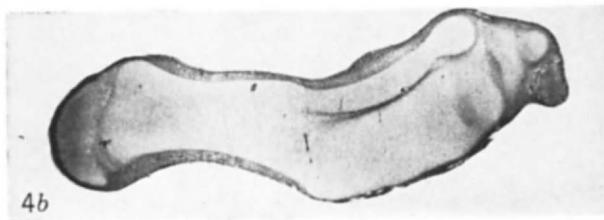
5. *a*, *b*. Transverse sections of the sternal plates from an 8-day budgerigar embryo. The ribs and pectoral muscles were removed and the ventro-lateral body wall was cut in half down the mid-line, one half (*a*) being fixed as a control, and the other (*b*) being explanted *in vitro* with its dorsal surface upwards. *a*. Control plate fixed immediately after dissection; the plate is nearly flat and has no keel. *b*. Explanted plate after 2 days' cultivation. The normal curvature of the corpus sterni (concave to the clot) and a half-keel have developed *in vitro*; note that the keel has a double bend, due to mechanical factors, so that the proximal part is vertical but the distal part horizontal. (After Fell, 1939, by courtesy of the Royal Society.)

6. Frontal section of a sternal rudiment from a 13-day mouse fetus after 11 days *in vitro*. When explanted the rudiment consisted of two mesodermal bands which were widely separated, and attached rib stumps; during cultivation the bands moved together, fused and chondrified. The intercostal regions then underwent chondroblastic hypertrophy and ossified, thus producing a segmental structure as *in vivo* (after Chen, 1952b, by courtesy of the Editor, *Journal of Anatomy*).

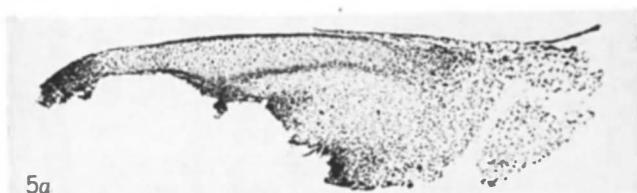
PLATE II



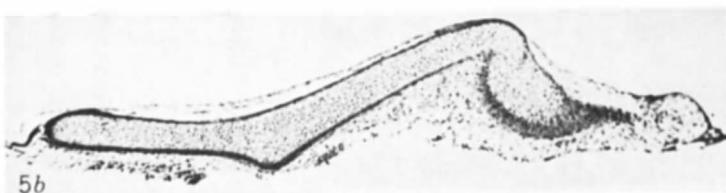
4a



4b



5a



5b



6

blastic and an inner osteoblastic layer, forms as *in vivo*, and a sheath of bone is deposited on the cartilage. In the normal skeleton, however, the osteoblastic layer soon becomes vascularized and trabeculae of bone develop between the vessels so that the total thickness of the bone is much increased. Vessels and accompanying tissue also invade and destroy the cartilage of the shaft which is replaced by marrow. In the cultures, on the other hand, where there is no vascular system, the periosteal bone thickens but remains a simple sheath, which is much narrower than the trabecular bone of the normal skeleton, while the hypertrophic cartilage persists and is not replaced by a marrow cavity. The terminal cartilage, on the other hand, continues to enlarge, as *in vivo*, by cellular multiplication and the formation of more matrix, and consequently its growth is less affected by *in vitro* conditions than is the shaft. The terminal cartilage differentiates almost normally, forming a small-celled epiphysis sharply demarcated from an intermediate zone of flattened cells which merges with the hypertrophic region.

Mammalian long-bone rudiments have been cultivated successfully. Verdam (1936, 1937. Quoted from Gaillard, 1953) has grown the skeletal primordia of rat limbs, but this work will be considered in a later section. Recently the cartilaginous rudiments of human fetal long bones in culture have been studied by Zaaijer (1953) who noted the curious fact that while explants isolated from a young embryo (6½ weeks) developed *in vitro* in a normal organotypic way, those from older fetuses only did so if enveloped by epidermis; in its absence they regressed. This phenomenon is not seen in avian explants, where encapsulation is fatal owing to the impervious layer of keratin which is rapidly formed by the epithelium. On the other hand, enclosure by epidermis is necessary for the development of the urodele limb bud in culture (Wilde, 1948).

If explanted long-bone rudiments are fractured experimentally they undergo repair *in vitro* (Niven, 1931; Bucher, 1952). When the break is made at a very early stage of chondrification, the two fragments fuse perfectly so that it is difficult to see the site of the fracture, but if the cartilage is broken when it is already well differentiated, it does not heal and repair is effected by the bone only. Fractures in older chick or mammalian long bones, in which there is already a marrow cavity, unite by the deposition of new bone. Bucher and Weil (1951) and Weil (1951) have shown that repair *in vitro* can be hastened by the addition to the medium of a commercial bone extract (ossopan), which also improves the differentiation of intact rudiments (Weisshaupt, 1950).

The behavior *in vitro* of the sternal rudiment presents some interesting features; it has been investigated in the budgerigar (*melopsittacus*) by Fell (1939) and in the mouse by Chen (1952b). In the normal avian embryo, as already stated, the sternum first appears as two plates

of condensed mesoderm in the dorso-lateral body-wall. The two plates, attached dorsally to the ribs, move in a ventral direction, meet in the mid-line and fuse; the keel develops along the line of fusion between the two masses of pectoral muscle. If the ventro-lateral body wall with the sternal plates from which the ribs and pectoral muscles had been removed, was explanted on a clot in a watch glass, the two plates moved together as *in vivo* (this movement will be considered later), fused and formed a small keel. Closure of the plates was never completed at the back, where they were always separated by a triangular gap; each plate acquired a short posterior lateral process, but the hindmost part of the sternum remained defective. Moreover the groove for the articulation of the coracoid did not develop further *in vitro* and soon disappeared. As with the avian limb blastemata, with more prolonged cultivation the anatomy of the sternum became increasingly abnormal.

Experiments were also made with single sternal plates, explanted at the same stage of the development (Plate II, 5a, b). When first removed from the embryo, the plates were flat, but in spite of the absence of the viscera and regardless of whether the dorsal or ventral surface was uppermost on the clot, the plates assumed their normal curvature. Each also produced a half keel which formed two right-angled bends, so that the proximal part was vertical as in the normal rudiment, but the distal portion lay flat on the clot.

The mouse sternum also develops well in culture (Chen, 1952b). Normally it arises as two mesodermal bands in the dorso-lateral body wall of the fetus (Chen, 1952a); these bands with their attached ribs move to the mid-line and fuse. The mesoderm chondrifies and a series of ossification centers appear in the intercostal segments of the rudiment. When the body wall containing the sternal bands with stumps of their associated ribs, was cultivated *in vitro* (Chen, 1952b) the bands approached one another, fused, chondrified and underwent a typical segmental ossification (Plate II, 6). As in the avian sternum *in vitro*, however, the posterior part of the explanted rudiment remained incomplete.

Chen (1953) utilized the organ culture technique to investigate the controversial question of whether the segmental structure of the mammalian sternum is due to intrinsic factors or whether it is imposed on the rudiment by the influence of the ribs. To settle this point, Chen removed the rib stumps from the sternal bands before explantation; as before, the sternal bars moved together, fused and chondrified but they ossified as a continuous rod with no trace of segmentation. In other experiments (Plate III, 7) he removed some of the ribs and left the rest; hypertrophy of the chondroblasts and ossification were inhibited only where ribs were attached. These results showed clearly that the ribs

prevent ossification in the costal regions and are thus directly responsible for the segmental structure of the mammalian sternum.

The development *in vitro* of the patella of the embryonic chick has been studied by Niven (1933) who found that the explanted rudiment acquired a fairly normal shape but failed to develop the concave surfaces for the articulation of the condyles.

d. *The Relative Importance of Intrinsic and Extrinsic Factors in Determining Skeletal Form*

In the results described in previous sections, we have seen that skeletal rudiments possess a considerable capacity for self-differentiation when isolated in culture. The blastemata of the avian limb buds form the rudiments of the long bones and of the articulations in the absence of the musculature; even when explanted before a condensation has appeared, the presumptive sternal tissue forms a recognizable sternal plate. When the primordia of the long bones are isolated *in vitro*, they acquire a fairly normal shape and so also does the patella. The sternal rudiment in culture develops a keel in the absence of the pectoral muscles and the corpus sterni assumes its normal curvature in the absence of the viscera; similarly the mammalian sternum acquires much of its normal anatomy *in vitro*. These findings are clear evidence that the general form of the cartilaginous skeleton in the higher vertebrates is determined by factors inherent in the early rudiment, a view first expressed by P. D. F. Murray as a result of his classical experiments on chorio-allantoic grafts of fragments of chick limb buds (Murray and Huxley, 1925; Murray, 1926).

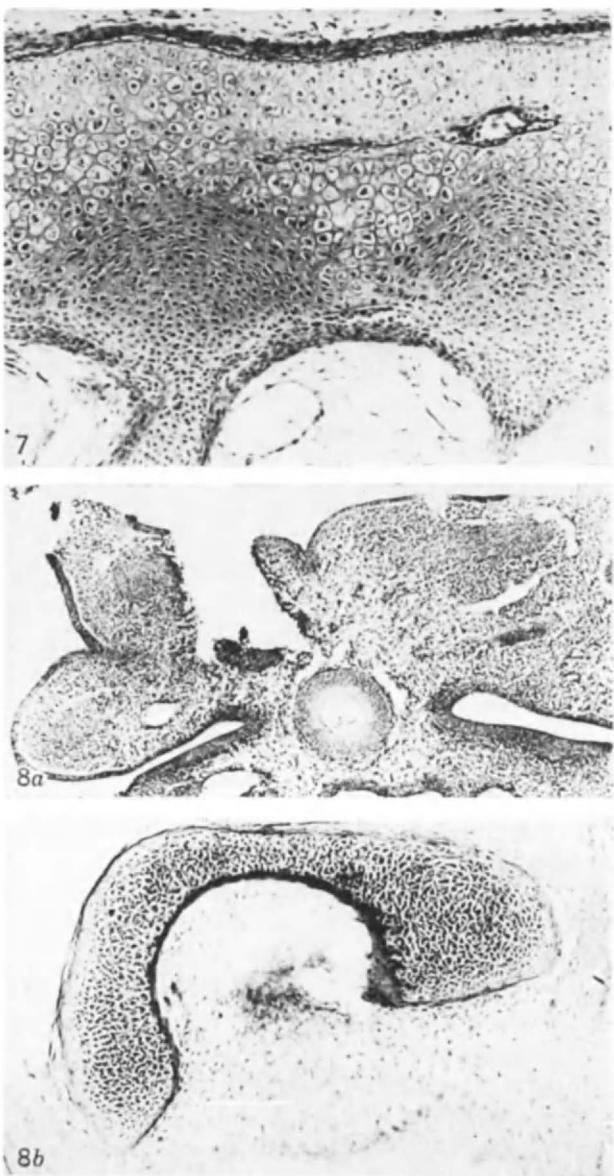
When we consider the rudiments in culture more closely, however, we notice many imperfections in their anatomical development. Thus the individual bone primordia which appear in the explanted blastemata of the chick limb buds remain abnormally short and thick; although articular surfaces are formed, no joint capsule develops and later the surfaces fuse and obliterate the articulation. The keel produced by the avian sternum *in vitro* is abnormally short and in explants of single sternal plates, only the proximal part of the keel is vertical; in both avian

PLATE III

7. Frontal section of a sternal rudiment from a fetal mouse explanted at the same stage as that shown in Plate II, 6, and cultivated for 10 days. At the time of explantation the rib stumps were removed from one sternal band and left attached to the other. Segmentation has appeared only on the side associated with ribs and the opposite side has ossified as a continuous rod (after Chen, 1952b, by courtesy of the Editor, *Journal of Anatomy*).

8. a. Frontal section through the mandible of a 3-day chick embryo. On the left a fragment of mesoderm has been removed which included the whole chondrogenic area. b. The excised fragment after 8 days' cultivation *in vitro*. A cartilage rod, more than 1 mm in length, has developed in culture (after Jacobson and Fell, 1941, by courtesy of the Editor, *Quarterly Journal of Microscopical Science*).

PLATE III



and mammalian sterna, the posterior part is missing or imperfect. The coracoid groove in the bird's sternum soon disappears *in vitro* and the patella does not form the concave articular surfaces for the femoral condyles. Moreover, in all the skeletal explants, more prolonged cultivation results in the gradual loss of normal anatomical features which were distinct at an earlier stage.

These facts show that while the general shape of the cartilaginous skeleton may develop in response to intrinsic factors, extrinsic influences are concerned in providing the right conditions for the normal expression of inherent potencies and for maintaining the normal structure once it has developed.

As we have seen, skeletal primordia do not complete their anatomical development in culture. For the rudiment to attain its normal shape, a certain relationship between rate of growth and rate of differentiation is required. Anatomical differentiation occurs mainly in the procartilaginous and early cartilaginous stage by means of differential growth, and when once the rudiment is fully chondrified little further structural change takes place. There is no blood circulation around the rudiment developing in culture and therefore its growth *in vitro* is far below that *in vivo*. On the other hand, chondrification is retarded very little by conditions *in vitro*, so that a severe imbalance between rate of growth and rate of differentiation is produced. Thus the explanted rudiment chondrifies before it has time to achieve its final shape by differential growth at the procartilaginous stage, so that its anatomical development is arrested.

Certain structures require appropriate mechanical conditions for their normal development. An example of this is provided by the keel of the avian sternum; as we have seen, when a single sternal plate is cultivated, the keel begins to form at right angles to the plate, but lacking the necessary mechanical support, it is unable to continue its right-angled growth and acquires a second bend so that the distal part of the keel develops horizontally lying on the surface of the clot.

Environmental factors are responsible for preserving the characteristic shape of a skeletal rudiment. This is shown by the fact that explanted primordia which develop a comparatively normal form during the first few days *in vitro* lose much of their characteristic shape on more prolonged cultivation and become increasingly distorted. It is probable that not only a blood circulation, but functional activity is required for the maintenance of normal anatomical structure, once this has differentiated.

Certain features of the cartilaginous skeleton seem to be determined entirely by extrinsic factors. This is probably true of negative articular surfaces; as described above, the articular surfaces for the condyles fail to develop in the isolated patella (Niven, 1933) while in the avian sternum the coracoid groove soon disappears (Fell, 1939). It may be

that the formation of a negative surface requires the presence of the corresponding positive surface and is not a phenomenon of self-differentiation.

The clearest example of structure being determined by extrinsic forces is provided by Chen's experiments on the segmentation of the mouse sternum, which showed conclusively that the segmental arrangement of the ossification centers is imposed upon the sternal rudiment by the associated ribs.

The gross morphology of bone probably depends much more upon environmental influences than does that of cartilage.

e. *Morphogenic Movements*

In early embryogenesis, mass migration of cells and even of entire structures are not uncommon, and the *in vitro* technique has been used to investigate one example of this phenomenon.

As already mentioned, the avian sternum originates as two mesodermal plates lying obliquely in the dorso-lateral body-wall (Fell, 1939); accompanied by the pectoral muscles, the rudiments move towards the mid-ventral line where they meet and assume a horizontal position. The question to be solved was how this displacement was effected.

The plates did not come in contact by mere expansion, as this would not cause them to rotate from an oblique, dorso-lateral situation to a vertical lateral, and finally to a horizontal ventral position. That they were not pushed into place by elongation of the attached ribs was demonstrated by the experiments described above, in which the ventro-lateral body wall with the sternal primordia, but without the ribs, was explanted in a watch glass; in these cultures the plates moved together and united in the normal way. That an actual displacement took place *in vitro*, was proved by cine-photography and by measurements made on serial photographs and camera lucida drawings of living explants.

In normal development *in vivo*, the entire dorso-lateral tissue of the thoracic wall, including muscles, connective tissue and epidermis as well as the sternal rudiments, moves towards the mid-line and the original ventral body wall degenerates (Fell, 1939). The ventral edges of both the sternal plates and the pectoral muscles are continuous with a fringe of undifferentiated ameboid cells which appear to be invading the mesenchyme of the ventro-lateral body wall. It seemed possible that the plates were carried into position by this general downgrowth of undifferentiated cells, a type of phenomenon which is not uncommon in tissue cultures where pieces of differentiated tissue are often displaced by the streaming of adjacent cells. To investigate the possibility, the ventro-lateral body wall, including the sternal plates was cut in half down the mid-line and the two halves were arranged back to back, i.e. with the

costal margins of the plates in contact (Plate IV, 10a, b). The two halves were quickly united by fusion of the soft tissues, and so formed a single sheet once more; the two sternal plates, however, moved apart, often to a considerable distance. In controls in which the two halves were oriented normally, i.e. with the ventral cut edges of the body wall in contact, the plates approached one another in the usual way.

Other experiments were made in which the ventral body wall including only the extreme ventral edges of the sternal plates, was explanted *in vitro* and was marked with four patches of sterile carbon. The patches moved towards the mid-line, although the explant as a whole spread outwards; in controls taken from the lateral mesoderm and similarly marked, the carbon patches moved apart.

Similar experiments have recently been made by Chen (1953) on the sternal rudiments of fetal mice; his findings were in complete agreement with the observations described above.

From these results it was concluded that the union of the sternal rudiments in both birds and mammals is probably due mainly to the active ameboid migration of neighboring undifferentiated cells towards the mid-ventral line. It would be interesting to know whether the same mechanism operates in other morphogenic movements in skeletal development.

3. THE SPECIFICITY OF CHONDROGENIC AND OSTEOPGENIC CELLS

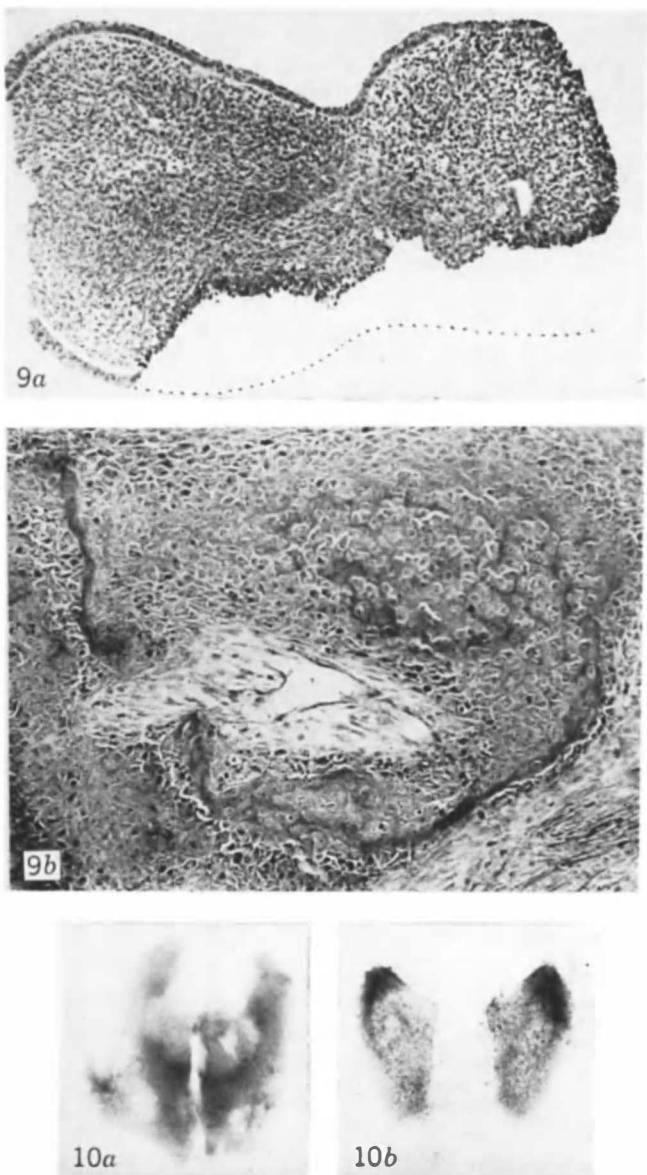
In the adult animal, the developmental specificity of the connective tissue is not always sharply defined. Thus in callus formed round a healing fracture, both cartilage and bone appear, the one often merging imperceptibly with the other, while under pathological conditions, ectopic cartilage and bone may differentiate almost anywhere in the body. It is a somewhat paradoxical fact that the potency of the early embryonic mesoderm should be much more rigidly determined. This was clearly shown by explantation experiments on the mandible of the early embryonic fowl (Jacobson and Fell, 1941).

PLATE IV

9. *a*. Frontal section through the proximal region of the right half of the mandible from a 4-day chick embryo; the part containing the osteogenic mesoderm has been excised for cultivation. *b*. The tissue removed from the mandible, after 6 days in culture; note the active ossification center that has appeared *in vitro* (after Jacobson and Fell, 1941, by courtesy of the Editor, *Quarterly Journal of Microscopical Science*).

10. *a*, *b*. Photographs of a living culture of the ventro-lateral body wall from an 8-day budgerigar embryo. The body wall has been cut in half down the mid-ventral line and the two halves arranged with the costal margins of the sternal plates facing each other. *a*. Shortly after explantation. *b*. Same, after 48 hours' growth; the costal margins are united by a sheet of connective tissue, but the sternal plates have moved widely apart. (After Fell, 1939, by courtesy of the Royal Society.)

PLATE IV



In this investigation, the morphogenic destiny of different regions of the lower jaw from 3-4 day chick embryos was studied by excising selected regions and allowing them to develop in tissue culture; the rest of the jaw was then fixed and sectioned to ascertain the exact site of the piece removed. In this way the boundaries of the chondrogenic and osteogenic areas at different stages of development could be precisely determined.

The chondrogenic cells destined to form Meckel's cartilage, were found to originate from a small proliferation center immediately beneath the buccal epithelium of the branchial arch. In the 3-day mandible, the presumptive fate of this region (Fig. 5) was already determined, and when it was excised and grown *in vitro*, it produced a rod of cartilage (Plate III, 8a, b). By the 7th day the chondrogenic center is exhausted and when the area was explanted at this stage, only connective tissue differentiated in the culture.

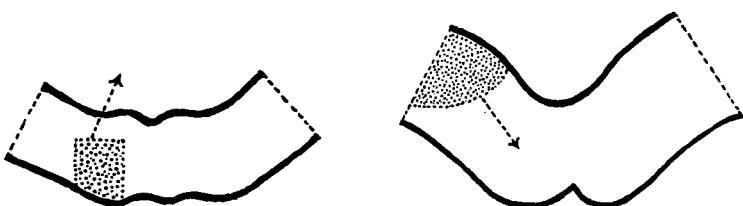


FIG. 5. The site of the proliferation center beneath the mouth epithelium in the mandible of a 3-day embryonic chick. The arrow indicates the direction of growth. (After Jacobson and Fell, 1941, by courtesy of the Editor, *Quarterly Journal of Microscopical Science*.)

FIG. 6. The site of the proliferation center beneath the epithelium lining the first branchial cleft in the mandible of a 4-day chick embryo. The arrow indicates the direction of growth. (After Jacobson and Fell, 1941, by courtesy of the Editor, *Quarterly Journal of Microscopical Science*.)

The osteogenic cells which give rise to the os angulare, spleniale, and supra-angulare are derived from a single proliferation center which appears at about the 4th day immediately beneath a slightly thickened patch of epidermal ectoderm at the proximo-lateral end of the mandible (Fig. 6). When excised and grown in culture, the osteogenic mesoderm produced bone, showing that its potency was already determined (Plate IV, 9a, b). The cells which form the os dentale originate at about the 5th day from a separate center in the disto-lateral part of the mandible, beneath a thickened patch of mouth epithelium corresponding to the rudiment of the mammalian enamel epithelium; this region also was self-differentiating *in vitro*.

These results demonstrated the morphogenic specificity of the chondrogenic and osteogenic mesoderm in early development. Although the

cells composing the two types of proliferation center appeared identical, yet when grown in the same environment in tissue culture, those of the chondrogenic region formed only cartilage and those from the osteogenic centers, only bone, while explants from adjacent areas formed no skeletal structures of either kind.

In the embryonic chick, there are two main types of cartilage: hypertrophic cartilage which is always associated with periosteal ossification and secretes alkaline phosphatase (Robison, 1923), and small-celled cartilage which neither ossifies nor produces phosphatase. The future differentiation of these two types of cartilage is already determined at a stage when no histological difference between them can be detected. This was shown experimentally in the following way (Fell and Robison, 1930). In the mandible, the distal part of Meckel's cartilage belongs to the small-celled type, while the adjacent quadrate becomes hypertrophic and ossifies. Experiments were made in which Meckel's rod and the quadrate were explanted *in vitro* at a stage when both were small celled and histologically identical. Growing under exactly the same environmental conditions, Meckel's rod merely enlarged, remained small celled and secreted no phosphatase, while the quadrate underwent chondroblastic hypertrophy, ossified and produced phosphatase as *in vivo*. Thus the histogenetic potencies of the two types of cartilage were distinct and were manifested in the standardized environment of tissue culture.

The nature and function of the periosteum has been much debated and some investigators have gone so far as to maintain that it has no specific bone-forming properties. Results obtained with tissue culture however, show that in embryonic and early post-embryonic life, at least, it is undoubtedly an osteogenic organ.

We must first consider the origin of the periosteum, and what causes a chondrogenic perichondrium to be transformed into an osteogenic periosteum. There is reason to think that this transformation is not in itself a phenomenon of self-differentiation. In normal embryonic life, osteoblasts always differentiate from the inner layer of the perichondrium as soon as chondroblastic hypertrophy begins but not before. Lacroix (1947) found that when fragments of the growth cartilage of a young rabbit were implanted into the brain, bone was formed in association with the grafts; he therefore suggested that the cartilage may induce the formation of osteogenic tissue. Further circumstantial evidence in support of this view is provided by an abnormal type of ossification sometimes seen in cultures of blastemata from 4-5 day chick embryos. As already stated, in such explants hypertrophy of the cartilage cells is often retarded, incomplete, or suppressed. In the same explant the humerus may undergo chondroblastic hypertrophy but not the radius and ulna; although environmental conditions are the same for the whole explant,

periosteal ossification occurs only round the hypertrophic cartilage of the humerus. Sometimes hypertrophy appears very late in development in the interior of a previously small-celled bone-rudiment; if the hypertrophic region is completely surrounded by small-celled cartilage, no osteoblasts differentiate, but if in one small area it reaches the surface, periosteal ossification takes place at this point only.

Fischer (1931) investigated the effect of adding hypertrophic cartilage, from which he claimed to have removed all the superficial tissue, to unorganized cultures of perichondrium. He states that the implanted cartilage induced the formation of more cartilage in the perichondrial culture, but his photographs suggest that the "induced" tissue was not cartilage but young bone. Further experiments are required, however, before any conclusion can be reached.

When once the periosteum has differentiated, it readily ossifies when stripped from the bones of embryonic chicks and cultivated *in vitro* (Fell, 1932). If removed from the shaft of a 6-day rudiment, it forms a nodule of bone in culture, and sometimes also a separate nodule of small-celled cartilage. It is probable that the bone develops from that part of the membrane which surrounded the hypertrophic segment of the shaft and in which the inner osteoblastic layer had differentiated, and that the cartilage arises from the more distal part of the tissue where no osteoblasts have been formed and the cells are still chondrogenic. Good ossification centers also appear in cultures of periosteum stripped from 10-day embryonic limb bones (Plate V, 11).

The writer has obtained excellent ossification from the periosteum of chicks on the point of hatching (unpublished results). Care must be

PLATE V

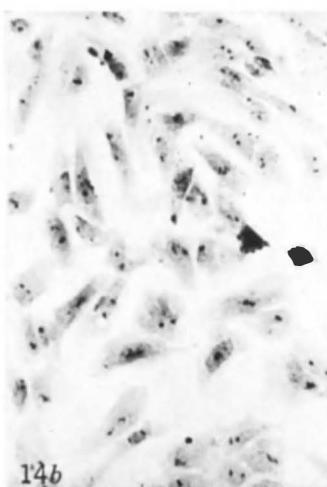
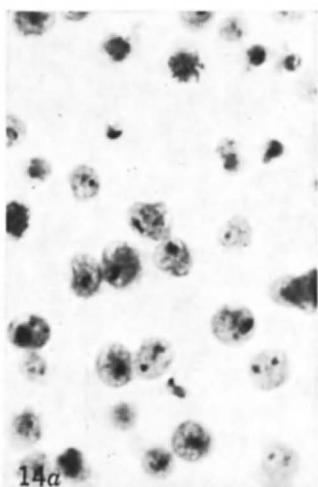
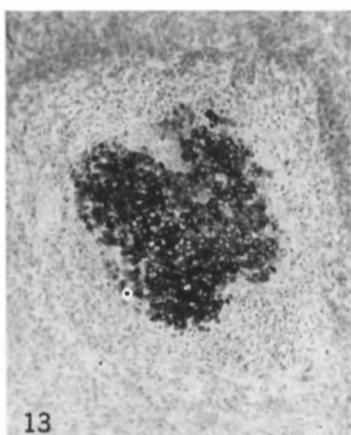
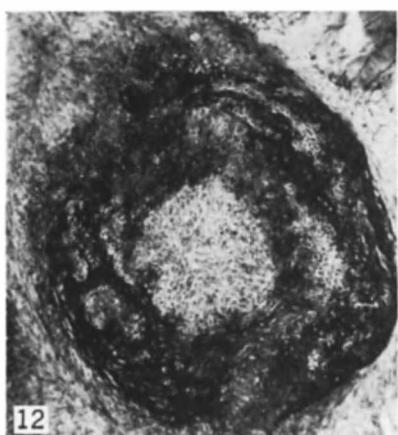
11. Section of a culture of periosteum stripped from the tibia of a 10-day chick embryo, after 6 days' growth *in vitro*. Note the compact ossification center which appeared during cultivation.

12. Section of an endosteal culture. A fragment of bone from the tibia of a 4-day (hatched) chick was grown *in vitro* for 6 days; the original bone was removed and rejected and the zone of outgrowth, mainly composed of endosteal osteoblasts, was cultivated for a further 8 days. Note the mass of new bone that has developed in culture.

13. Endosteal culture prepared in the same way, from another fragment of the same tibia as that from which the specimen shown in 12 was derived. Cartilage has differentiated in the interior of the tissue and merges with the surrounding bone. The slide has been stained with toluidine blue; note the intense metachromatic coloration of the cartilaginous matrix in contrast to the unstained intercellular substance of the bone.

14. *a.* Smear of a cell suspension of a completely disintegrated blastema from the limb-bud of a 4-day chick embryo. *b.* Similar suspension after 24 hours' cultivation on a plasma and embryo extract clot. Under these conditions the cells assumed a fibroblastic form and did not differentiate. (After Moscona and Moscona, 1952, by courtesy of the Editor, *Journal of Anatomy*.)

PLATE V



taken, however, to preserve intact the delicate osteoblastic layer underlying the thick, tough fibrous coat, otherwise no bone is formed and only fibrous tissue differentiates.

Endosteal osteoblasts from the bones of late embryos ossify well *in vitro*. Such cultures are prepared as follows (Fell, 1932). Fragments of the tibia or femur, cleared of periosteum and marrow, are explanted in ordinary hanging drop preparations. Cells emigrate from the cut ends of the Haversian spaces and proliferate in the medium until by the 5th or 6th day a broad halo of unorganized tissue has been formed around the original fragment. The explanted bone is then removed and rejected and the zone of growth transplanted to fresh medium. The central hole thus produced is soon filled by ingrowing cells and ossification begins in the interior of the dense sheet of tissue which mainly consists of endosteal osteoblasts. A week after the old bone has been excised, it is replaced by a nodule of new bone (Plate V, 12).

Although in the early stages of skeletal development, chondrogenic and osteogenic cells are strictly determined to form cartilage or bone respectively, this is not true of older skeletogenic tissue. Thus in the endosteal cultures, cartilage often develops as well as bone (Fell, 1933). The larger and denser the endosteal culture, the bigger is the area of cartilage which forms in the center of the tissue. The chondrifying region is usually easily seen in life as a clear, refractile mass which contrasts with the opaque, fibrous osteoid tissue and bone by which it is surrounded. If the culture is examined histologically, a patch of hypertrophic cartilage giving an intense metachromatic stain with toluidine blue or thionin (Plate V, 13), is seen merging gradually with the surrounding bone; the histological picture closely resembles that often encountered in sections of callus in healing fractures.

It is interesting that this chondrification is sometimes reversible. If a translucent, chondrified area is watched for several days, it may begin to diminish in size and gradually acquire the fibrous texture of the adjacent osteoid tissue; in section, the tissue is found to be histologically intermediate between cartilage and bone. As already mentioned, the cartilage cells in the endosteal cultures are always of the hypertrophic type which, like osteoblasts, secrete phosphatase (Robison, 1923); also the matrix is seldom very highly differentiated and the metachromatic material consists of little more than thin capsules around the chondroblasts. In view of these facts, it is perhaps not so surprising that such primitive cartilage can be directly transformed into bone.

Islets of hypertrophic cartilage may also develop in the periosteal bone formed in explants of entire bone rudiments. This was first noted by Roulet (1935) and has often been observed by the present writer.

From the results described above, it is seen that even *in vitro* the

older tissue has greater morphogenic versatility than the very young embryonic material; so far, however, bone or cartilage has not been produced in cultures of non-skeletogenic tissue, so that *in vitro* we have nothing comparable to ectopic ossification *in vivo*.

4. THE EFFECT OF MECHANICAL FACTORS ON SKELETAL TISSUE *in vitro*

It is a familiar fact that *in vivo*, the structure of the skeleton can be drastically modified by mechanical forces; this is equally true of skeletal tissue *in vitro*, and has been demonstrated experimentally by Weiss and his collaborators and by Glücksmann.

Weiss and Amprino (1940) explanted the chondrogenic mesoderm of the sclerotic from 4-day embryonic chicks, into a mixture of plasma and embryo extract; as the medium was clotting, it was stretched with a needle until coagulation was complete. When the needle was withdrawn, the clot adhered to the coverslip, retaining its deformation and the resulting elastic tensions within it. In this way the chondrogenic tissue embedded in the stretched coagulum was subjected to tension during its subsequent growth. If the tension was excessive, the explant became drawn out into a thin layer which formed stout bundles of collagenous fibers along the lines of stress but no cartilage; moderate tension did not inhibit chondrification, but the resulting cartilaginous plate was abnormally thin.

The influence of mechanical factors on the differentiation of chondrogenic cells was demonstrated in another way by Moscona and Moscona (1952). They removed the skeletal blastema from the limb buds of 4-day chick embryos, and by a technique of enzymic digestion (Moscona, 1952) disintegrated the tissue into a suspension of single cells (Plate V, 14a). If deposited on a plasma clot so that they were not in intimate contact with one another, the chondrogenic cells merely spread out on the medium in the fibroblastic form and remained undifferentiated (Plate V, 14b); if placed in a fluid medium, however, they reaggregated into small clumps which redifferentiated into nodules of cartilage (Plate VI, 14c).

If folds are produced in explants of the differentiated scleral cartilage (Weiss and Dorris, 1936; Weiss and Amprino, 1940; Glücksmann, 1938, 1942) the arrangement of the cells is greatly disturbed. At the convex surfaces which are under tangential strain, the perichondrium is dense, with cells and collagenous fibers running parallel to the surface; on the concave side the perichondrium loosens into a stroma from which fibers radiate into the interior of the cartilage along the lines of tension produced by compression of the plate at this point.

The effect of mechanical factors on osteogenic tissue and bone *in vitro*, has also been studied. Glücksmann (1938) subjected cultures of

endosteal bone to stress by implanting them between fragments of adjacent ribs which were still attached to each other by intercostal muscle; during cultivation the ribs were drawn together by degeneration of the muscle and by the formation of a common fibrous capsule, and thus exerted pressure on the implanted endosteal culture. The structural effect of the pressure depended on the stage of development of the implant at the beginning of the experiment. If ossification were at an early stage, a "compression structure" was produced, i.e. the explant became greatly elongated in a direction parallel to the ribs and the calcified trabeculae of bone which were subsequently formed, had the same orientation. On the other hand, if the endosteal culture was already well calcified when laid between the ribs, it acquired a "resistance structure" in which the bony trabeculae were oriented at right angles to the ribs and prevented their further approximation.

In another investigation, Glücksmann (1939) applied stresses to metatarsals and phalanges of chick embryos by placing these rudiments in contact with each other in such a way that during their subsequent growth, they applied pressure to one another at various points and in various directions. The results showed that cartilage appeared at the site of pressure in both the epiphysial perichondrium and the diaphysial periosteum. For example, in one combination a bowl of small-celled cartilage developed in the periosteum of one rudiment in response to pressure exerted by another; the cartilage fitted the impinging epiphysis like an acetabulum.

When the elongation of an unossified cartilaginous rudiment was obstructed by barrier cartilages placed at either end (Glücksmann, 1942), it became very bent. Little or no bone developed on the convex side, but an abnormally large amount was formed on the concave surface where

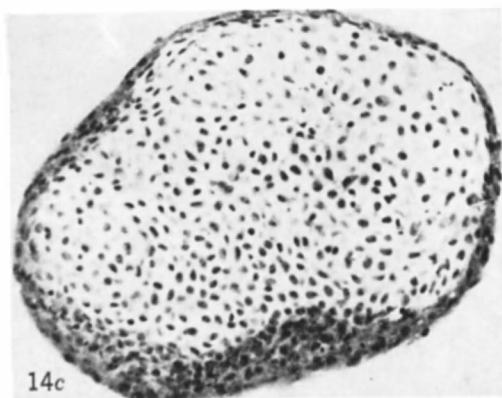
PLATE VI

14. *c.* Similar suspension to that shown in 14 *a, b.* The cells were allowed to aggregate in a fluid medium and were cultivated for 4 days; they have differentiated into a nodule of cartilage. (After Moscona and Moscona, 1952, by courtesy of the Editor, *Journal of Anatomy*.)

15. The effect of vitamin A on fetal mouse bones (Fig. 7). *a.* Section of control radius from a late mouse fetus after 7 days' cultivation in normal medium; the rudiment has enlarged slightly, and remains healthy. *b.* Opposite radius from the same fetus after 7 days' cultivation in medium containing about 2000 I.U. vitamin A per 100 cc. Note the enormous shrinkage of the entire rudiment and the extensive absorption of the bone. (After Fell and Mellanby (1952), by courtesy of the Editor, *Journal of Physiology*.)

16. The effect of insulin on bone rudiments of the embryonic chick. *a.* Living femur from a 6-day chick embryo after 7 days' growth in normal medium. *b.* The opposite femur from the same embryo after 7 days' cultivation in medium containing 0.16 unit/ml of insulin. Note the short shaft and greatly enlarged ends. (After Chen, 1954, by courtesy of the Editor, *Journal of Physiology*.)

PLATE VI



the natural elasticity of the perichondrium pulled the membrane away from the cartilage, leaving a space which became filled with bone. When the same experiment was performed on older rudiments, they were only slightly bent and ossification was always greater on the convex than on the concave side; in these explants the periosteum remained firmly attached to the osseous sheath and the increased bone formation on the convex side followed tension stresses running parallel to the surface of the cartilage.

In another series of experiments, Glücksmann altered the degree or direction of the normal tension stresses in an ossifying rudiment, by removing all or part of one epiphysis. During normal growth, the epiphyses expand, not only in a direction parallel to the long axis of the shaft, but also at right angles to it; since the periosteum is attached at its distal end to the epiphyses, their enlargement subjects the membrane to tension stresses in two directions. By cutting off one epiphysis, the lateral component of these forces was removed and tension greatly diminished. Since tension appears to promote ossification, this operation might be expected to reduce the amount of bone formed; the result confirmed this expectation. When half the epiphysis was excised, no bone developed on the operated side, but was normal on the opposite surface. These observations support the view that tension stresses encourage bone-formation and that the histological structure of the developing bone is oriented along the lines of tension in the osteogenic tissue.

The experiments described in this section have shown that mechanical factors influence the structure of cartilage and bone *in vitro* as they do *in vivo*. As Glücksmann (1942) points out: "It is clear, therefore, that the effect of mechanical stresses on skeletal tissue developing under the extremely simplified conditions of culture *in vitro* are essentially the same as those produced by similar mechanical factors on skeletal tissue developing *in vivo* where the situation is complicated by the presence of muscles, blood vessels and nerves. This affords evidence that many of the structural effects resulting from mechanical stresses *in vivo* are due to the direct action of the stresses on the skeletal tissue itself."

III. Physiology

1. THE EFFECT OF THE CULTURE MEDIUM ON GROWTH AND DIFFERENTIATION

The usual culture medium for skeletal tissue is a mixture of plasma and embryo extract in concentrations and proportions which vary according to the tissue to be grown.

The age of the embryo from which the extract is made is of great importance. Gaillard (1935, 1942) was the first to demonstrate this point

by critical experiments. He cultivated a strain of osteoblasts from the os frontale of a 16-day chick, in Carrel flasks until all trace of the original bone had disappeared. Each explant was then divided into two parts. Both halves were first explanted in a clot containing the press juice of 7-day chick embryos with a supernatant fluid phase of the same embryo extract. The control half was maintained in this medium throughout the culture period, the supernatant press juice being renewed at intervals, but the experimental half received as its supernatant fluid press juice from embryos of steadily increasing age up to the 18th day of incubation, then serum from a young hatched chick and finally from a nearly adult bird. When the cultures were examined histologically, the control series showed little or no differentiation, but those treated with the "ascending range of body-fluids" developed thick bundles of collagen and sometimes normal bone. This striking histological difference between the control and the experimental series of cultures was not correlated with any difference in growth which had nearly ceased in both sets by about the 5th day, while bone formation began after this stage.

Verdam (1936, 1937) showed that intact bone rudiments of rat embryos differentiated very well in response to the "ascending age range method." Thus the metacarpals of early rat embryos when grown with extract of younger fetuses, rapidly lost all histological structure, but when cultivated with extracts of embryos of increasing age, differentiation was almost normal.

Miszurski (1938) found that although the tibiae of chick embryos grew best in extract from 7-day embryos they failed to ossify, whereas bone formed readily in the presence of extract from 13-day chicks, an observation which the present writer has often confirmed. Miszurski found that extract of a 19-day embryo when bicentrifuged and suitably diluted, gave both good differentiation and a better growth than the 7-day embryonic juice, but that it was toxic if used in too high a concentration.

The long-bone rudiments of the chick grow and develop in dried, reconstituted plasma and fresh embryo extract, though the cultures are slightly inferior to controls in fresh plasma (Carpenter, 1950). These rudiments also enlarge and differentiate quite well in the absence of embryo extract, on plasma clotted with thrombin, but better results are obtained when extract is added (Chen, 1954).

Calcification of osteoid tissue is usually slower and less complete *in vitro* than *in vivo*. Paff (1948), using femur rudiments of the chick, reports that more bone and better calcification was obtained at a pH of 7.0–7.2 than at pH 7.8–8.0.

Interesting observations have been made by Verne, Verne-Soubiran and Verne (1940) on the effect on calcification, of plasma from dogs

whose bile was drained by means of a gall-bladder fistula. When the limb bones of early chick embryos were grown in such plasma, ossification was inhibited although hypertrophic cartilage differentiated; controls in normal dog plasma ossified in the usual way. If bones from 12–14 day embryos were fractured and the broken ends placed in apposition, only a membranous union took place in the bile-free plasma while the controls in normal medium were repaired by the deposition of new bone. When fragments of the parietal bones from chick embryos were cultivated in bile-free plasma, the bony trabeculae became decalcified and assumed a fibrous appearance comparable to that seen in *osteitis fibrosa*. Similar changes took place more slowly *in vivo*, in the bones of the operated dogs, but the exact nature of the humoral conditions responsible was not known.

The differentiation of skeletal blastemata from chick limb buds can be modified by diluting the culture medium and thus reducing its nutritive properties (Fell and Landauer, 1935). In these experiments the skeletal rudiments from both leg buds were explanted *in vitro* at various stages of early embryonic life. One of each pair was grown in normal medium (equal parts of embryo extract and plasma) and the other in a growth-restricting medium (5 parts of Pannett and Compton's saline, with a trace of embryo extract to clot the medium, and 4 parts of plasma). As described above (p. 418), under the best conditions of culture, growth of the explanted rudiments is greatly retarded, but in the growth-restricting medium the inhibition was much exaggerated, although the tissue remained healthy. The results showed that there is a critical period in the early development of the limb skeleton during which conditions which restrict the growth of the cartilage also prevent the hypertrophy of the chondroblasts and associated periosteal ossification; after this period the same conditions fail to prevent hypertrophy and ossification. Thus in 39 explants from limb buds ranging in length from 0.48–2.19 mm, 5 (13%) ossified in the normal medium and 1 (3%) in the growth-restricting medium; of 37 pairs of rudiments from leg buds of 2.24–3.81 mm, 27 (73%) ossified in the normal medium and only 10 (27%) in the growth-restricting. On the other hand, out of 15 pairs of explants from buds ranging from 3.86–5.24 mm, all ossified in both media.

Experiments were then made in which the osteogenic mesoderm of the mandible from embryos with leg buds ranging from 0.69–2.8 mm were cultivated in the growth-restricting media; the blastemata of the leg buds were grown in the same watch glasses. Of the 18 mandibular explants, 17 formed bone in the growth-restricting medium, but none of the limb blastemata did so. This showed clearly that membrane bone could develop under conditions which completely prevented periosteal

ossification. It is probable that in the latter, the primary effect of the growth-restricting conditions was on the cartilage, the cells of which were unable to hypertrophy in the normal way. As mentioned previously (p. 423), there is evidence that the hypertrophic cartilage may induce the differentiation of osteoblasts in the perichondrium; if this is true, it would explain why the same environmental influences may inhibit periosteal but not membranous ossification *in vitro*.

Recently Wolff, *et al.* (1953) have grown skeletal rudiments and other organs on an entirely synthetic medium composed of agar, saline, glucose, various amino acids and sometimes vitamins. The development *in vitro* of the avian syrinx was studied by means of this technique. When the syrinx was explanted before cartilage had appeared, it formed four or five pairs of tracheo-bronchial arches in latero-median succession; that true growth had taken place was demonstrated by the presence of mitosis and by an increase in total nitrogen. Tibiae from 8-day embryos were also cultivated in a synthetic medium to which novocaine had been added as a stimulatory substance. The rudiments elongated, increased in weight and continued to differentiate, but there was no increase in total nitrogen; in the presence of novocaine, the catabolism of nitrogen was partly compensated by the assimilation of amino acids.

While growth in synthetic medium is inferior to that in normal nutrients, nevertheless these pioneer experiments are of great interest and importance and no doubt further research will improve the growth-promoting properties of synthetic media.

2. THE EFFECT OF VITAMIN A ON SKELETAL TISSUE *in vitro*

So far, very little work has been done on the effect of vitamins on skeletal tissue in culture. The action of vitamin A, however, has been studied in some detail (Fell and Mellanby, 1952). When a young animal is fed on a diet containing excess vitamin A, the skeleton is severely affected. Bone and cartilage are rapidly resorbed, and although new bone is deposited, it is not sufficient to compensate for the resorption, and spontaneous fractures occur. It was not known whether these changes were due to the direct action of the vitamin on the bone and cartilage, or whether the effect was secondary and perhaps mediated through the endocrine system.

Experiments were therefore undertaken to see whether excess vitamin A would affect skeletal tissue *in vitro*, where the mediation of other systems would be excluded. Two main series of experiments were made. The first was done on the limb-bone rudiments of early embryonic chicks, to study the effect of the vitamin on development, and the second was made on the long bones of fetal mice near term, in order to observe

the action of the vitamin on bones already at an advanced stage of ossification.

The femur, tibia, humerus, radius, and ulna of 6-day chick embryos were grown by the watch-glass method. Of each pair of rudiments, one was cultivated on normal medium as a control and the other on the same medium to which had been added about 1500 I.U. of vitamin A per 100 cc. During the first two days there was no obvious difference between the experimental and control explants; both enlarged and continued to develop. After this stage, the growth of the experimental rudiment declined and then stopped, while that of the control reached 3-4 times its original length. In the high concentration of vitamin A, the cartilage became soft and gelatinous and often greatly distorted, but nevertheless periosteal ossification continued and the soft tissue attached to the rudiment proliferated even more profusely than in the controls. Histological examination showed that in the high A medium, the primordium differentiated into the three zones characteristic of ossifying cartilage, viz. the diaphysial region of hypertrophic cells, intermediate zones of flattened cells and small-celled epiphyses. The matrix, however, underwent a striking change; it lost its normal metachromasia with such stains as toluidine blue, while at the same time assuming a bright pink color with van Giesen's stain which leaves normal cartilage matrix almost colorless. The intercellular partitions were much narrower than in the controls. These changes appeared first in the diaphysis but later spread to the epiphyses also. The chondroblasts usually appeared healthy and often underwent mitosis.

When such explants were transferred to normal medium they were found to have little capacity for recovery (Herbertson, 1955). If the rudiment were not too adversely affected, the changes were arrested and the epiphyses continued to enlarge, but in the diaphysis the depleted matrix was not restored nor did it regain its metachromatic staining properties. On the other hand the periosteal bone often proliferated extensively when the explants were transplanted from high A to normal medium.

It was interesting that the different rudiments did not all respond to excess vitamin A to the same extent. Thus the effect was greatest in the femur and tibia, slightly less severe in the humerus and least in the radius and ulna. As we shall see later, hormones produced a similar differential action.

Recently (Fell and Mellanby, unpublished) the effect of high vitamin A on the explanted blastemata of 4-day limb buds has been investigated. It was even more drastic than on the older rudiments. Procartilage or early cartilage differentiated during the first two days in culture, then rapidly disintegrated. The same phenomenon was observed in the otic

capsule of explants of the 4-day otocyst; the capsule began to form in the treated cultures, then quickly disintegrated and disappeared, while the epithelial part of the ear primordium continued to differentiate normally.

The well developed bones of late fetal mice responded to excess vitamin A (1500–3000 I.U./100 cc) with astonishing speed and intensity (Fig. 7; Plate VI, 15a, b). The original rudiments consisted of a stout

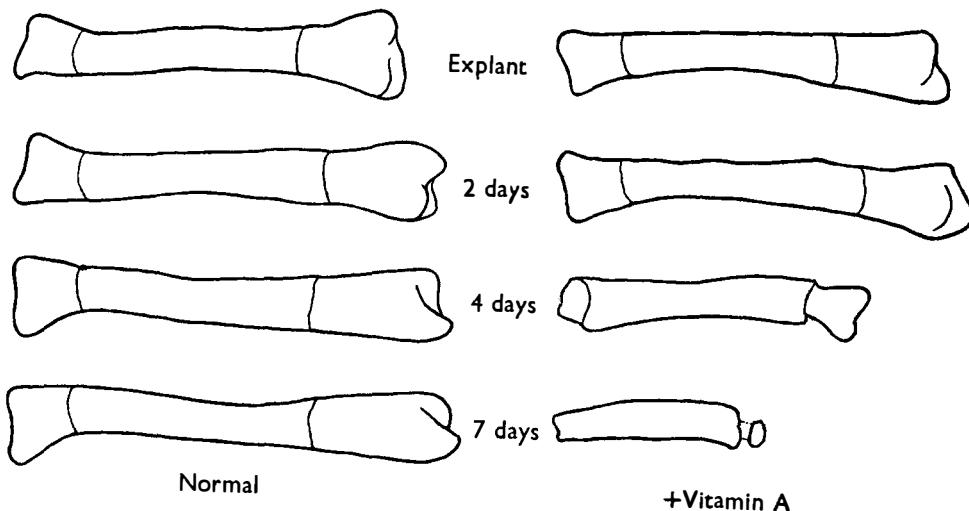


FIG. 7. Camera lucida drawings of living radii from a late mouse fetus, one grown in normal medium and the other in medium containing about 2000 I.U. vitamin A/100 cc. During 7 days' cultivation the control radius enlarged somewhat and the bone and cartilage remained intact. That grown in medium with excess vitamin A, also enlarged during the first 2 days, then suddenly the cartilage began to shrink and the bone to be absorbed; by the 4th day the cartilage had almost disappeared. Sections of these explants are shown in Plate VI, 15 a, b. (After Fell and Mellanby, 1952, by courtesy of the Editor, *Journal of Physiology*.)

bony diaphysis with a marrow cavity and large cartilaginous ends. As with the chick bones, no effect was seen during the first two days; then suddenly the terminal cartilages began to shrink, the bone became rarefied but, as before, the adherent soft tissue grew profusely. If the bones were examined histologically at this stage, the intercellular partitions of the cartilage were seen to have dwindled and to have lost their metachromasia, but the cartilage cells looked healthy and often showed plentiful mitosis. After 10 days' cultivation in the presence of 3000 I.U. of vitamin A per 100 ml, the bone rudiment had almost vanished; only a few shreds of bone and sometimes a small nodule of cartilage remained, lying in a broad sheet of ameboid cells. The controls in normal medium were healthy and intact.

The healthier and more active the tissues appeared, the more drastic

was the effect of the vitamin. Thus a high dose (6000 I.U./100 ml) was less effective than lower concentrations, as the former was slightly toxic. Similarly if the explants were killed by heating them in Tyrode to 45° C, vitamin A produced no change. As with the avian material, the younger and more cellular the explant, the more complete was its destruction by the vitamin.

In the experiments described above, vitamin A, as the acetate or alcohol, was added to normal plasma. It seemed desirable to compare the action of the "artificial" hypervitaminosis with "natural" hypervitaminosis in which the vitamin was introduced into the plasma by feeding the donor fowl on a diet with a high content of vitamin A. Accordingly, blood plasma was drawn from fowls suffering from hypervitaminosis A, the concentration of vitamin was estimated and the same quantity of the acetate or alcohol added to normal medium. Fetal mouse bones were grown in media prepared from the two plasmas. The results showed that while qualitatively the effects of the two media were the same, i.e. both caused resorption of bone and cartilage and loss of metachromasia from the cartilaginous matrix, quantitatively they differed greatly. The "artificial" hypervitaminosis was far more potent and rapid in its action than the "natural." This difference in activity was associated with differences in the extractability of the vitamin in the two plasmas; thus nearly all the "artificially" added vitamin could be recovered by shaking the plasma with petrol ether, but that introduced "naturally" by feeding the donor fowl could only be extracted with petrol ether if the proteins had first been denatured with 50% alcohol.

From these results it was concluded firstly that vitamin A acts directly on skeletal tissue, secondly that its effect is due to altered physiological activity of the cells and not to cellular degeneration, and thirdly that in the blood stream the vitamin is in a less active state than when added directly to blood plasma.

3. THE EFFECT OF HORMONES ON SKELETAL TISSUE *in vitro*

The action of hormones on skeletal tissue *in vitro* has been studied rather more widely than that of vitamins, but much remains to be done.

The effect of the anterior pituitary and of the growth hormone has been investigated by Verdam (1946; quoted from Gaillard, 1953). In his first experiments he cultivated fragments of the pituitary from an adult rat near explants of the metacarpals from rat fetuses, and found that the health and differentiation of the metacarpals was much improved. Similar results were obtained when the pituitary was cultivated separately for 24 hours in a liquid medium which was then removed with a pipette, dialyzed to remove low molecular catabolites, and added as a supernatant fluid to cultures of metacarpals. As compared with controls

grown with supernatant fluid composed of serum, embryo extract and physiological saline, the experimental explants formed a larger zone of hypertrophic cartilage, some endochondral bone and a longer sheath of periosteal bone. Other controls grown with the incubated fluid in which no pituitary explants had been cultivated, degenerated.

Finally Verdam investigated the influence on the metatarsals of some commercial hormonal preparations; of these, one contained mainly thyrotrophic and gonadotrophic hormones, one contained prolactine and the third growth hormone. The first two had no effect on the explanted bones, but the preparation of growth hormones improved differentiation of the cartilage and ossification, but did not significantly increase growth.

The influence of the sex hormones on explants of the syrinx of duck embryos has been demonstrated by Wolff and Wolff (1952). In the normal duck, the syrinx is sexually dimorphic, that of the male being characterized by asymmetry of the cartilages which are better developed on the left side than on the right, while those of the female syrinx are symmetrical. The syrinx was removed from duck embryos at different stages and cultivated in a mixture of agar, serum, embryo extract and Tyrode. If the rudiment was explanted at the 9th day when the sexual differentiation of the gonads was established, it developed *in vitro* according to the sex of the embryo from which it was taken. If explanted before that stage, however, the syrinx differentiated according to the male type, whatever the sex of the embryo from which it was obtained; this indicates that the male type is the asexual or neutral form and that it is self-differentiating. When the early rudiment was cultivated in medium to which the female hormone had been added, however, it acquired the female shape regardless of the sex of the donor, but curiously enough the addition of a massive dose of an androgen (testosterone propionate) had the same feminizing effect as the female hormone.

Extensive experiments have been made by Chen (1954) on the effect of insulin on cultures of limb-bone rudiments from 6-day chick embryos. When insulin is injected into the egg, the elongation of the long bones in the embryo is greatly retarded (Landauer, 1947). Chen added insulin (0.16 unit/ml) to the plasma and embryo extract of the medium, and found that the explanted rudiments developed very abnormally; the ends enlarged greatly, while the shaft remained very short and was often bent (Plate VI, 16a, b). In histological sections it was found that the hypertrophy of the diaphysial cartilage cells was much retarded and sometimes almost inhibited, although periosteal ossification was usually good. Plenty of cartilage matrix was formed with the normal metachromatic staining reaction. Often a double shaft developed in the treated explants, especially in the radius; this was due to down-growth of chondrogenic tissue from the two epiphyses alongside the diaphysis

and outside the periosteal bone.

Chen discovered that much more drastic effects were obtained if embryo extract were omitted from the medium and the plasma clotted with thrombin instead. As mentioned earlier in this chapter, the controls grew and differentiated quite well in the absence of the extract, although their growth rate was somewhat reduced. When embryo extract was omitted, a recognizable effect could be obtained with a dose of insulin of only 0.0016 unit/ml. Further investigation showed that the embryo extract inactivated the hormone.

As with experiments with vitamin A, different rudiments responded to insulin in different degrees. Thus the humerus was the most severely affected and the radius and ulna showed the least change.

Recently, Mellanby and Fell (1955) have examined the effect of L-thyroxine on the limb-bone rudiments of 6-day embryonic chicks. One series of experiments was made on the whole blastemata dissected from 4-5 day limb buds. One of each pair was explanted in medium containing 0.156 or 0.016 mg per liter of L-thyroxine and the other in normal medium; after about a week's cultivation they were fixed and sectioned. As described on p. 406, the explanted wing blastema formed a humerus, radius, ulna, and usually part of the pectoral girdle, while that of the leg produced a femur, tibia, fibula, often part of the metatarsus and pelvis. As in the earlier experiments, in normal medium the tissue chondrified readily but only a minority of the rudiments underwent chondroblastic hypertrophy and ossified; certain bone primordia differentiated more readily than others, however, and out of 22 control explants of wing and the same number of leg blastemata, 11 humeri, 4 tibiae, 2 femora but no radii or ulnae formed hypertrophic cartilage. In the opposite rudiments grown in medium containing thyroxine (0.156 or 0.016 mg/l) the incidence of hypertrophic cartilage was much higher; among the 22 explants, all the humeri, 12 tibiae, 7 femora, 3 radii but no ulnae showed hypertrophy.

In the second group of experiments, isolated rudiments from 5½-6½ day embryos were used. As before, one of each pair was grown in normal medium and the other in a clot to which thyroxine (0.156 mg/l) had been added. Here again, the various rudiments from the same embryo reacted differently to the same environmental conditions. The hormone had a harmful effect on the femur and tibia; growth, especially of the shaft was retarded, degeneration appeared among the hypertrophic cells and the intercellular partitions became abnormally thin, although the staining reactions of the matrix were normal; periosteal ossification was as good as and often better than in the controls. The differentiation of the humerus was at first stimulated, but by the 8th day changes similar to, but less pronounced than those seen in the femur and tibia, had some-

times appeared. The radius and ulna showed no ill effects and were often larger and better developed than their controls in normal medium.

From these results it is seen that the effect of a given dose of thyroxine depends on the stage at which the rudiments are explanted and on the particular rudiment exposed. The action of the hormone *in vitro* resembles that *in vivo*; both in cultures and in the animal, skeletal differentiation is stimulated and different rudiments are affected in different degrees. Willier (1924) grafted pieces of the thyroid on to the chorio-allantoic membranes. The embryos became emaciated and the limbs were abnormally short; the legs were more susceptible than the wings, an observation that agrees with the finding that *in vitro* the femur and tibia are more adversely affected than the wing bones.

It is interesting that when three different agents—vitamin A, insulin, and thyroxine—were applied to the limb bones of 6-day chick embryos under identical conditions of culture, each produced its characteristic histological effect, and that no two types of effect were the same. The action of these substances had two features in common, however: the shaft was always more affected than the epiphyses and different rudiments responded to the same agent in different degrees. The least affected bones were always the radius and ulna, which may perhaps be correlated with the fact that *in vivo* they develop more slowly than the other primordia and their cartilage does not reach such an advanced stage of hypertrophy. It would seem that each bone has its physiological individuality which determines its responses to environmental influences.

IV. Conclusion

The observations described in this chapter show that tissue culture has contributed to our knowledge of skeletal structure and physiology in many ways. In the writer's view, one of its most important uses in the future may be in the field of hormone and vitamin research, where the method can provide us with information which cannot be obtained in any other way. So far it has served to demonstrate that several hormones and a vitamin act directly on skeletal tissue, and that each agent produces a specific histological effect. The next step will be to investigate the biochemical changes underlying these histological alterations; promising results are already being obtained by means of microchemical techniques and the use of radioactive tracers. The development of a satisfactory synthetic medium for organ cultures, so ably begun by Wolff and his collaborators, is also bound to play an important part in future research on the physiology of cartilage and bone *in vitro*.

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CHAPTER XV

THE GROWTH OF BONE

H. A. SISSONS

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I. Body Growth and Bone Growth

Growth data can be very easily given quantitative expression, with the result that there have been many attempts to formulate empirical mathematical equations relating change in the weight or size of growing organisms to age. Some of the results of these attempts are presented in D'Arcy Thompson's stimulating work "Growth and Form" (1942), which also shows how the form of a great variety of biological systems can be related to the growth mechanisms which bring them into being. Nowhere is the relationship between growth and form more evident than in the skeleton, and the purpose of the present paper is to collect data on some aspects of normal and pathological bone growth, and to indicate how they influence the structure—both gross and microscopic—of the bones concerned.

Because of the ease with which skeletal measurement—either from preserved specimens or from growing individuals—can be carried out, information relating to the skeleton is frequently included in studies on general growth. It is therefore of interest to examine some of the laws which have been found to describe general body growth, and to see how

far they can be applied to the growth of the skeleton as a whole and to the growth of its component units.

When growth data are presented in graphical fashion, the growth of an animal or plant is usually found to follow an S-shaped curve, as shown in Fig. 1. Such a curve summarizes the general knowledge that after a

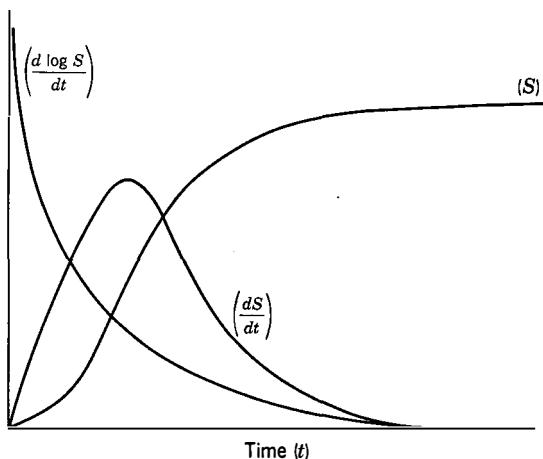


FIG. 1. General curves of growth, showing:

- Change in size (S) during growth.
- Change in growth rate (dS/dt) during growth.
- Change in specific growth rate ($d \log S/dt$).

period of active growth, the size of any particular multicellular organism approaches a limiting value. The differential of such a curve (dS/dt), measuring the *absolute* growth rate at any moment, increases to a maximum and then becomes progressively less, diminishing more and more slowly as the organism increases in size. As Medawar (1945) points out in an excellent summary of the relationships between size, shape, and age, if the logarithm of size, rather than size itself, is plotted against time—thus expressing the growth increment relative to the size of the organism at any moment—a curve is obtained whose differential ($d \log S/dt$), which is known as the *specific* growth rate, diminishes progressively from the commencement of growth. Such considerations remind us that the growth of an organism shows a general resemblance to the growth of a tissue culture, where an initially *logarithmic* growth (with constant *specific* growth rate) is succeeded—as the environment of the culture medium becomes changed—by growth characterized by an ever diminishing *specific* growth rate.

It is found that the growth of bone conforms generally to the ideas outlined above. This can be very easily shown (see Figs. 2 and 3) from a consideration of human growth in height, stature being quite an exact measure of skeletal size, and thus of overall bone growth. Figure 2 shows

the general shape of the height curve over the period of growth, a smooth curve being fitted to the heights of males obtained for this purpose from the data of Roberts (1878) and of Simmons and Todd (1933). The initial *logarithmic* part of the S-curve is seen in human growth only during foetal life, and is not included in the present figure, so the curve

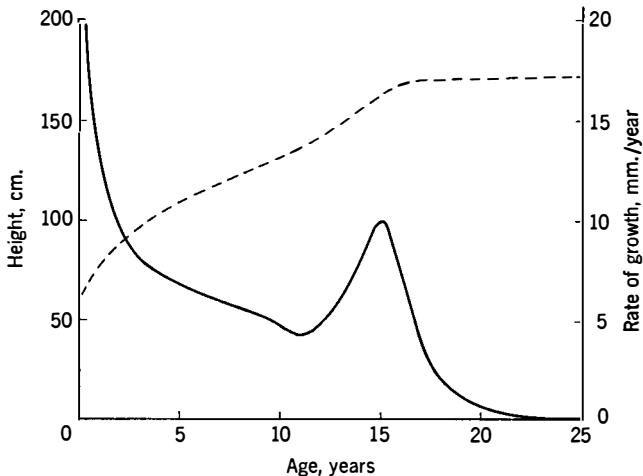


FIG. 2. Skeletal growth as shown by change in height.

(i) - - - Heights of males during the growth period.

(ii) ——— Rate of increase in height (mm/year) during same period.

(The diagram shows smoothed curves to fit the data from Roberts (1878) and Simmons and Todd (1933)).

shows merely a rapid increase at the onset, slowing as full height is reached at an age of about 16–17 years. The differential curve for the rate of growth shows the same general decline that was seen in the ideal curve of Fig. 1, but this is temporarily interrupted by a peak corresponding to the rapid growth of the adolescent period, this being preceded by a small depression where growth is significantly retarded for a short time. In Fig. 3 the same curve for growth in height is compared with a curve showing increase in body weight over the same period (See D'Arcy Thompson for similar curves from different sources). When the cube of the height is compared with the weight (for bodies of similar shape, the weight—i.e. the volume—varies as the cube of the linear dimensions) it can be seen that there is a general correspondence between the two curves over the growing period, but that the increase in height ceases long before body weight approaches its limiting value. This, of course, reflects the dependence of skeletal growth on the presence of epiphyseal growth plates at the ends of the long bones, cessation of growth and the disappearance of these structures occurring after puberty.

This very brief outline indicates that two rather special features of general skeletal growth, at least in the human, when compared with the ideal growth curves shown in Fig. 1, are the fluctuations of growth rate

associated with adolescence and the early cessation of skeletal growth that is associated with "closure" of the epiphysial plates of the long bones.

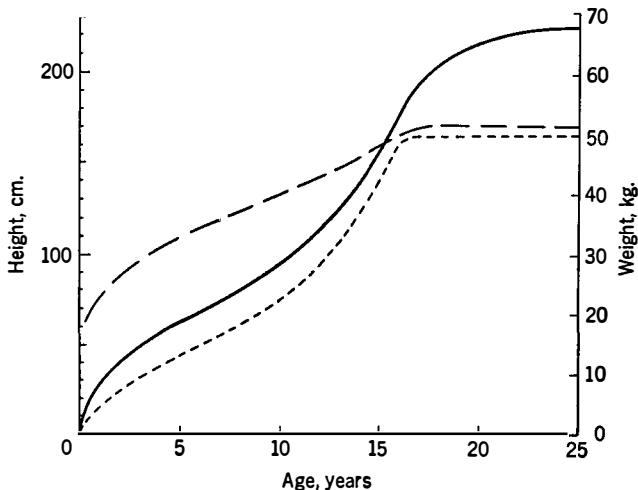


FIG. 3. Growth in height compared with growth in weight.

- (i) —— Height
 - (ii) —— Weight
 - (iii) - - - Cube of height, plotted on an arbitrary scale to allow comparison with the Height and Weight curves.
- (The diagram shows smoothed curves to fit the data for males from Roberts (1878) and Simmons and Todd (1933)).

The changing proportions of the skeleton during development—the relative increase in length of the lower limbs, and the relative decrease in the size of the head, to name two obvious human examples—indicate that individual bones can show considerable variation from the overall rate of growth. Such variation is only an example of the differential growth that has been found to exist between various parts of the developing organism. A special study of this aspect of growth has been made by Huxley (Huxley, 1932; Reeve and Huxley, 1945) who has analyzed growth data from many widely differing types of biological material. His studies, for example those on the changing proportions of the claws of lobsters and crabs during development, lead him to put forward an equation

$$y = b + x^k$$

to relate y and x , the magnitudes respectively of a particular organ and of the rest of the body. If this equation of Huxley's is a proper description of biological growth, the changing proportions of differentially growing systems can be shown to result from differences in the *specific* (i.e.

(*logarithmic*) growth rates of the various parts, the ratios between these specific growth rates remaining constant over long periods of growth and development. Huxley has applied this principle to an analysis of the growth of individual bones of the hind limb in sheep, and to the growth of the facial and cranial regions of the skull in sheep dogs and baboons. He has also shown that variations in *specific* growth rates do not occur at random throughout the body, but are arranged in a regular manner (i.e. as relating to the different segments of a limb) resulting in a continuous gradient of growth for the body as a whole or for a particular region of it.

A number of authors have questioned the exactness of Huxley's equation as a description of differential growth, D'Arcy Thompson (1942) suggesting, for instance, that simpler types of mathematical relationship may equally well describe the biological data analyzed. However, Huxley's study of differential growth is cited here to emphasize, in connection with the growth of the skeleton, not only the detailed biological coordination of skeletal growth with the growth of the body as a whole, but the similar coordination between the differentially growing parts of the skeleton themselves.

II. Structural Aspects of Bone Growth

1. DEVELOPMENT OF KNOWLEDGE

The rigid nature of calcified bone tissue makes its interstitial growth impossible, and in all situations increase in the size of a bone is brought about by the deposition of new tissue on the surfaces of the existing bone. The persistence, in spite of the subsequent "remodeling" of the newly formed bone, of tissue formed at an earlier time enables a close relationship to be traced between growth and structure of bone, the tissue containing—both on the gross and microscopic scale—many indications of its earlier outlines.

The lack of any general interstitial enlargement was appreciated early in the development of knowledge of bone growth (for a more detailed account of this topic, see Keith, 1919). In 1727, the versatile biologist Stephen Hales recorded an experiment, in which by measuring the leg bone of a young chicken 2 months after drilling two holes in the shaft to act as markers, he showed that the bone grew by addition of new tissue at its ends. The holes were no further apart, although the bone had lengthened considerably. This observation on the absence of interstitial growth of bone was confirmed by Duhamel (1742) in France and by John Hunter. A considerable number of later workers, particularly after the discovery of X-rays made it possible for the position of metallic markers inserted into bones to be followed throughout the period

of growth, have testified to the correctness of the observation. Dubreuil for example, in 1913, using young goats and rabbits in which such markers had been inserted into the shafts of long bones, showed by serial radiographs that growth in length occurred only at the epiphyseal plates, and that there was no evidence of interstitial elongation of the bone of the shaft. Haas (1926) came to the same conclusions from experiments on dogs and rabbits, and Bisgard and Bisgard (1935) from experiments on goats.

Another type of experimental "marker" which is of considerable importance in regard to the development of knowledge on bone growth is provided by madder feeding. It appears that the initial observation in this field was made by one John Belchier, a surgeon, who observed at the dinner table that the bones of pigs which had been fed on madder

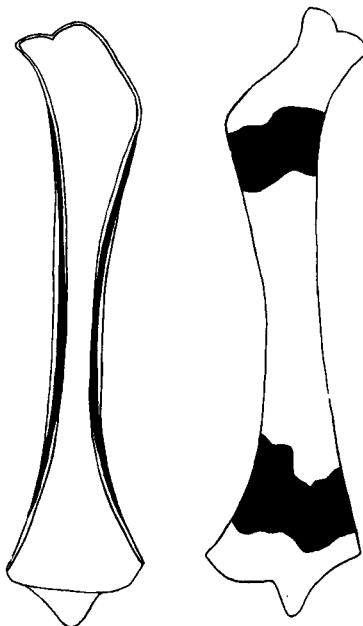


FIG. 4. Showing a longitudinal section and the external aspect of a femur in a young pig which, prior to death, had been fed for a month on a diet containing madder and for a month on a normal diet. The bone formed during the period of madder feeding is indicated in black. (From John Hunter, redrawn after Keith (1919)).

were colored red with this dye. Belchier carried out some limited experiments on the subject, but it was Duhamel, a French squire with pronounced scientific interests, who related the results of madder staining to the mechanism of bone growth. In a series of experiments carried out between 1739 and 1743 and described in the *Memoires de l'Academie Royal des Science*, he showed that madder colored those parts of the

skeleton which were being formed at the time of its administration. Bone formed after the withdrawal of madder from the animal's diet was normally colored, and in certain regions this newly formed bone covered over the older bone which retained its red coloration (see Fig. 4). When madder feeding had been suspended for some weeks before the experimental animal was killed, the bone at the ends of the shafts of long bones were found to be uncolored, and Duhamel was aware of the implications of this in connection with the mechanism of longitudinal growth. From the layer of white bone ensheathing the mid-shaft region he correctly inferred that a long bone grew in thickness—as did a tree—by the progressive development of new bone on its outer surface. He could not, of course, explain the increase in diameter of the marrow cavity by such a mechanism, and could think only that this resulted from expansion of the shaft, i.e. from interstitial growth. It was John Hunter who provided the proper explanation for this phenomenon. In the years 1760–1770 not only did he repeat the “marker” experiment of Hales and Duhamel demonstrating the terminal location of longitudinal growth in a long bone, but he carried out experiments with madder feeding at his experimental farm at Earl's Court. From these, and from a consideration of the way in which the proportions of a growing bone are maintained, despite the absence of interstitial expansion, Hunter realized that two fundamental processes must be concerned in the growth of bone—the deposition of new bone—such as occurs in the periosteal region of the shaft of a long bone, and the absorption of pre-existing bone, which must occur on the surface of the expanding marrow cavity. He saw, too, that this process of absorption must be at work on the external surfaces of the expanded metaphyses of long bones as these increase in length. It was in the course of his madder-feeding experiments that Hunter made the observation—which sounds surprisingly modern today—that living bone “is constantly changing its matter.”

More recently, Brash (1934) has used madder feeding in the pig as an aid to the study of bone growth. Again, a period of madder feeding was followed by a return to a normal diet for some weeks, and the details of growth studied for a number of individual bones, including both long bones and bones of the skull. From the same experimental material Payton (1932) has recorded information on the relative amounts of bone formed at the ends of each of the long bones of the limbs, and the rates of longitudinal growth of these bones.

The type of observation described distinguished the longitudinal growth of bone at the ends of the shaft from the periosteal growth in thickness only in that the former is a more rapid process (see Fig. 4, where the considerable length of new bone at the ends of the shaft compares with the thin periosteal layer on the rest of the surface). Applica-

tion of the microscope to the problems of bone growth made it possible to ascribe the growth mechanisms to cellular processes. It became evident, from the researches of Goodsir (1845), Koelliker (1852), and Virchow (1860) that the formation of bone was the function of a specialized cell that we now know as the osteoblast. In situations such as the periosteal region of the shaft of a long bone these cells form bone by surface accretion on the pre-existing cortex, while at the ends of the shaft they form bone on a scaffolding provided by the continuous proliferation of the cartilaginous epiphyseal plate. The first type of bone formation has become known as *membranous ossification*, the second as *endochondral ossification*. An important reason for distinguishing the two types of bone formation is that under normal circumstances endochondral ossification can occur only when an epiphyseal plate is present, which—for most mammalian species—is during the period of active growth terminating with skeletal "maturation." Endochondral ossification is the mechanism concerned with the rapid development in length and bulk of the growing skeleton, while bone formation occurring independently of cartilage continues after the final skeletal proportions are attained and is part of the process of constant structural remodeling of bone.

Soon after the microscopic observation of osteoblasts and the appreciation of their role in bone formation, Koelliker (1873) described the cellular mechanism of bone destruction, the existence of this process, it will be remembered, being Hunter's important contribution to the subject of bone growth as part of the process of continued "remodeling." Koelliker observed large multinucleated giant cells or "osteoclasts" in relation to microscopic areas of bone destruction, and he showed, by mapping out the distribution of these cells over the external surfaces of the bones of calves, that they were present at precisely those sites (chiefly metaphyseal, for the long bones) where remodeling, and hence bone destruction, should—and in fact did—occur.

We can now proceed with a more precise description of the cellular processes on which bone growth depends.

2. ENDOCHONDRAL OSSIFICATION

The general histological features of this process, first adequately described by Müller (1858) have recently been summarized by Lacroix (1949) and by Ham (1950). In the mammalian bone endochondral ossification consists of a coordinated sequence of cellular processes. These are the multiplication, growth and degeneration of cartilage cells in the epiphyseal plate, the vascularization of the degenerated—or "hypertrophic"—cartilage by blood vessels and connective tissue from the adjacent shaft of the bone, the formation of a network of bone trabeculae on

the framework of the unresorbed cartilage by the ingrowing connective tissue cells, and finally the remodeling, or progressive structural modification, of this bony tissue. Under normal circumstances all these processes proceed in equilibrium, with the result that the metaphysial bone trabeculae extend continuously into a receding zone of "hypertrophic" cartilage, so extending the length of the bony shaft. The histological structure of the growing region at the lower end of the femur in a rabbit, and its various components, is illustrated in Fig. 5 and Plate I.

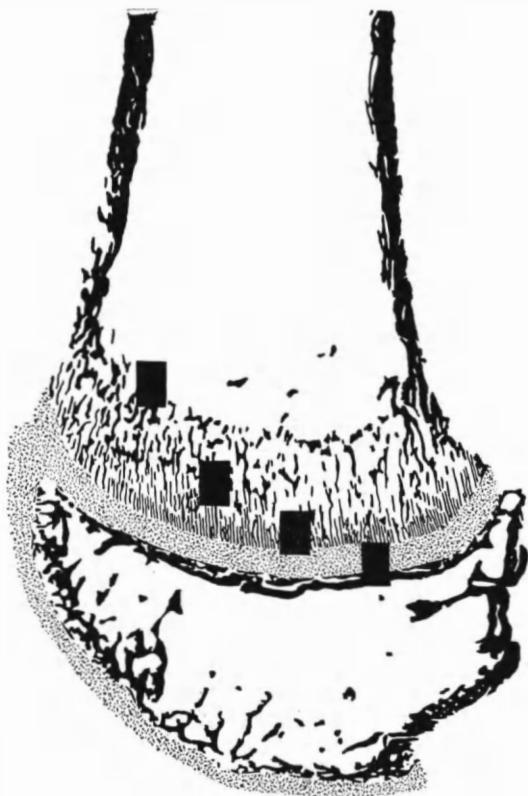


FIG. 5. Section through the growing region at the lower end of the femur in a young rabbit ($\times 7\frac{1}{2}$). The four black rectangles indicate the sites of the fields illustrated in Plate I.

Relatively little is known of the factors determining the normal growth of epiphyseal cartilage tissue. The cartilage cells are arranged in longitudinal columns, and descriptively, these columns, and thus the epiphyseal cartilage as a whole, are regarded as being divided into a number of zones (see Dodds and Cameron, 1934), corresponding to the various phases of the maturation process. Mitoses of cartilage cells are found only in that part of the epiphyseal plate remote from the surface of vascularization. The maturing cartilage cells are displaced by

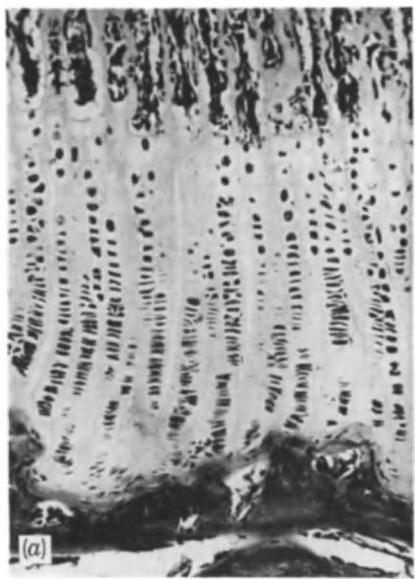
the continued proliferation of cells in this region, and their progress along the cartilage columns of the plate is associated with great increase in size and with thinning of the intercellular cartilage matrix to form thin partitions. Thus the growth of the epiphyseal cartilage plate, in contrast to that of bone itself, is a process of *interstitial* and not *appositional* growth. Under normal conditions there is a parallel between the rate of growth of an epiphyseal plate and its thickness. The rapidly growing plate of a young animal, for instance, is thicker than that of one at a later period when the rate of growth is reduced. The final stage of "maturation" of the cartilage cells which, in mammals, proceeds to their death and disintegration, is associated with calcification of the intervening cartilage matrix. McLean and Bloom (1940), using undecalcified sections where the bone salt is outlined by its reaction with a solution of silver nitrate, have studied details of this calcification, while Follis and Berthong (1949), and Pritchard (1952) have described histochemical changes associated with the maturation of the cartilage cells. The calcified cartilage is almost immediately vascularized from the adjacent metaphysis, and the degenerated cartilage cells completely destroyed. The transverse cartilage partitions, and some of the longitudinal ones, are resorbed in the process, capillary blood vessels and connective tissue cells extending into the cell spaces of the hypertrophic cartilage. The work of Dodds (1932) indicates that osteoclast giant cells play an important part in the resorption of calcified cartilage, although the vascular endothelium and adjacent connective tissue cells also appear to be able to produce this form of chondrolysis.

The open latticework of vascularized calcified cartilage, which is sometimes referred to as the *primary spongiosa*, immediately becomes the site of progressive deposition of bone, this being formed by osteoblasts which develop from connective tissue cells accompanying the blood vessels in their invasion of the cartilage. The continued production of the

PLATE I

- Endochondral ossification. Photomicrographs of the 4 marked areas in Fig. 5.
- (a) Iron hematoxylin ($\times 100$). The lowest of the marked areas, showing the columnar arrangement of the proliferating and maturing cartilage cells of the epiphyseal plate.
 - (b) Eosin-azure ($\times 100$). The next of the marked areas, showing the growing surface of the cartilage plate, and the adjacent *primary spongiosa*. Cartilage matrix stains deeply, while the thin layers of bone which can be seen on the cartilage lattice in the upper part of the field are unstained.
 - (c) Eosin-azure ($\times 100$). The marked field from the central part of the metaphyseal spongiosa. Remodeling has reduced the number of trabeculae present, while the thickness of these structures is being increased through osteoblastic formation of lamellar bone.
 - (d) Eosin-azure ($\times 100$). The highest of the marked areas in Fig. 5, showing conspicuous osteoclast giant cells on the surfaces of bone trabeculae of the metaphyseal spongiosa remote from the cartilage plate.

PLATE I



(a)



(b)



(c)



(d)

primary spongiosa by the growing epiphyseal cartilage plate leaves the resulting bone trabeculae, which show conspicuous inclusions of cartilage matrix, further and further behind the advancing front of vascularization. The *secondary spongiosa*, as the tissue is now called, undergoes extensive remodelling (see Plate I, c and d)* by continued bone formation and osteoclastic resorption as this displacement continues. The longitudinal struts of the primary spongiosa usually correspond closely in number and arrangement with the comparable structures of the non-vascularized part of the cartilage plate. The newly formed metaphysial bone trabeculae of the secondary spongiosa, too, show the same pattern of arrangement at first, but undergo progressive reduction in number as bony remodeling takes place. The present writer (Sissons, 1953) has provided some experimental evidence regarding the duration of the various cellular processes concerned in the formation and maturation of the developing bone trabeculae in this situation. The shaft bone that eventually replaces the metaphysial traveculae is almost entirely devoid of trabecular structures, and in contrast to the *cancellous* bone of the metaphysis it consists of *compact* bone, made up—in most species—of numerous longitudinally-arranged Haversian systems or “osteones” (see Amprino and Godina, 1947, and also Chapter I of the present volume, for structural details of compact bone.)

In many bones, as Lacroix (1949) has described, the periphery of the epiphyseal cartilage plate and of the immediately adjacent metaphysial spongiosa is marked by a cylindrical collar of membrane bone—the *perichondrial ring*. This structure, by continued bone apposition at one margin and continued resorption at the other, keeps pace with the growth of the epiphyseal plate and is seemingly displaced with it relative to the shaft.

The proliferation and growth of the epiphyseal cartilage tissue is the “moving force” of endochondral ossification, on which the longitudinal growth of the whole system depends. This is very clearly shown by the transplantation experiments of Lacroix (1949), where isolated blocks of epiphyseal cartilage, transferred to an extraskeletal site, continued to grow and to undergo “hypertrophy” and actually induced the surrounding connective tissue to vascularize and ossify the *primary spongiosa* so formed. The transplanted epiphyseal cartilage even became surrounded by a miniature perichondrial ring, formed by the adjacent connective tissue. Lacroix’s experiments indicate that the hypertrophic cartilage provides something in the nature of a chemical stimulus to the cells taking part in the succeeding processes of endochondral ossification.

Changes in endochondral ossification in the course of evolution have been reviewed by Haines (1942). He finds the most primitive type of epiphyseal mechanism to be that present in bony fishes, where the

* Plate I, see p. 453.

epiphyses themselves are wholly cartilaginous, but present a "growth zone" consisting of first flattened and then hypertrophic cartilage cells. These changes in the growth cartilage are followed by vascularization from the adjacent shaft, and endochondral bone formation occurs very much as in more highly developed forms. In reptiles, the columnar arrangement of the growth cartilage is more marked, although in these and in other primitive forms endochondral ossification differs from the mammalian pattern in that cartilage columns are eroded and vascularized in groups, the *primary spongiosa* thus corresponding much less accurately in its pattern with the calcified cartilaginous scaffolding which precedes it.

In reptiles and birds, the cartilage of the developing epiphysis is penetrated by conspicuous canals consisting of vascular connective tissue and concerned with the nutrition of the otherwise avascular cartilaginous tissue. The vessels of these canals sometimes communicate with those of the metaphysial bone marrow, but the canals apparently do not persist as permanent structures. In mammals, they are less conspicuous, and disappear after the appearance of bony epiphyseal centers of ossification.

There are a number of detailed studies of endochondral ossification in birds, including those of Lubosch (1924), Fell (1925), and Wolbach and Hegsted (1952). The same general processes found in mammals are seen, but cells of the growth cartilage do not undergo the same degree of "maturation" prior to vascularization, and considerable amounts of cellular cartilage tissue are included in the metaphysial spongiosa prior to its resorption. Indeed, each group of penetrating blood vessels from the metaphysis is lodged in a tunnel surrounded by unresorbed cellular cartilage.

According to Haines, the absence of endochondral ossification in some fish—such as Dipnoi—and in Urodela and Anura, does not indicate a primitive condition, but has occurred as an evolutionary change from precursors possessing this type of bone formation. While secondary centers of ossification are characteristic of mammalian epiphyses, occasional more primitive forms show them. Their absence in no way changes the mechanism of endochondral ossification at the ends of long bones.

3. MEMBRANOUS OSSIFICATION

As seen on the surface of the shaft region of a growing bone, membranous ossification is histologically a far simpler process than endochondral ossification. It involves the direct appositional production of new bone on a pre-existing surface, this, indeed, being the final stage in endochondral ossification. The formation of bone matrix and its calcification, as shown by McLean and Bloom (1940), usually take place

simultaneously. Cell division of the osteoblasts concerned in the formation of membrane bone is not usually conspicuous, but new cells are continually being formed and many of them eventually become included in the lacunae of the bone as mature osteocytes. Osteoclastic resorption of bone (see Plate II, a, b, c) is just as important in the growth of bone formed by membranous ossification as elsewhere. It is of interest that the surface of the actual bone tissue, whether it be the periosteal surface, the endosteal surface, or that in Haversian canals, is involved by successive cycles of bone deposition and bone resorption. The new bone tissue formed in any one of these intermittent periods is marked from the adjacent tissue by *cement lines*, whose outlines thus give an indication (see Petersen, 1930) of the previous cycles of activity of the tissue. In Paget's disease of bone, the very irregular "mosaic" pattern of cement lines is the structural reflection of irregular and intermittent bone formation and bone destruction in this condition. The intermittence of osteoblastic bone formation is also well shown (see Sissons, 1949) by the varying density of newly-formed periosteal bone under certain other pathological circumstances.

Although it does not come within the scope of the present survey of bone growth, it is of interest to remember that in the mature skeleton, the maintenance of normal bone structure depends on a balance between the opposed processes of osteoblastic bone deposition and osteoclastic resorption, and that under pathological conditions departure from the normal structure—as stressed by Baker (1950)—can only be brought about by the mediation of these processes.

4. RADIOACTIVE ISOTOPES AND BONE GROWTH

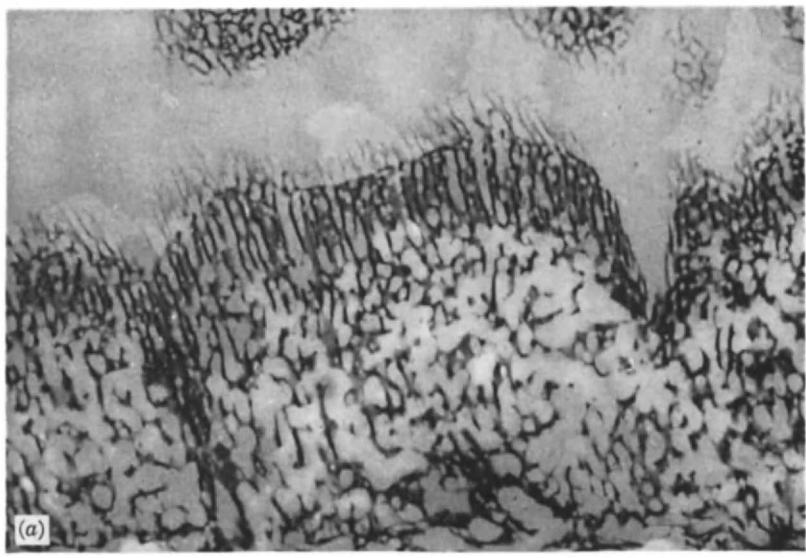
Because of their predominant localization in the inorganic material of newly-formed bone, and the comparative ease with which they can be recognized in autoradiographs (see Bourne, 1952), certain radioactive isotopes, such as Ca^{40} , P^{32} , and Sr^{89} , when administered to young growing animals in doses insufficient to retard growth, can provide the same type of evidence regarding bone growth as did

PLATE II

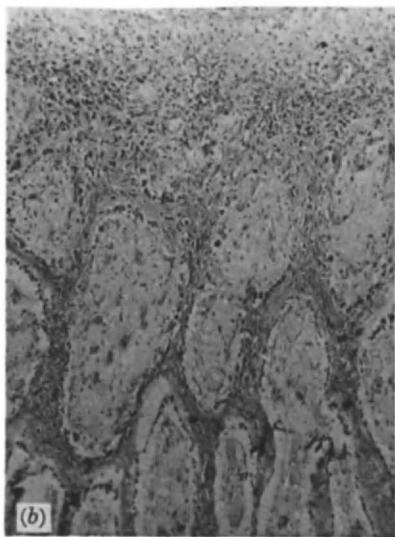
Membranous ossification. Preparations from bone formed by the abnormal displacement of the periosteum of the shaft of a long bone in the dog.

- (a) Microradiograph of a 60μ section showing the general structural pattern of the bone formed on the surface of the shaft under the periosteum, the latter passing across the upper part of the field ($\times 8$).
- (b) A histological preparation from the most superficial, and hence most recently formed, bone tissue. Note the conspicuous osteoblasts on the surfaces of the thin trabeculae of non-lamellar bone ($\times 87$).
- (c) A histological preparation from the remodeled bone from the deeper part of (a). Note the greatly increased thickness of the trabeculae, now of lamellar bone, and the conspicuous osteoclasts present on their surfaces ($\times 87$).

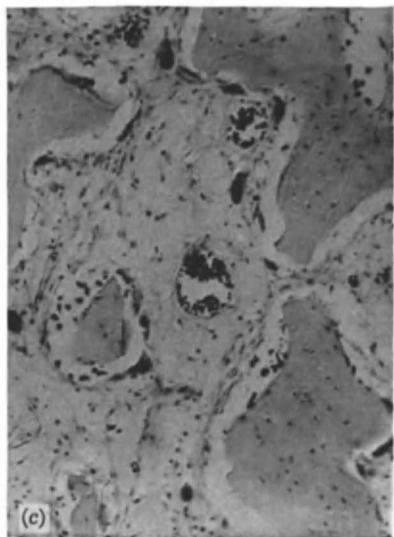
PLATE II



(a)



(b)



(c)

the early madder-feeding experiments of Duhamel and Hunter. That is to say, they can mark the sites of active bone formation at any given moment and allow them to be traced as growth proceeds. The work of Leblond *et al.* (1950) (and see Chapter XII) using P³² in the rat, of Tomlin, Henry, and Kon (1953) using Ca⁴⁵ in the rat, and of Vaughan and her colleagues (Kidman *et al.*, 1952; Jowsey *et al.*, 1953) using Sr⁸⁹ and Sr⁹⁰ in the rabbit, clearly confirms and extends the previously available evidence on longitudinal bone growth in the epiphyseal plate region and on the remodeling in the various regions of the shafts of long bones. In the femur of the rat, for example, the work of Tomlin *et al.* (see Fig. 6) shows that the diameter of the shaft is increased by complete remodeling of the posterior wall, where endosteal resorption completely removes the original cortex as periosteal growth proceeds. Anteriorly the original cortex is retained and is covered over by periosteal and endosteal new bone. See Chapter XII for an account of the use of radioactive isotopes in studying bone growth.

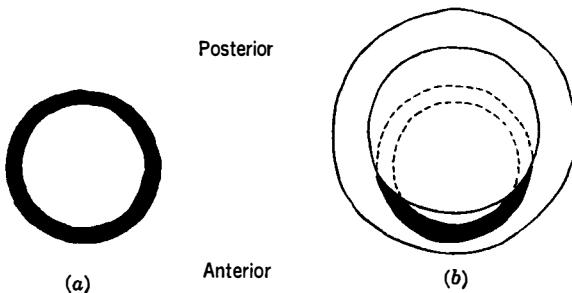


FIG. 6. Growth and remodeling of bone.

- (a) Outline of a transverse section through the mid-shaft of a rat femur at 30 days of age.
- (b) Outline of a similar section at 200 days.

Bone formed prior to 30 days of age (as indicated by autoradiographic studies) is shown in black, and the dotted outline indicates the part of the original shaft destroyed in the remodeling process.

(Drawn from data of Tomlin *et al.*, 1953.)

III. Physiological Control of Normal Bone Growth

It is evident from transplantation studies on isolated bones (Fell and Robison, 1929; Murray and Huxley, 1925; Murray and Selby, 1930; Willis, 1936) and on blocks of epiphyseal cartilage (Lacroix, 1949) that the proliferating tissue of the epiphysis has considerable autonomous powers of growth. In the course of normal development this undergoes important modification from the continued influence of local and general factors. Dietary, endocrine, and other less precisely specified factors all play their part in sustaining or modifying bone growth, and considerable research has been carried out in recent years in an attempt to explain

the precise role of each factor. Information on the necessary physiological conditions for normal growth is not infrequently obtained by study of the changes in growth associated with the absence of a specific factor.

1. DIETARY AND RELATED FACTORS

Normal growth can only proceed under conditions of optimal dietary intake, and departure from this results either in a specific deficiency pattern or in general retardation or cessation of growth, including skeletal growth. It is of interest that, as shown by Smith (1931), skeletal growth can continue under conditions of general restriction of food intake where the total body weight is stationary, or even falling. On the whole, however, changes in skeletal growth parallel those of body weight for a wide variety of dietary disturbances.

The restricted dietary intake and the other metabolic disturbances frequently associated with acute or chronic general illness leave their mark on growth. In 1933 Harris pointed out that factors of this sort resulted in the transient arrest of bone growth with the production of "transverse lines of growth arrest" which were apparent in X-rays of the growth regions of long bones. Subsequent work (Cohen, 1948; Follis and Park, 1952) has shown that the non-specific factors of general illness can influence the processes of endochondral bone formation in a number of ways. If cartilage proliferation and osteoblastic bone formation are equally affected, the result is simple retardation or cessation of bone growth. Cartilage columns are short, and metaphysial trabeculae are few, and a line of arrested growth may not be produced. If cartilage proliferation continues while osteoblastic bone formation is suppressed—and according to Follis and Park such a state of affairs is not infrequent—the primary spongiosa goes on increasing in thickness although it does not undergo replacement by bone, and forms a calcified lattice which may be visible radiologically. If, on the other hand, cartilage proliferation ceases, and osteoblastic bone formation continues, the surface of the cartilage plates becomes covered over with an almost continuous layer of bone of increasing thickness. If growth is later resumed, this dense layer of bone becomes separated from the epiphysial plate by normal trabeculae of newly formed bone tissue, and may persist for a long time as a "transverse line" indicating the previous arrest of longitudinal growth.

In contrast to the pattern of general retardation described above, certain of the cellular processes of endochondral bone growth are more specifically involved in certain vitamin deficiencies.

Here vitamin D is outstanding, the essential skeletal abnormality of rickets being failure of calcification of newly developed tissue (see

Chapter XIX), whether this is the hypertrophic cartilage of the epiphyseal plate or the bone tissue produced in a variety of situations by continuing osteoblastic activity. In the growing epiphyseal plate, vascularization of the uncalcified cartilage fails to occur and the plate becomes irregularly thickened.

In vitamin C deficiency, on the other hand, the fundamental abnormality is failure of bone matrix formation (see Chapter XVIII), the proliferation and calcification of cartilage are not primarily affected, although the formation of bone in the adjacent metaphysis is prevented.

There is some evidence that vitamin A has a specific effect on bone (see Chapter XVII), not only as a necessary factor for growth and maintenance of a normal structure, but also when present in excess. Wolbach (1946) has put forward the view that vitamin A accelerates the histological sequence of endochondral ossification and in particular of bony remodeling in many situations, and he ascribes the skeletal changes of vitamin A deficiency to the retardation of these normal growth processes. He and his colleagues (Wolbach, 1947; Maddock, Wolbach, and Maddock, 1949; Wolbach and Hegsted, 1952; Wolbach and Hegsted, 1953) have demonstrated the effects of excessive vitamin A administration on the growth and structure of bone in a variety of species, including rats, guinea pigs, chickens, and ducks. As well as general retardation of growth, bone rarefaction, sometimes resulting in fracture, is produced. Wolbach consistently interprets the results as "acceleration of all the processes of bone growth," although it is sometimes difficult to attach a precise meaning to this phrase.

2. ENDOCRINE FACTORS

While the role of nutritional factors, including vitamins, appears to lie in providing a background for optimal skeletal growth, hormonal factors exercise a more positive control over the rate of skeletal growth. They are responsible, for instance, for the adolescent changes in growth rate illustrated in Fig. 1, and in particular, influence the pattern of skeletal "maturation"—this term relating to the times of appearance of epiphyseal centers of ossification and the times of cessation of epiphyseal bone growth which in most species is marked by the disappearance of epiphyseal plates. A wide variety of clinical and experimental data indicate the influence of the hormones of the pituitary, the thyroid, the adrenals, and the gonads on skeletal growth and development. Clinical data relating to endocrine abnormalities and their effect on growth are by no means fully understood. This field, however, has recently been reviewed by Wilkins (1953), while Schinz *et al.* (1952) summarizes information on the radiological evidence relating to bone development in a wide variety of disorders of this type. More detailed reference is

made to individual hormones in other chapters of the present volume, while a considerable amount of experimental data has been reviewed by Silberberg and Silberberg (1949) (see Chapter XX) and by Simpson, Asling and Evans (1950) (see Chapter XXI).

For many years, the association of giantism and acromegaly on the one hand, and dwarfism on the other, with pituitary abnormalities have pointed to an important pituitary control of growth. Skeletal growth in pituitary dwarfs is usually normal for the first few years of life, suggesting that in the human a pituitary influence is not essential for growth at this stage. After this, skeletal growth and maturation becomes greatly retarded, the patient showing marked diminution of stature and also evidence of absence of other pituitary factors such as the gonadotrophic hormones. Erdheim (1916) has described some of the structural skeletal changes in pituitary dwarfism. Experimental studies of the effect of hypophysectomy in bringing about cessation of bone growth (Aschner, 1909; Smith, 1926; Dandy and Reichert, 1938; Asling *et al.* 1950; Becks and Evans, 1953) and of pituitary extracts in promoting such growth (Evans and Long, 1921; Ray, Evans, and Becks, 1941; Greenspan *et al.*, 1949) have given rise to the concept of a specific *growth hormone* of the pituitary.

Proliferation of cartilage cells in the epiphyseal plate of the pituitary dwarf, or of the experimentally hypophysectomized animal ceases, and the plate becomes thinner than normal. "Hypertrophy" of the cartilage cells cannot take place, and vascularization of the plate ceases, while metaphysial trabeculae become reduced in number. In experimental animals the structure of the plate resembles that seen when normal cessation of growth occurs, except that the plate persists and the "fusion" of the bony epiphysis with the shaft does not occur. Administration of growth hormone reactivates cartilage proliferation, and the normal sequences of endochondral ossification are resumed.

Hypothyroidism, too, has long been known to produce retardation of bone growth, and delay in the appearance of epiphyseal centers of ossification (see Chapter XII). The hypothyroid dwarf differs from the pituitary dwarf in that the epiphyses that are present usually show a stippled and fragmented appearance known as *epiphyseal dysgenesis* (see Wilkins, 1941). The skeletal abnormalities of clinical hypothyroidism are paralleled by experimental studies in a wide variety of species, where the thyroid has either been surgically resected (see, for example, the results of von Eiselberg, 1894 (goat); Hofmeister, 1894 (rabbit); Simpson, 1924 (sheep); and Ray *et al.*, 1950 (rats)) or destroyed with radioactive iodine (Lusted *et al.*, 1953).

The histological changes in growing epiphyseal plates in the absence of thyroid hormone differ markedly from those described in relation to pituitary growth hormone. From the observations of Langhans (1897),

Looser (1924, 1929) in cretins, and of Hofmeister (1894), Coryn (1938), Silberberg and Silberberg (1943), and Becks *et al.* (1948, 1950) in experimental hypothyroidism, it appears that proliferation of cartilage cells is somewhat reduced, but that the "maturation" of the cartilage cells does not occur and the vascularization of the abnormal cartilage is very much retarded. The cartilage plate becomes thickened, and its calcification irregular.

There is some clinical evidence, such as that of Schlesinger and Fisher (1951), of increased skeletal growth and accelerated skeletal maturation in children in hyperthyroidism or receiving excessive doses of thyroxine. Experimentally, excess thyroid hormone does not appear to be a potent stimulus to bone growth, although a number of conflicting experimental results have been put forward with regard to its effects.

Evans and his colleagues, from an extended series of experimental studies on the effects of thyroxin and pituitary growth hormone on bone growth in rats, which were reviewed in 1950 by Simpson, *et al.*, and again in 1954 by Ray *et al.* suggest—particularly from the results of experiments where these hormones, separately and together, were administered to thyroidectomized-hypophysectomized rats—that the main influence of thyroxine is to advance skeletal maturation, while that of pituitary growth hormone is to stimulate skeletal growth without advancing skeletal maturation.

Gonadal and adrenal hormones also influence skeletal growth. Simpson *et al.* (1944) have shown that testosterone has a stimulating effect on epiphyseal growth, and that its influence is synergistic with that of pituitary growth hormone. It has been suggested (see Wilkins, 1953) that adrenal androgen is important, not only in influencing bone growth at adolescence, but in helping to bring about skeletal maturation in both girls and boys. Experimental studies, however, on the effects of androgens on skeletal maturation are lacking. The administration of large doses of estrogen, in contrast (see Gardner and Pfeiffer, 1943) has a retarding effect on bone growth, and in some species the metaphysial bone formed during the prolonged administration of estrogen is abnormally dense. Certain adrenal hormones, too, have a retarding influence on bone growth. Thus the administration of adrenocorticotropic hormone (ACTH) (Becks *et al.*, 1944) or of cortisone (Follis, 1951a) produce retardation and even arrest of bone growth. Follis has described how, in the rat, the administration of cortisone produces, in addition to retardation of growth, interference with resorption of the resulting metaphysial spongiosa, producing a zone of dense unresorbed bone adjacent to the epiphyseal plate: in other species (Follis, 1951b) only retardation of growth was found. These effects are similar to those pro-

duced by excessive estrogen administration. Sissons and Hadfield (1954) have shown that, even in rat, the administration of cortisone in amounts sufficient to severely retard longitudinal bone growth, is only sometimes accompanied by the production of dense metaphysial bone. The reverse change, i.e. increased *resorption* of metaphysial trabeculae was sometimes found, resulting in a diminution of the density of the metaphysial bone. This change is much more pronounced in rabbits, where under cortisone administration metaphysial trabeculae can be completely resorbed and the thinned epiphysial plate covered over by an almost complete transverse layer of bone.

Cortisone has been shown to have its inhibiting effect on general and skeletal growth in the chick embryo (Karnofsky, Ridgway, and Patterson, 1951) as well as in the intact animal. The developing egg, incidentally, is a particularly convenient system for the investigation of growth inhibiting agents, and a specific effect on skeletal growth and development has been shown to occur with a number of different types of inhibiting substances, when introduced into the egg at a sufficiently early stage of development. Thus Landauer (1947), and later Duraiswami (1950) showed general interference with skeletal growth and development following the injection of insulin. Changes resembling those of achondroplasia have been produced by a number of substances including thallium sulfate (see Karnofsky, Ridgway, and Patterson, 1950).

With regard to endocrines, it can be appreciated that a number of complex interrelationships must be concerned in the control, both normal and abnormal, of bone growth and maturation. Considerable interest at present centers on these interrelationships, and on the exact metabolic systems or endocrine pathways by which any particular hormone influences skeletal growth, although little definite evidence on these topics yet exists.

IV. Bone Growth Under Pathological Conditions

Interference with normal bone growth can usually be related to abnormality of the controlling physiological factors. Thus in the previous section the disordered or retarded growth occurring under a variety of abnormal dietary and hormonal circumstances has been discussed. In the present section, a number of additional causes of abnormal growth are considered, where the factors operating are either extrinsic or related to less obvious controlling mechanisms.

1. GENETIC FACTORS

Apart from their influence in determining general body size, and thus skeletal growth, genetic factors play an important part in determin-

ing the specific growth and development of the skeleton. A number of familial conditions exist, and are inherited in definite Mendelian fashion, where one or more mutant genes is responsible for abnormality of these processes. Some of the conditions involve localized abnormalities of the skeleton, as seen in brachydactyly and syndactyly, while others, such as osteogenesis imperfecta, the osteochondrodystrophies, and multiple exostoses, involve the skeleton as a whole.

A good example of the generalized type is provided by achondroplasia, which has been found by a number of workers (see Cockayne, 1933; Mørch, 1941) to be inherited in man as a single dominant factor. It takes the form of an interference with all the processes of endochondral bone growth, resulting in great shortening of the long bones of the skeleton.

A number of other human bone diseases involving bone growth are thought to be of genetic origin, but information concerning them is not complete. In fact, the detailed genetic study of such abnormalities in man is beset by many difficulties, and the exact influence of genetic factors on skeletal growth can be better analyzed in animal material. Human achondroplasia, for example, is paralleled by similar conditions in cattle, dogs, and rabbits. In the last-named species Brown and Pearce (1945) have not only studied the genetic aspects of the condition (unlike achondroplasia in other species, the condition in the rabbit is recessive) but have described very fully the anatomical changes produced, with particular reference to those in connection with the greatly retarded growth of bone. A number of rather different genetic conditions affecting the development and growth of cartilage have been described in the rat and the mouse (see Grüneberg, 1947), these sometimes resulting in secondary effects on other organs, such as the lungs or the central nervous system, according to the distribution and time of onset of the skeletal abnormalities.

A genetic factor, by interfering with a normal growth process, may actually produce results which add to our understanding of the process itself. This appears to be the case with the mutant gene in the mouse known as "*grey lethal*," which was described by Grüneberg (1935). Its skeletal effects have been summarized by him (1947) and have more recently been described in some detail by Bateman (1954). These authors suggest that, in addition to a number of other effects, the "*grey lethal*" gene specifically interferes with osteoclastic resorption of bone and thus allows continued accumulation of bone at the sites where such erosion normally occurs. In the abnormal animals the external contours of most bones are changed, and metaphysial trabeculae persist in what would normally be the marrow cavities of the shafts of long bones. Bateman is aware that complete dependence cannot be placed on an argument

which seeks to establish the sites of bony erosion by an assumption that this process only, is affected by the genetic mechanism under consideration, but the reported correspondence between the sites of excessive bone formation in the "*grey lethal*" animals on the one hand, and the known sites of osteoclastic erosion in normal animals on the other, makes the hypothesis an interesting and profitable one. It is, moreover, in keeping with the abnormal osteoclasts described by Barnicot (1947) in "*grey lethal*" mice.

It has been assumed that any genetic factors influencing skeletal growth do so by directly affecting some metabolic or enzymic process in the developing skeletal tissues. But this is not always the case, the interference with skeletal growth sometimes being secondary to an effect on the hormonal environment of the developing organism. A case in point is the condition of hereditary pituitary dwarfism in the mouse (see Grüneberg, 1947), where the primary genetic effect is thought to involve the anterior lobe of the pituitary which fails to develop normally. Other endocrines such as the thyroid show marked hypoplasia, which is presumed to be the result of absence of pituitary hormones. About 16–18 days after birth, bone growth becomes severely retarded and skeletal maturation progressively delayed.

3. MECHANICAL FORCES INFLUENCING BONE GROWTH

The role of pressure and tension in determining bone structure through influence on its cellular processes of growth has long been debated, and the subject has been well summarized by Murray (1936). Under normal circumstances intrinsic factors are of overwhelming importance in determining the form of bones. Even when, as described by Triepel (1922a, b) for the human femur, the bone structure is such that its mechanical properties are precisely suited to the forces to which it is subjected, the mechanical forces play only a reinforcing role in determining the normal structure. Wolff's law (1899), a generalization relating the structure of a bone to the stresses and strains acting on it, while valid, is not to be regarded as wholly mediated by adaptive changes to mechanical forces. But under abnormal circumstances, as shown, for example, by the studies of Weidenreich (1922) on the effect of disuse on the bones of the foot, changes in external forces may have important effects by influencing the slow process of bony remodeling in the parts of the skeleton concerned.

The evidence relating to the effect of pressure on the growing epiphyseal plate itself is of a different kind. In experiments designed to alter the naturally occurring stresses of weight bearing, and to determine any resulting differences in skeletal growth, Fuld (1901) using dogs, and Colton (1929) using rats, made these normally quadruped animals

assume a bipedal habit by amputation of the forelimbs soon after birth. Relatively slight changes, if any, in the absolute or relative lengths of the bones of the hind limbs were obtained. The reason for this is clear from more direct investigations of the effects of pressure on developing epiphyseal plates. Thus Strobino, French, and Colonna (1952) in experiments where the tibial epiphyseal plates in growing calves were compressed by a system of pins and springs, showed that forces of 500–560 lbs. (equivalent to pressures of 40–45 lbs/square inch on the epiphyseal plate itself, and thus outside the limits of physiologically sustained forces) were required to measurably retard longitudinal growth of the plate.

Such compression of epiphyseal plate tissue is not infrequently used, clinically, when it is desired to stop the increase in length of a growing bone. Steel staples are inserted round the periphery of the epiphyseal plate in order to rigidly unite the bony epiphysis to the metaphysis. The experimental results cited above are in keeping with clinical experience in this respect, for it is known (see Blount and Zeier, 1952) that the growing epiphyseal plate can straighten and break steel staples which have been inserted across it, unless these are sufficiently strong and numerous.

3. OTHER PHYSICAL AGENTS INFLUENCING BONE GROWTH

A number of experimental procedures have demonstrated that the cells of the growing epiphyseal plate are more sensitive to a variety of damaging agents than those of the adjacent bone. The effect of local cold (Scow, 1949), or of the raised tissue temperature associated with the dissipation of ultrasonic vibration in bone (De Forest *et al.*, 1953), is to cause selective damage to the epiphyseal plate tissue, with premature fusion of the plate and arrest of growth. The same destruction of the epiphyseal plate tissue, with retardation or complete arrest of growth, sometimes follows physical trauma as in a fracture, or in experimental damage (see Imbert, 1951) to the same region.

Ionizing radiations are important damaging agents to the growing epiphyseal plate, as has been known since the observations of Perthes (1903), Tribondeau and Recamier (1905), and Forsterling (1906), on the effects of X-rays on the growth of long bones. These observers noted stunting of growth following irradiation, while later workers have studied the relationship of dosage to growth retardation, and have described the cell changes produced in the growing region after irradiation. From the work of Dahl (1936), of Spangler (1941), and of Barr, Lingley, and Gall (1943), it appears that a dose of between 1800–2600 r of X-rays is required to completely and permanently arrest epiphyseal growth. From some limited growth data provided by Dahl (1936), and

by Regen and Wilkins (1936), it would appear that with doses of this order some growth occurs in the days following irradiation, but that growth completely ceases within 10–20 days. With smaller doses of X-rays (see Hinkel, 1942, 1943; Reidy *et al.*, 1947; Heller, 1948) temporary retardation, and not complete arrest of growth is produced, growth at a normal rate being resumed after an interval of from 2 to 10 weeks.

In 1920, Segale reported some histological observations on irradiated bones, and described, in rats killed 8 days after irradiation, irregular outline of the various zones of the epiphyseal cartilage plate. The columnar arrangement of cartilage cells was lost, and their nuclei were degenerated or pyknotic. Subsequent histological studies (Dahl, 1936; Bisgard and Hunt, 1936; Gall, Lingley, and Hilcken, 1940; Hinkel, 1943; Barr, *et al.*, 1943; and Heller, 1948) indicate that such degenerative changes of cartilage cells, with associated changes in the intercellular matrix, can be produced by doses as small as 300–400 r. Gall *et al.* (1940) comment that even with a small dose of 600 r, histological evidence of damage persists for as long as 19 weeks—a surprising finding in view of the much shorter period of growth retardation which was noted above as a finding of several workers.

Post-irradiation damage is a condition where—as in rickets and hypothyroidism—the normal relationship between rate of growth and epiphyseal plate thickness does not hold. The irradiated plates, during their period of retarded growth, are irregularly thickened, sometimes to as much as twice their normal width. Thus it is clear that irradiation has interfered with the cellular processes of cartilage vascularization and resorption, as well as with cartilage proliferation and maturation.

Ionizing radiations from “internal” sources following the administration of radium, or radioactive isotopes such as Sr⁸⁹, which selectively localize in the skeletal tissue, produce similar effects to external sources of irradiation (Heller, 1948). Disproportionate damage to epiphyseal plate tissue is often produced because of the selective localization in the newly formed calcified cartilage and bone in this region. Longitudinal bone growth can be temporarily or permanently arrested, metaphysial trabeculae destroyed, and the continuity of structure between the metaphysis and the epiphyseal plate interrupted, as shown in Fig. 7 (ii). Cartilage cells show the same degenerative changes as were described following irradiation from external sources: osteoblasts are affected more frequently because of the high local dosage attained on the bone surfaces. Resumption of longitudinal growth may occur after variable periods of time, but the original site of the damaged epiphyseal plate is usually marked by a transverse zone of devitalized bone and cartilage which becomes separated from the epiphyseal plate by the subsequently formed bone (Fig. 7 (iii).)

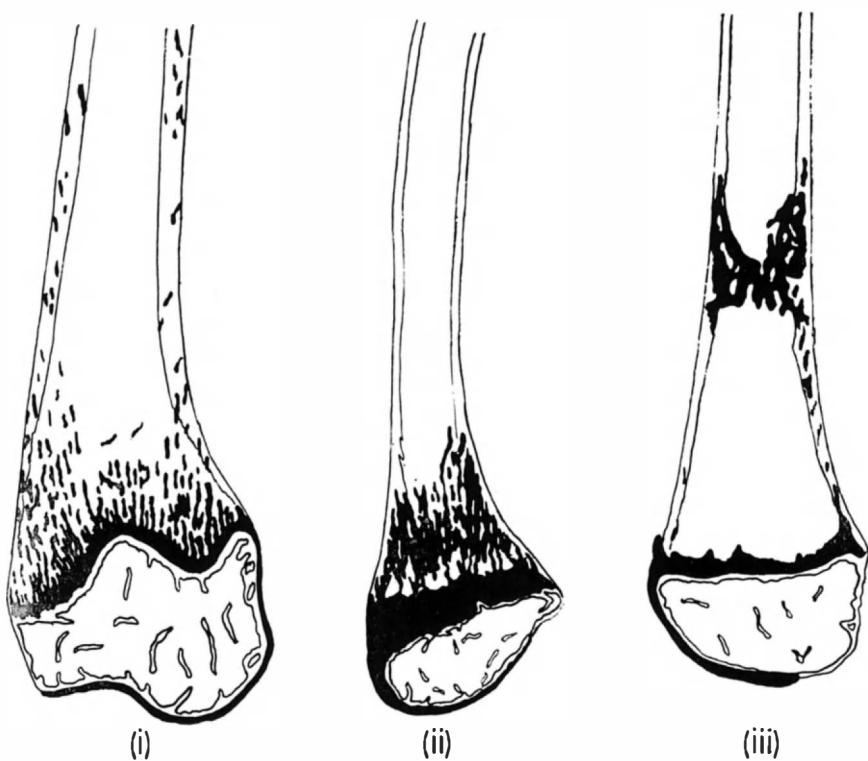


FIG. 7. Effect of "internal" irradiation on bone growth ($\times 11$).

- (i) Longitudinal section through the growing region at the lower end of the femur in a normal rat.
- (ii) A section through the same region in an animal 11 days after administration of $2.9 \mu\text{c/g}$ of Sr^{89} . Note the increase in thickness of the cartilage plate, and the resorption of metaphysial trabeculae adjacent to the plate surface.
- (iii) A section through the lower end of the femur in an animal 5 months after the administration of $2.9 \mu\text{c/g}$ of Sr^{89} . The site of the growth zone at the time of injection is marked by an area of devitalized bone and cartilage some distance along the shaft.

(Figures redrawn from Heller, 1948.)

4. VASCULARITY AND BONE GROWTH

One would expect that a significant alteration to the vascularity of the metaphysial surface of the growing epiphysial plate would produce an alteration in its rate of growth. A number of experimenters have attempted to demonstrate effects of this sort (Ollier, 1867; Johnson, 1927; Ferguson, 1933; Wu and Miltner, 1937) by ligating or otherwise destroying the nutrient vessels of a long bone, but with surprisingly little effect. After the usual procedures the circulation is so quickly reestablished through collateral pathways in the shaft that the vascularity of

the growing tissues is not affected. Similarly, clinical attempts to influence peripheral circulation and bone growth by sympathectomy (Harris and McDonald, 1936; Barr, Stinchfield, and Reidy, 1950) have had little result. Recently, however, Trueta (1953) has described the effects of interfering with the circulation at the growing end of a long bone by permanently blocking the medullary cavity of the shaft. Using rabbits, he finds such a procedure to result in *increased* growth of the epiphyseal plate, and argues that an increased collateral circulation through vessels entering the bone in the metaphysial region is responsible by bringing about an *increase* in vascularity of the metaphysial surface of the plate. Such a mechanism awaits direct demonstration, but Trueta is able to make a comparison between his experimental results and the occasional association of bony overgrowth with osteomyelitis of long bones. He points out that in this condition bony overgrowth occurs only in those cases where the shaft—but not the epiphyseal plate itself—is involved, and where a mass of new bone has obstructed the shaft vessels. The same factor, he suggests, operates occasionally following the fracture of a long bone during the period of growth, also resulting in temporary overgrowth of the affected bone.

5. MISCELLANEOUS FACTORS INFLUENCING BONE GROWTH

The growing tissue of the epiphyseal plate is occasionally obliterated by extension to it of an adjacent inflammatory lesion in osteomyelitis. The same cessation of growth and premature fusion of the epiphysis to the shaft, which was described as resulting from trauma and other physical damage, then occurs.

Under circumstances of prolonged immobilization of a limb, particularly where some other pathological process such as joint tuberculosis is also at work, or where the limb is completely paralyzed as in poliomyelitis, the same type of premature epiphyseal arrest may occur (Gill, 1944; Kestler, 1947; Ross, 1948; Parke, Colvin, and Almond, 1949). The resulting structural changes have been recorded by the present writer (Sissons, 1952) but little is known about the precise factors interfering with the function of the epiphyseal plate under these circumstances.

Scoliosis is another condition where epiphyseal bone growth, this time in vertebral bodies, is abnormal. Here, too, little is known of the factors responsible. The wedged vertebrae of advanced cases are clearly produced by unequal epiphyseal growth, but despite some experimental studies—such as that of Bisgard and Musselman (1940)—on the production of scoliosis, it is not known whether the bony changes are primary or the result of forces influencing the bone from without.

6. ENDOCHONDRAL OSSIFICATION APART FROM CIRCUMSTANCES OF BONE GROWTH

A number of pathological conditions are characterized by the occurrence of active endochondral ossification under circumstances where it is not usually found. The best example of this is to be seen in the callus which develops in response to a fracture. The callus consists, in part, of cartilage produced by proliferation of osteogenic cells and by the differentiation of granulation tissue which forms in the damaged area, and this cartilage undergoes the usual sequences of calcification and endochondral ossification resulting in its replacement by bone. The same general factors would appear to influence the endochondral ossification of fracture callus as control this process in normal bone growth. Thus Blunt *et al.* (1950) and Sissons and Hadfield (1951) have shown that cortisone administration inhibits cartilage proliferation and vascularization in developing fracture callus very much as it does in the normal epiphyseal cartilage plate.

The tissue of some cartilage tumors, too—particularly those of slow growth—is found to undergo the usual sequences of calcification and endochondral ossification, its vascularization and bony replacement being effected by non-neoplastic stromal tissues. Other instances of this not infrequent behavior of abnormal cartilage are provided by osteoarthritis—where bony replacement of articular cartilage occurs—and acromegaly—where there is reactivation of endochondral ossification, as shown by Erdheim (1931) and by Waine, Bennett, and Bauer (1945), in the normally quiescent cartilage of ribs.

V. Conclusions

Certain generalizations are possible from the work surveyed in previous sections of the present account. It is evident, for example, that widely contrasting histological appearances of the epiphyseal plate and adjacent metaphysial tissues are found under different conditions of growth retardation. It would further appear that these structural differences can be explained according to the particular stage or stages of the sequence of cellular events of endochondral bone growth that are affected. Thus a number of different factors including pituitary growth hormone, dietary intake, and mechanical compression, primarily influence the proliferation of the epiphyseal cartilage itself. Variations in growth rate produced by these factors are reflected in the thickness of the epiphyseal plate, an increased rate of growth being associated with a thickened plate, and retarded growth with a thinned plate, in each case without other important histological abnormality. Such a relationship between rate of growth and plate thickness does not, however, apply

to the effects of irradiation, vitamin D deficiency, or thyroid deficiency, the retardation of growth produced by these agents—which influence the maturation and vascularization of the epiphyseal cartilage as well as its proliferation—being associated with a plate of increased thickness. Excessive amounts of cortisone or estrogen, as well as vitamin C deficiency, affect the metaphysial bone trabeculae as well as influencing epiphyseal cartilage proliferation.

It can be seen that the subject of bone growth, although one where the broad outlines are known, still poses many unsolved problems. It is anticipated that information from a number of diverse fields, including histology and comparative anatomy, endocrinology, and pathological anatomy—as well as from experimental studies, will all contribute to a better understanding of the processes concerned.

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CHAPTER XVI

REPAIR AND TRANSPLANTATION OF BONE

A. W. HAM and W. R. HARRIS

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I. The Repair of Bone

1. INTRODUCTION

The succession of histological changes that occur in and about a fracture, and which eventually result in its healing, are described in almost every textbook of pathology and surgery. It might be expected that by this time the accounts of bone repair in these books would be as uniform, simple, and accurate as, for example, the accounts they contain of the process of acute inflammation. But the accounts of bone repair are not uniform; indeed, many are bewilderingly involved, being attempts to reconcile the many (and often irreconcilable) controversies that have arisen in this field over the last one hundred years. Accordingly, in a

book such as this, some attempt should be made to explain the reasons for the present-day confusion about this important matter as well as to deal with the process of bone repair in a fairly comprehensive fashion.

2. DEFINITION OF TERMS AND DESCRIPTION OF STRUCTURES TO BE DISCUSSED

A long bone characteristically has a tubular form. It consists of bone tissue. Its exterior, except at articulating surfaces, is *covered* with a membrane, the *periosteum*, and its cavity is *lined* with another, the *endosteum*.

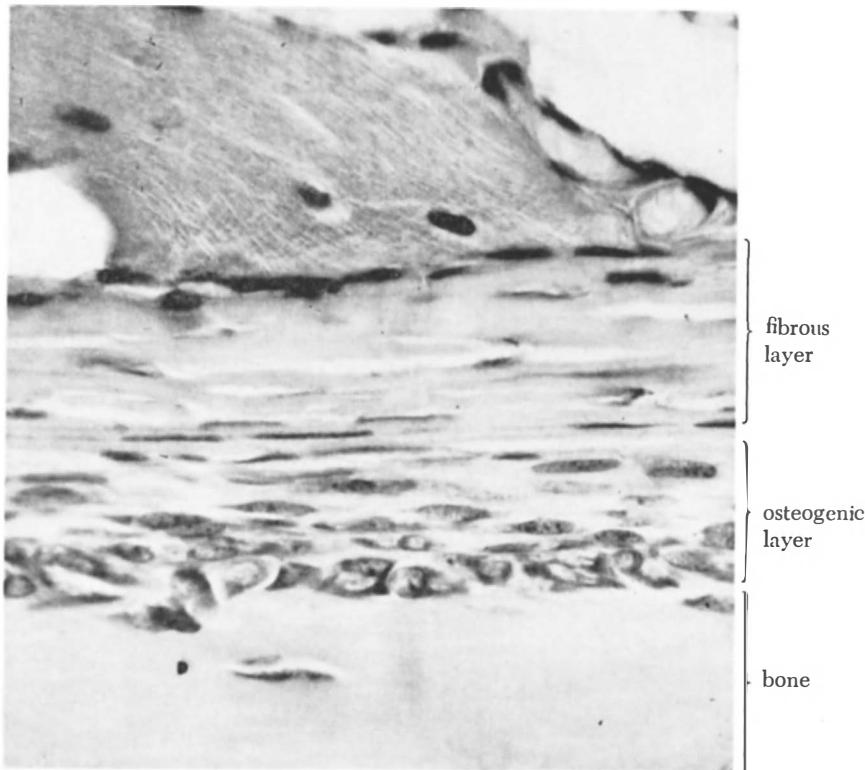


FIG. 1. High-power photomicrograph of section of rabbit's rib showing active periosteum. Muscle fibers, taking origin from the fibrous layer of the periosteum, may be seen above.

a. *The Periosteum*

This membrane has two layers. The outer consists chiefly of collagenic fibers, which are woven into a tough membrane (Fig. 1). The cells that are sparsely scattered throughout this membrane have flat, thin, dark-staining nuclei and it is generally agreed that at least most of them are fibroblasts (Fig. 1).

The inner layer of the periosteum is applied directly to the surface of the bone. In the bones of the young this layer, in contrast to the outer layer, is cellular. When growth is over, however, this layer becomes less noticeable and in the bones of the old it may consist of so few cells that it cannot be identified as a separate entity.

In young growing bones, as noted before, the inner layer of the periosteum is so cellular that it is easily recognized. The cell types that the periosteum contains manifest themselves clearly if the periosteum is stimulated by an injury as, for example, in Fig. 1. In the upper part of this illustration a few muscle fibers are seen taking origin from the fibrous layer of the periosteum. Between the fibrous layer and the surface of the bone, two types of cells can be seen. The first type is fusiform in shape and in contrast to the fibroblasts of the fibrous layer cytoplasm as well as nuclei may be seen in them. These cells are generally termed *osteogenic* cells for reasons that will be explained later. All transitions are to be seen between these and cells of a second type that are disposed directly against the bone. These are *osteoblasts* and are characterized by large amounts of basophilic cytoplasm. Although some are somewhat fusiform in shape, most are generally nearly as wide and thick as they are long. They commonly demonstrate prominent Golgi images (Fig. 1). The osteoblasts seen in Fig. 1, have not yet begun to deposit new bone intercellular substance on the shaft of bone that they cover, but their appearance suggests that it would not have been very long before they would have begun surrounding themselves with new bone intercellular substance and so adding to the diameter of the shaft.

The inner layer of the periosteum, which in a stimulated state contains both osteogenic cells and osteoblasts, is generally termed its *osteogenic layer* because it is the layer that generates bone. This layer is also called the cambium layer by some authors.

A controversial problem must now be mentioned. It has already been pointed out that all transition stages between osteogenic cells and osteoblasts can be seen in the inner layer of stimulated periosteum. With the light microscope what appear to be transition stages between the fibroblasts of the outer layer and osteogenic cells of the inner layer, can sometimes also be seen in stimulated periosteum. The question then arises as to whether or not the fibroblasts of the outer layer are the mother cells of the osteogenic cells of the inner layer which, in turn, are the mother cells of the osteoblasts that abut on the bone.

In considering this question it should be pointed out that the conclusion that the fibroblasts are the mother cells of the osteogenic cells is not the only one that could be drawn from observing what appear to be transition stages between fibroblasts and osteogenic cells in growing periosteum. That the osteogenic cells form fibroblasts rather than the reverse,

seems to us to be a much more logical view, and that they may do so, and thicken the fibrous layer of the periosteum in reparative processes, seems very probable. That the osteogenic cells of the periosteum are, in a sense, the mother cells of the membrane as a whole and can give rise, on occasion, to fibroblasts on one side, and osteoblasts on the other, seems to us to be a much more acceptable hypothesis than the one that postulates the very differentiated-appearing fibroblasts of the outer layer to be the mother cells of all types in the membrane.

b. *Bone Tissue*

The substance of bone, apart from the membranes that cover and line it, consists of two components, intercellular substances and cells. The more abundant of these is the former and it consists of non-living organic material which, under normal conditions, is impregnated with mineral salt. The second component of bone substance consists of cells termed *osteocytes*; these are scattered through the intercellular substance in little spaces known as *lacunae*. All of the osteocytes that are in bone were once osteoblasts situated on a bony surface and there they surrounded themselves with intercellular substance and became buried in the material they produced. Although osteocytes are entombed in rock-like intercellular substance they do not die, as might be expected, because they receive a meager diet through tiny canaliculi that permeate the calcified intercellular substance. The canalicular mechanism, however, is a very inefficient one for bringing nutrition to cells, and taking waste products away from them, and accordingly it does not function effectively over more than a very short distance, hence all osteocytes must be situated perhaps no more than one tenth of a millimeter from a capillary, if they are to remain alive (Ham, 1953). That arrangements could be made throughout the substance of dense bone to satisfy this requirement must certainly have challenged Nature's ingenuity, but the challenge was met by having compact bone built up in units termed *Haversian systems* (described in detail elsewhere in this book). These permit osteocytes to be close to capillaries by having the limited number of successive layers of bone of which each is composed arranged around the innumerable longitudinally disposed capillaries that permeate the substance of compact bone. In these layers of bone substance that are disposed around the central blood vessel of each system the osteocytes lead a precarious existence. It does not take much imagination to visualize that any interruption of the blood supply in a Haversian system would soon lead to the death of the cells in the layers of bone that surround the vessel of that system. An appreciation of this fact makes it understandable why so many osteocytes on each side of a fracture line die when the Haversian vessels are ruptured by

a fracture, and why, moreover, the cells of a compact bone transplant die soon after the transplant is cut.

c. *Endosteum*

The marrow cavity of a long bone is lined by a single layer of contiguous cells which vary from osteogenic to osteoblastic in type. These cells are descendants of the osteogenic cells in the periosteal bud which originally invades the cartilage model of the bone-to-be. Cells of a similar nature line the Haversian canals of compact bone. In Haversian systems that are in the course of construction the lining cells are osteoblastic in type but in systems that have been completed the lining cells may be of a flattened osteogenic type. The endosteal cells that line Haversian canals are descendants of the osteogenic cells of the inner layer of the periosteum because it is from the periosteum that Haversian systems are added to the exterior of a bone as it grows in width (Ham, 1953).

d. *Marrow*

The cavity of a long bone is filled with bone marrow; this may be fatty or red depending on the species of animal, its age, and the particular bone being studied. Marrow begins to form in the cartilage model of a bone soon after it is invaded by a periosteal bud, and it is obvious that the cells that are in the bud, and which can form bone, can also form marrow. Although the lines of differentiation that develop in bone marrow are designed to provide for the production of reticulo-endothelial phagocytic cells, and for various kinds of blood cells, the mother cells from which these types arise retain their potentiality to form osteogenic cells as well. Accordingly, as we shall see, the undifferentiated cells of bone marrow can participate in bone repair and indeed can be an excellent source of bone-forming cells (Fig. 5).

e. *Fragments and Callus*

When a long bone is broken into two parts each part is thereafter called a *fragment*. The new tissue that forms at and around the site of the break to join the two fragments together again is said to be the *callus* or *callus tissue*. In successful repair the callus at first is generally composed of both cartilage and bone but sometimes only of bone, and eventually it consists only of bony tissue. In unsuccessful repair the callus may be composed of dense fibrous tissue (fibrous union). The callus, in a successful union, is sometimes considered to have two parts, an internal and an external callus. The former term refers to the callus tissue that develops between the marrow cavities of the two fragments. The external callus refers to the mass of new tissue that forms around the exterior of the two fragments in the region of the break. Some authors also describe a *temporary* callus and a *permanent* callus. These terms, in our opinion, are not justified.

Having now defined and described the various structures with which we must deal in describing the healing of a fracture, we shall now describe the changes that occur in them on succeeding days after a bone is broken. For our example of a fracture we shall choose the rib of the rabbit. This reason for selecting this bone is that a rabbit's rib can be fractured without breaking the skin; this obviates complications induced by operative wounds, saw cuts, sutures and possible infections. Furthermore the fractured rib does not require splinting. There are, however, certain disadvantages about basing too general conclusions on the sequence of events that are seen in the healing rabbit's rib as will be pointed out later.

3. THE REPAIR OF A FRACTURE

a. *Immediate Effects of a Fracture*

The shaft of a long bone contains in its substance almost innumerable longitudinally disposed small blood vessels. These Haversian vessels, at the site of a break, are all torn. Furthermore, although it is conceivable that in some fractures in which the bone is cracked, but the periosteum is not torn, that some of the larger vessels in the periosteum and marrow cavity that cross the line of fracture, may remain intact, many periosteal and marrow vessels are torn in every fracture, and probably all those periosteal and marrow vessels that cross the line of the fracture are torn in most fractures. The tearing of the Haversian, periosteal, and marrow vessels at the line of fracture has two important effects; first, the vessels bleed for a time, and secondly, the circulation stops in them back to sites where they anastomose. We shall consider the second of these effects first.

b. *Effects of Cessation of Circulation in Torn Vessels*

Most Haversian canals contain only a single vessel and so when the flow of blood in one is interrupted, the osteocytes in the Haversian systems, previously supplied by the vessel, die back to the site where the vessel anastomose with some other vessel in which blood is still circulating. The number of anastomoses between adjacent Haversian vessels does not seem to have been investigated to any extent and it probably varies a good deal in different bones and in animals of different species. In the rabbit's rib, dead bone, as indicated by the lacunae which formerly contained osteocytes becoming empty (Figs. 2 and 4), can be observed for distances ranging up to a millimeter or more from the site of the fracture. The line between living and dead bone, which is seen in each fragment some distance from the site of the fracture is a most irregular line. This is probably to be explained by the fact that the vessels in different Haversian systems anastomose at different sites along their

course, with some having anastomoses closer to, and others more distant from, the line of the fracture.

The tearing of periosteal vessels causes local damage in the periosteum but may also assist in causing the death of bone because the Haversian vessels are supplied by branches from these vessels. The tearing of marrow vessels, and subsequent cessation of circulation in them causes some death of marrow tissue on each side of the line of fracture (Fig. 2).

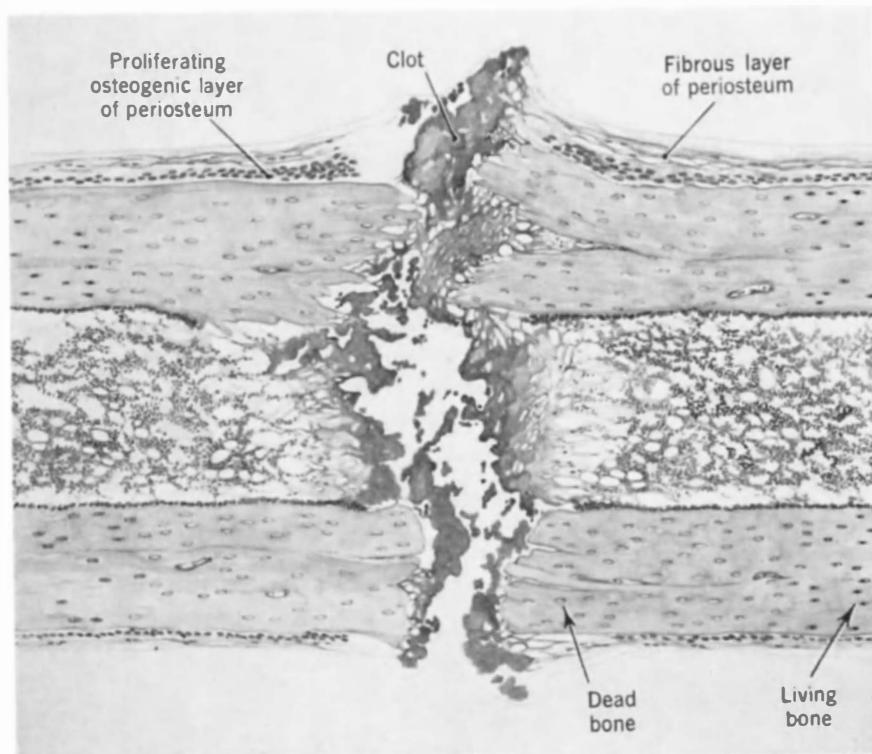


FIG. 2. Drawing of the microscopic appearance of a longitudinal section cut through a fractured rabbit's rib in which healing had taken place for 48 hours. The drawing was made sufficiently out of perspective to permit both a considerable area and cell detail to be shown.

c. *The Role and Fate of the Blood Clot*

The torn vessels bleed into and about the fracture area and this results in a blood clot forming between the fragments and around the site of the fracture (Figs. 2, 4, and 5). The function and fate of the blood clot will now be discussed in detail.

In most textbook accounts of fracture healing, the blood clot is given an important role. Although various accounts differ considerably, most suggest that the first, and an extremely important, step in the healing of a

fracture, is the replacement of the blood clot by granulation tissue, and that this is followed by a second step in which there is some sort of mysterious transformation of the granulation tissue into a cartilaginous or bony type of tissue so that a true callus is formed.

Figures 2, 4, 5, and 6 were prepared to show the fate of the blood clot in healing fractures of the rabbit's rib. Figure 2 shows a clot 2 days after a fracture and at that time it can be seen that there is no sign whatever of the clot being invaded by granulation tissue. Figure 4 shows that at 5 days, although great waves of new callus tissue are sweeping toward the break from the osteogenic layer of the periosteum, the clot for the most part is still as it was in the beginning. Some proliferating cells are beginning to invade the clot where it projects into the muscle but the new callus tissue, *which by this time has become cartilaginous, is not invading the clot*, but pushing the clot ahead of it, with a sharp line of demarcation between it and the clot, as it advances toward the break (Fig. 4, sharp line of demarcation). At the end of a week there is still no sign of the clot being invaded except by cells from the marrow (Fig. 5) and even at the end of 2 weeks, when callus tissue, developed from the osteogenic layer of the periosteum of both fragments has swept from one fragment to the other to achieve union, there is no sign that the invasion of the clot by granulation tissue, and its subsequent conversion into osteoid tissue, has had anything to do with the formation of the external callus.

It seems extraordinary that observations on the blood clot in the healing rabbit's rib could be so at variance with the usual textbook account of fracture healing. There are, however, some reasons for this state of affairs and they will now be discussed.

First, a good many of those who describe the healing of a fracture must surely reason to some extent by analogy. Anyone familiar with the phenomenon of the organization of fibrin by granulation tissue, and its importance in the healing of certain types of injury to ordinary connective tissue, would be disposed to assume that a similar phenomenon was an essential step in the process of bone repair. Secondly, something comparable does occur in the region between the two marrow cavities of the fragments. The clot in this site may be invaded by undifferentiated marrow cells and blood vessels (Fig. 5). This is not an important factor in the healing of most fractures but the fact that something comparable to the organization of fibrin does occur in this site is probably just enough to perpetuate the concept of the organization of the blood clot being the first, and a very important step, in the repair of a fracture. Thirdly, in fractures of cancellous bone, repair may be chiefly effected by osteogenic and marrow cells growing into and through a clot; this, however, does not give rise to a temporary callus, which must undergo a transformation

into a cartilaginous or bony type of tissue; instead it is osteogenic from the beginning and from the beginning constitutes permanent callus.

To sum up: The study of the healing of a fractured rabbit's rib gives no indication that the replacement of the blood clot by granulation tissue is an early and important step in the formation of the external callus. Actually, as Figs. 4 and 5 show, the blood clot for the most part is a bystander rather than a participant in the process, and a bystander who seems to be in the way of the participants at that. And even in fractures of cancellous bone where blood clots are invaded to a greater extent by the cells that will bring about repair, there are no mysterious transformations of granulation tissue in the clot because the cells that invade it are osteogenic from the first, and form bone just as they would if there had been no clot to impede their progress.

Having considered the role of the blood clot, we shall now study the source and nature and growth of the tissue that becomes the callus that repairs a broken bone.

4. THE ORIGIN, GROWTH AND NATURE OF CALLUS TISSUE

In the following account we shall describe, from what may be seen in longitudinal sections cut through healing fractured ribs of rabbits, the changes that occur around a fracture and which result in the formation of a proper callus and hence in the repair of the bone.

a. *Changes in the Osteocytes of the Fragments*

The study of sections made 2 or 3 days after a bone has been fractured show clearly that all the osteocytes near the line of the fracture are dead. The evidence for this is that the lacunae that contained them become empty except for a little debris (Figs. 2, 4, and 5). In some instances the osteocytes become rounded and dark staining before they dissolve.

Even if the osteocytes near the fracture line did not die it is difficult to see how they could escape from their prisons and participate in the repair of bone. Yet years ago, following Macewen's work, it was argued that the cells of bone were largely responsible for the repair of bone. This view persisted a long time and perhaps still persists to some extent and so we shall briefly discuss it.

Since a microscopic examination of healing fractures provides such convincing evidence that the osteocytes near the line of fracture all die, and since they could scarcely escape from their prisons and contribute to bone repair even if they lived, the evidence submitted as proving that bone cells contribute to bone repair must obviously have been of the most indirect kind. Actually, at first, it depended on investigators seeming to prove that periosteum was not osteogenic and so it was concluded that callus tissue was derived from the bone itself. As this matter was

further investigated, it became apparent that the callus tissue that formed to repair a fracture did not come from osteocytes along the line of the fracture, but from what these investigators considered to be the bone cells that were directly under the periosteum. But the membrane that these investigators termed periosteum is the membrane that histologists consider as only the fibrous layer of the periosteum and the bone cells to which these investigators attributed repair are the cells that histologists

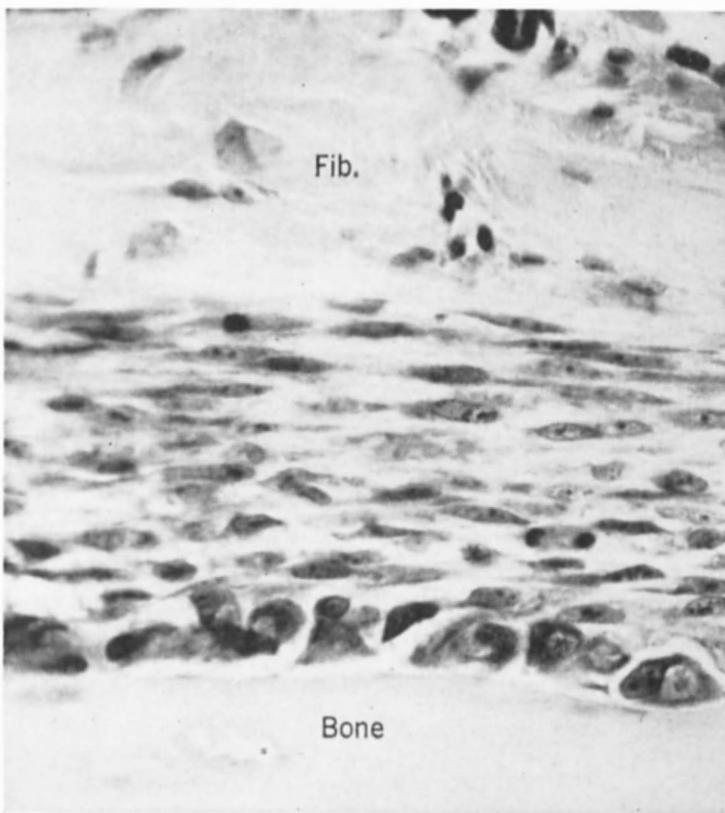


FIG. 3. High-power photomicrograph showing the thickened and proliferating osteogenic layer of the periosteum near the fracture, 24 hours after it occurred. Three mitotic figures may be seen among the osteogenic cells. The osteoblasts that are applied to the surface of the bone have prominent Golgi images but have not yet deposited new bone intercellular substance on the shaft. (Fib. = fibrous layer of the periosteum.)

describe as those of the osteogenic or cambium layer of the periosteum. For anyone who is interested in this controversy, which persisted for a long time, accounts of various stages are given by Macewen (1912), Keith (1919), Gallie and Robertson (1920), and Ham (1930, 1934). The controversy was perhaps useful because it helped elucidate the source

of callus tissue, but it was harmful in that it created a problem in terminology which has only ceased to be a nuisance in recent years.

b. *Periosteum and the Formation of the External Callus*

The periosteum is commonly torn at the site of a fracture and most of its cells die for a short distance back from each side of the break. As soon as 24 hours after a fracture, however, signs of activity are to be observed in the living periosteum a little farther from the line of fracture (Figs. 2 and 3). This activity is always greater on the side of the rib that abuts on the chest muscles than on the side that faces the pleural cavity, and it will now be described.

Figure 3 illustrates what may be seen in such a site only 24 hours after a rib is broken. It will be observed, first, that there is no sign of activity in the fibrous layer of the periosteum; this layer, however, is being lifted away from the bone by the activity in the osteogenic layer of the periosteum. Here, the cells are actively dividing by mitosis (three mitotic figures are to be observed in this figure), and becoming larger. For both these reasons the osteogenic layer of the periosteum is much thicker than it was before the fracture.

Although cell proliferation is the prominent feature at this time, signs of cell differentiation are also to be seen. The cells that directly abut on the bone have become plump, deeply-stained osteoblasts with prominent Golgi images, and are all prepared to begin surrounding themselves with intercellular substance and so forming bone which will be deposited on the surface of the fragments that they cover.

Over the next few days the osteogenic cells continue to proliferate and this results in a collar of callus tissue being formed around each fragment, close to the site of fracture (Fig. 4). That this collar arises from the osteogenic cells of periosteum is easily seen by the fact that the outer and unchanged fibrous layer of the periosteum is lifted farther and farther away from the bone by it. The first sign of differentiation of the osteogenic cells of the collar into osteoblasts has already been described and as early as 2 days after the fracture a deposit of new bone can sometimes be seen to have been added, more or less evenly, to the outer surface of the fragment in the deepest part of the collar and at some little distance away from the fracture line. But, although the first bone that is deposited on the fragments is generally a thin and more or less even layer, that which is subsequently added on the following days is irregular, being arranged in the form of trabeculae, with vascular spaces between them (Fig. 4). This latter bone is formed because further osteogenic cells of the collar differentiate into osteoblasts and these, in turn, become osteocytes.

The collars continue to enlarge rapidly in size because of the continued proliferation of the osteogenic cells of which they are primarily

composed. We have already noted that in the deepest part of the collar, osteogenic cells become osteoblasts and form bone. But farther away from the original fragment, out in the rapidly growing collar, the osteogenic cells differentiate, not into osteoblasts, but into chondroblasts and these, in turn, soon form cartilage. Accordingly, the deepest part of the collar becomes bone; the next layer outward becomes cartilage; and the most superficial layer of the collar (except for the fibrous layer of the periosteum) continues to be composed of rapidly growing osteogenic cells (Figs. 4 and 5).

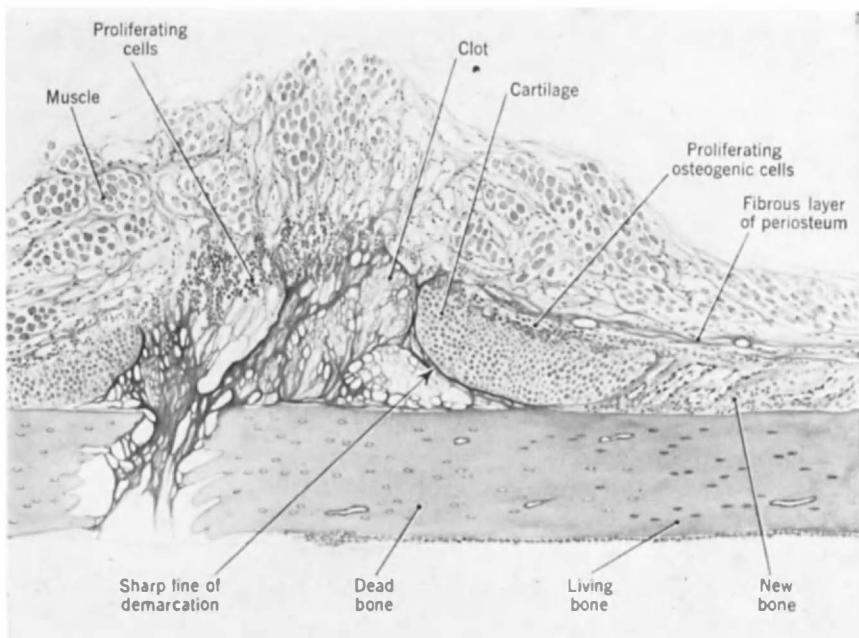


FIG. 4. Drawing of the microscopic appearance of a portion of a section of a fractured rabbit's rib that had healed for 5 days. The cells of the thickened osteogenic layer of the periosteum have formed both cartilage and bone and have lifted the fibrous layer of the periosteum away from the bone. Notice the sharp line of demarcation between the cartilage and the clot.

It should be observed that there is no sharp line of demarcation between the cartilage and the bone that forms in the callus (Fig. 5, lower right), they merge imperceptibly into each other, as was described by Ham in 1930. This is a very different appearance from that seen when bone is replacing the cartilage of the callus. (Fig. 6); in the latter there is a sharp line of demarcation, as will be explained later.

The development of cartilage in a callus should not be regarded as a curious phenomenon; it should be expected. The osteogenic cells of the periosteum of a long bone, which give rise to the callus, are direct

descendants of the cells that once comprised the chondrogenic layer of the perichondrium of the cartilage model of that bone, and it is not surprising that they should again manifest their capacity to form cartilage when they subsequently are called upon to participate in the repair of a fracture. As Ham pointed out in 1930, the osteogenic cell has a dual potentiality, it can form either bone or cartilage, and which it forms depends primarily on the environment in which it differentiates. If osteogenic cells differentiate in a vascular environment they form bone, but if they differentiate in a non-vascular environment they form cartilage,

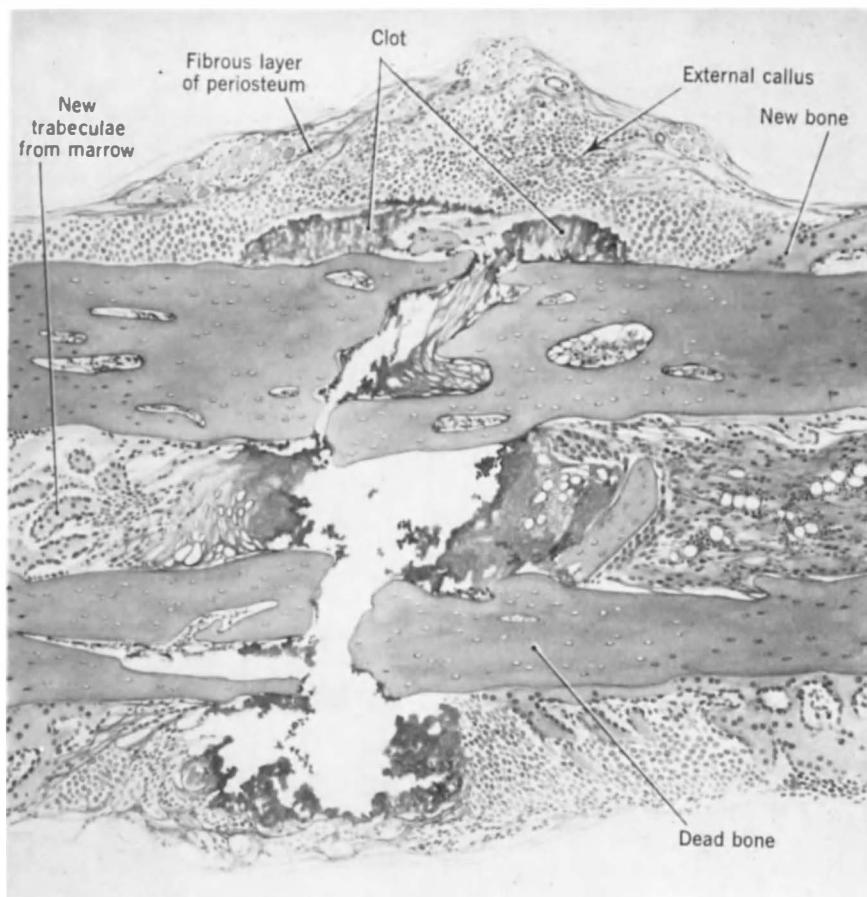


FIG. 5. Drawing of part of a section cut from a fractured rabbit's rib that had healed for one week. The osteogenic cells from the two collars have met above but they have not yet invaded or organized the clot. Some marrow cells are beginning to invade the clot at the left.

and if they differentiate in an environment which is neither one nor the other, they form a tissue that has some characteristics of both bone and cartilage.

The reason, we think, for the osteogenic cells forming bone in the deeper part of the callus is that blood vessels in this area are present and able to keep up with the growth of the osteogenic cells. But, as the osteogenic cells proliferate very rapidly in the outer aspect of the callus and advance toward the line of fracture, they outrun the blood vessels and hence must there differentiate in a non-vascular environment, and so they form cartilage (Figs. 5 and 6).

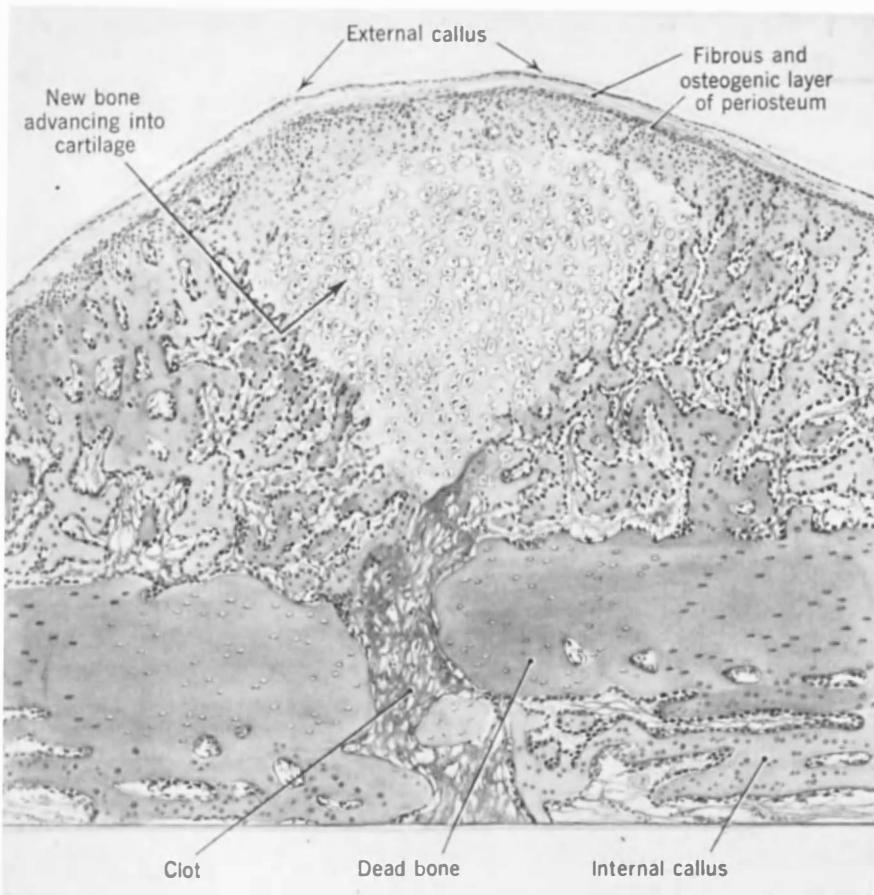


FIG. 6. Drawing of part of a section cut from a fractured rabbit's rib that has healed for 2 weeks. This drawing illustrates the external callus to advantage and shows that the cartilage in it is being replaced by bone along a V-shaped line. Some clot, still unorganized, can still be seen.

The presence of cartilage in callus tissue has been explained in other ways; one theory is to the effect that it forms where there is some movement. It seems to us that the rate at which a fracture heals is a very important factor as to whether cartilage will or will not be present in a

callus. If the callus grows slowly, so that blood vessels can keep up with the osteogenic cells, cartilage formation should be minimal. But, when the callus grows rapidly, as it does in the repair of a rabbit's rib, the osteogenic cells outdistance the capillaries and so produce considerable amounts of cartilage.

In the earlier stages of repair, it is clear and definite that the collars of callus that form around the two fragments have their origin from the osteogenic layer of the periosteum. But, as the collars continue to grow and approach one another, there is histological evidence for another possible origin of callus tissue. The fibrous layer of the periosteum fades out toward the summit of each collar, and the declivity between the two collars, which is therefore not separated from muscular tissue by a distinct fibrous layer of periosteum, is invaded by large numbers of cells the origin of which is somewhat obscure (Figs. 4 and 5). One view is that they are osteogenic cells that have emigrated from the collars, another is that they are fibroblasts from the connective tissue elements of the muscular tissue that abuts on this region. Since proper callus tissue (cartilage and/or bone) later appears in this site (Fig. 6), we must therefore ask whether or not fibroblasts, from the muscular tissue, can participate in building callus and form cartilage and bone.

There are many different views on this matter. Some authors see no fundamental distinction between the inherent abilities of fibroblasts and osteogenic cells to form bone and believe that the environment rather than the cell is the important factor in determining bone formation (Leriche and Pollicard, 1928; and Bertelsen, 1944, both review this matter). Others consider that osteogenic cells have an inherent tendency to form bone but that fibroblasts also can be induced to do so by certain as yet not clearly understood factors. We shall discuss this matter further under the heading "Bone Induction."

Fibroblasts and osteogenic cells, we think, are different from one another. We think they represent two closely related but different families of mesenchymal cells. Osteogenic cells, we think, are disposed to give rise to intercellular substance of a cartilaginous or bony type, while fibroblasts are disposed to give rise to the intercellular substances of ordinary connective tissue. As we shall discuss later, there is little evidence to show that ordinary fibroblasts are as capable of forming bone as readily as osteogenic cells. But we are not sure that in association with rapidly growing osteogenic cells, fibroblasts cannot change into osteogenic cells for reasons which we shall now outline.

Years ago it was more or less assumed that the nuclei of different kinds of cells in an animal body were different from one another so far as their genetic constitution was concerned. In recent years opinion has swung more to the view that the cells of an animal body have identical

nuclei but that cells are different from one another, and maintain these differences on reproduction, because of cytoplasmic factors. Of the cytoplasmic factors which might be responsible for imparting specific characteristics on cells, the ergastoplasm, which contains so much of the ribonucleoprotein of the cytoplasm, would seem to be the most likely suspect. The problem now arises as to whether or not contiguous actively growing cells of two closely related types, such as fibroblasts and osteogenic cells, could "infect" one another with their ergastoplasm so that fibroblasts, for example, would take on the nature of osteogenic cells; this to us seems to be much more of a possibility than that of fibroblasts spontaneously becoming osteogenic in type. Accordingly, while we do not think that fibroblasts by themselves are disposed to form bone, we should not be inclined to exclude the possibility of their becoming osteogenic when they grow in close contact later with osteogenic cells, particularly in the declivity between the two collars as the calluses approach one another.

As repair continues the two collars enlarge in size and approach one another (Figs. 4 and 5). Their enlargement is occasioned by the continuing proliferation of their osteogenic cells and to some extent also by proliferation of the younger cartilage cells they contain. The continued increase in the amount of substance in the collars causes them to advance toward one another because they are both fixed to the original fragments by new bony trabeculae at sites some little distance from the fracture line and so any enlargement of them between the points where they are fixed and the fracture line leads to them bulging toward the fracture line as well as outwardly. As has already been noted, the declivity between the collars, around the time when they are meeting and fusing, may be filled in with a mixture of fibroblasts from adjacent muscular tissue and emigrating osteogenic cells from the collars (Figs. 4 and 5), and this mixture of cells may assist in filling in the declivity between the collars with osteogenic tissue.

As has already been noted, there is no sharp line of demarcation between the cartilage and bone that first forms in the collars (the external callus) (Fig. 5, lower right), and the reasons for this have been explained. But, when the cartilage that has formed in the callus begins to mature, a sharp line of demarcation does appear between the central mass of cartilage and the new bone that lies to each end of it. In longitudinal sections cut through fractures the line of demarcation between cartilage and new bone is V-shaped as may be seen in Fig. 6. This V-shaped line of demarcation between cartilage and new bone represents the line along which the cartilage is becoming calcified and replaced by bone. The reason for calcification occurring along this V-shaped line is the proximity of the blood vessels in the spaces between the new bony

trabeculae, to the cartilage (Fig. 6). Along this line the cartilage cells presumably secrete phosphatase which brings about the calcification of the intercellular substance that surrounds them. This shuts the cells off from nutrition so they die and disintegrate leaving spaces into which capillaries and osteogenic cells from the new bony trabeculae penetrate. As the cartilage disintegrates, new bone is formed on such calcified remnants of it as remain, and so the new bony trabeculae, which shortly replace the cartilage, generally have cartilage cores. In short, the sequence of events that occur along the V-shaped line is almost identical with the sequence of events that occur on the diaphyseal side of an epiphyseal plate of a growing bone. Indeed, it seems probable that the wedge of cartilage may, because of interstitial growth within its substance, contribute to the extent of the bony callus as a whole, in the same way that an epiphyseal plate contributes to the growth of a bony diaphysis in length. And just as occurs in the epiphyseal plate of a long bone when growth is over, the cartilage of the external callus, under normal conditions, is all replaced by bone, when the growth of the callus is over.

The *internal* callus forms (1) from the endosteum of the marrow cavity, (2) to some extent from the endosteum of Haversian canals, and (3) from the undifferentiated mother cells of the marrow. As may be seen in Fig. 2, the endosteum begins to thicken 2 days after the fracture. At the end of a week some new bone has been formed by the endosteum from the marrow cavity and from the undifferentiated cells of the marrow (Fig. 5). The new bone from the latter source appears in the form of trabeculae, which may be seen to each side of the fracture line in the marrow cavity. These trabeculae are often continuous with trabeculae that have formed from the endosteum lining the marrow cavity. In some instances examples of marrow cells growing out into the clot between the two fragments can be observed (Fig. 5). Eventually trabeculae from each fragment meet and join with one another. In the rabbit's rib the internal callus is much less impressive than the external; this, however, may not be true of all bones and it is quite possible that in some bones and under certain circumstances the internal callus may be of greater importance than the external.

c. Remodelling of the Callus

By the processes that have been outlined the two fragments become firmly joined by an external callus of trabeculated bone and by an internal callus of trabeculated bone. The trabeculae in both calluses are joined to both living and dead portions of the original fragments (Fig. 6). This, although it provides a strong union, is not Nature's ideal, and a remodeling of the callus now takes place. In the remodeling process

the dead portions of the original fragments are slowly resorbed. As they are resorbed more and more trabeculae develop in this region and some of the trabeculae of the external and internal callus are resorbed. Then, when all the dead bone of the fragments is resorbed, the spaces between the trabeculae that have taken its place, are filled in by means of appositional growth, and so the cancellous bone that is in a direct line between the bone of the two fragments, gradually becomes converted to compact bone. When this occurs there is no further need for all the former trabeculae of the external callus or all those of the internal callus, and so many or most of these, under ideal conditions, are resorbed, and all that remains to show that a fracture has occurred in this region is slightly thickened and somewhat irregularly formed, compact bone in the shaft at the site of the original break (Fig. 7), and perhaps some trabeculae in the marrow cavity (Fig. 7).

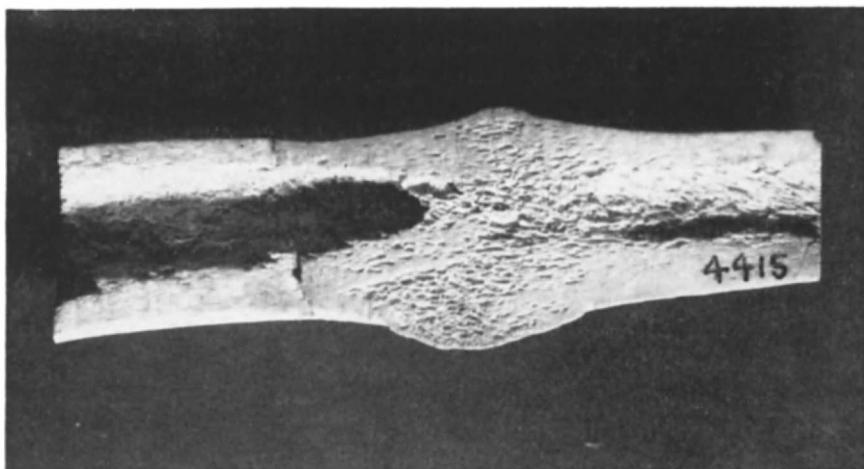


FIG. 7. Photograph of an old healed fracture. Specimen courtesy Department of Pathology, University of Toronto.

d. *The Healing of Fractures in Which Only One Fragment Remains Alive*

Fractures of the neck of the femur are of great interest for many reasons. They are of great clinical importance. To the student of bone they offer an example of a fracture in which only one fragment remains alive (for commonly the fragment to which the head of the femur is attached is cut off from its blood supply) and, in addition, they also offer an example of the healing of a fracture of a bone that is chiefly cancellous in type.

The healing of fractures of the neck of the femur has been investigated at length and in detail in dogs by Tovee and his associates (in press). In his experiments the neck of femur, close to the head, was cut

through at open operation. The head was then lifted free to make sure that it was separated from all blood supply. It was then placed back in position and pinned in place. After varying lengths of time, ranging from 2 days to 2 years, the specimens were recovered. The pins were then removed and the material was sectioned. It was the privilege of one of us (A. W. H.) to study most of the sections obtained. The general sequence of events, as indicated by the study of sections of specimens healed for different lengths of time, will now be described.

The bone and marrow of the heads die in a few days' time. The cells of the articular cartilage, however, which presumably are nourished from synovial fluid, generally remain alive. If good apposition between the fragments is obtained, a growth of osteogenic cells and capillaries, from the marrow and endosteum of the trabeculae of the living fragment, soon creeps over the line of fracture, and new trabeculae are formed in the dead fragment. These new trabeculae become cemented to the dead trabeculae of the head. Gradually the osteogenic and undifferentiated marrow cells, and the blood vessels from the living fragment, invade farther and farther into the dead head, replacing the dead trabeculae with new ones, and the dead marrow with new marrow. Under ideal circumstances, all the dead bone of the head and all the dead marrow are eventually replaced with new, and since the articular cartilage generally remains alive the histological picture of a fractured head, in which repair is excellent, is almost identical with that of a normal head.

e. *Healing of Fractures of the Parietal Bone*

In contrast to fractures of long bones, those of the parietal bone do not heal nearly so readily by bony union. Pritchard (1946) has investigated this matter in detail in rats. Our experience has been chiefly limited to the repair of drill holes of various diameters made through the parietal bones of rats. The sequence of events seen is similar qualitatively to that seen in the healing of long bones in that humps of bony callus develop from the osteogenic cells of the periosteum around the periphery of the drill hole. If the drill hole is of a small diameter the humps of callus tissue spread over it fuse, and so bony union is obtained. If, however, the drill hole is of a greater diameter the humps of callus tissue that form do not spread over the hole and so the central part of the hole is not repaired by bony union. In general, the growth of callus tissue is not so luxuriant as it is in fractures of long bones and it seems to be with great difficulty that growths of callus tissue from one side of a fracture ever meet and fuse with those from the other side.

It is of interest that in one animal Pritchard (1946) found that cartilage formed in the callus in the repair of the parietal bone. This

finding is illuminating because it shows that the osteogenic cells of the periosteum of these bones, which form in membranous environments and never pass through a cartilaginous stage, have the capacity to form cartilage should they proliferate in an environment conducive to leading differentiation along this line.

f. Non-union and Non-calcification

Before briefly discussing the problem of non-union of fractures we should like to emphasize that osteogenesis and calcification are different phenomena. Generally, under normal conditions, ossification—the formation of the peculiar organic intercellular substance of bone by osteoblasts—is followed by the calcification of the newly-formed intercellular substance. Since the two phenomena commonly occur almost simultaneously radiograms are commonly used to follow the course of osteogenesis in the repair of fractures. It perhaps should be appreciated that conclusions drawn with regard to osteogenesis by this method depend on the assumption that calcification is normal and it may not be normal under certain conditions.

For example, in 1938, Ham, Tisdall, and Drake, showed that fractures in animals, which were on a diet that deranged their calcium and phosphorus metabolism healed normally as judged from histological sections. But when radiograms were taken of these fractures they revealed little callus and hence the radiograms would normally be interpreted as indicating non-union; that is, that a bony callus had not formed. But when similarly treated animals were then put on a proper diet, radiograms showed that a callus appeared with extraordinary rapidity. The fact is, of course, that a bony callus was already present (as was proven by the histological sections) but it was uncalcified, and its sudden appearance in the radiograms was due to its becoming calcified rapidly when the mineral metabolisms of the experimental animals became normal. Accordingly, radiograms do not always give an accurate indication of whether or not osteogenesis has occurred.

If the two fragments of a fracture do not eventually become united by a bony callus, non-union is said to have occurred. In long bones non-union occurs when the two collars of osteogenic tissue from the osteogenic cells of the periosteum do not bulge toward each other and fuse, or when the growth of endosteum and undifferentiated marrow cells from the marrow cavities of the two fragments do not come together. There can be many reasons for non-union. In the bones of the old, for example, there may not be enough osteogenic cells in the periosteum to provide sufficiently abundant collars of callus, and the small collars may, as it were, grow tired before they meet and fuse. Anything that delays the growth and fusing of the collars encourages non-union. In this respect

it seems also that Nature in a sense is more or less content with any kind of repair and if fibroblasts by themselves invade the fracture area and repair the fracture with fibrous tissue, the stimulus for osteogenic collars to push the fibrous tissue aside and bring about bony union is probably not great. It is to be noted that bone transplants are commonly used in cases of non-union for they *stimulate* osteogenesis and *conduct* it from one fragment to the other. Thus they remedy the chief two defects that can cause non-union which are (1) a lack of osteogenic cell growth, and (2) the growth not being conducted from one collar to the other.

5. SUMMARY

The account given in the preceding pages of the sequence of events that occur to repair a fracture is based on what may be observed in histological sections of a standard type of fracture that has healed for different lengths of time and the observations made may be verified easily by anyone who makes a similar study. And, anyone who makes a similar study, will, we think, be as impressed as we are with the fact that the callus tissue, which repairs a broken bone, originates primarily and chiefly from the *osteogenic cells that normally cover and line bone surfaces*. These cells respond rapidly when a fracture occurs and they behave very differently, we think, from ordinary fibroblasts, in that they show an inherent disposition to form bone and cartilage. They are assisted in their work by undifferentiated cells from the marrow and perhaps they are also able, as callus growth is well advanced, to convert such fibroblasts as intermingle with them, to their own type. But there is no doubt that the osteogenic cells that normally cover and line bone surfaces are the cells to which the responsibility of repairing fractures has been allotted and if they are not present to do the work, or if they fail to produce a sufficiently substantial growth, non-union will result except in those fractures in which marrow cells play a very important role.

Bone repair, according to the views expressed above, is a simple and easily understood process. We might, therefore, ask why so many accounts of the process are so curiously complicated. One reason is that far too much stress is generally placed on the blood clot and its organization. Much of this emphasis, we think, is due to reasoning from analogy instead of from direct observations. Stressing the role of the blood clot has led to the concept of a temporary callus of granulation tissue being formed as a first step in the repair of a fracture. Having obtained this temporary callus of granulation tissue, many authors do not seem to know what to do with it. Some thereafter disregard it, some say it is replaced by true callus, and some visualize it as going through

a metamorphosis into true callus. All this, we think, is misleading. Our observations indicate that the blood clot has almost nothing to do with the formation of the external callus; if anything, it seems to be in the way of the cells that are trying to repair the fracture. And, if blood clot in the marrow cavity is invaded, in connection with the formation of internal callus, it is invaded by osteogenic cells which form true callus and not a temporary one that must later be replaced or changed. Another point on which accounts of fracture healing are often unduly complicated relates to whether fibroblasts or special osteogenic cells are chiefly responsible for callus. The fact that bone occasionally develops in various sites in the body by metaplasia seems to be sufficient evidence for some authors to assume that any fibroblast is as competent as forming cartilage and bone as the special cells that cover and line bone surfaces. There is no justification for this assumption and here again anyone who studies the repair of fractures under the microscope will have no doubts about the special role in the repair process of the cells that normally cover or line bone surfaces. Some authors, to complicate matters further, term these cells fibroblasts; this is not justified for they are a special breed of cells.

II. The Transplantation of Bone

Bone transplants are commonly used to fill gaps and defects in bone, to facilitate the healing of fractures that otherwise would not heal, or in which healing would be greatly delayed, and to bring about a bony ankylosis in joints damaged by disease.

The surgical use of bone transplants dates from a dramatic result obtained by Macewen of Glasgow in 1880. He transplanted tibial bone to a humerus, the shaft of which had been largely destroyed by osteomyelitis, and found, in due course, that the shaft of the humerus became restored.

Following Macewen's clinical and experimental work it was generally assumed that bone, transplanted from one site in the body to another, would not only continue to live, but would give rise to new bone in its new location. Accordingly it was believed that a bone transplant acted as a true graft, and so operations involving the transplantation of bone became known as bone-grafting operations.

As further work was done in this field it was shown that bone transplants cut from one individual and transplanted to another, often produced excellent results. It was also shown that bone obtained from other species and transplanted into man could at least sometimes achieve good results. It became apparent, moreover, that boiled bone could sometimes be used to advantage. These findings strongly suggested that the usefulness of a bone transplant was not entirely due to its functioning as a

true graft. Accordingly, innumerable histological studies were made on bone transplants to see whether they really lived or not. Unfortunately many of these experiments were of the sort that did not permit the histological appearances of the transplanted bone to be followed day by day. Instead, in many, transplantation was effected, and then at some very much later date, sections were cut from the site to which the bone had been transplanted. The finding of living bone at this later date was often taken to indicate that the transplant had lived. The net result of all these studies was that a good many surgeons continued to believe, and some still believe, that transplanted bone lives in its new site, and hence that bone transplants serve as true grafts.

The problem of whether bone transplants are true grafts or not is complicated further by the fact that bone transplants consist mostly of intercellular substance which, of course, is non-living material. If some of the intercellular substance of a transplant persists, and becomes incorporated in new bone at the site to which it is transplanted, does it mean that the transplant is a true graft—even if all of the cells of the transplant die? We think not; that a transplant can only be considered to be a true graft when living cells of the transplant live and flourish in a new site. Accordingly, whether bone transplants are true grafts or not should be decided on whether or not their *cells* continue to live and flourish after transplantation. We shall therefore examine the question of whether or not the cells of bone transplants continue to live and flourish. But before doing so we shall briefly discuss different types of transplants.

Bone transplants can be classified two ways.

First, they may be composed of either compact or cancellous bone. Compact bone transplants may be used in the form of solid pieces and of a substantial size or in the form of chips. Cancellous bone is commonly used in the form of chips. It should be noted here that compact transplants taken from young growing bones may consist of bone that is more of the nature of cancellous bone than compact bone (Ham and Gordon, 1952).

Secondly, bone transplants are classified as to whether they are cut from the individual into which they are being transplanted (auto-gogenous), or from another individual of the same species (homogenous) or from animals of another species (heterogenous).

Before discussing whether or not the cells of any of these different kinds of transplants live, some of the problems associated with homogenous and heterogenous transplants will be discussed.

It is common knowledge that except in the instance of identical twins each member of the human race is generally so different from all other members, that the tissues of one, transplanted to another, serve as

antigens. Almost invariably the antigens incite reactions which result in the destruction of the living cells of homogenous transplants. The same phenomenon occurs, even more emphatically, in the attempted use of heterogenous transplants. Yet both homogenous and heterogenous bone transplants have been employed with seeming success. How is this to be explained?

The immune responses which are generated when homogenous and heterogenous transplants of any type are made, react against the cells of the transplant and eventually destroy them. The amount of reaction that is incited by transplanting intercellular substances from one individual or from one species to another is probably not so great as that which is incited by living cells and in any event the reaction cannot act to kill the intercellular substance because it is non-living. Accordingly the usefulness of homogenous and heterogenous bone transplants is not due to a survival of their cells—for these would die as they would in transplants of any other homogenous and heterogenous tissue—but to the fact that they consist chiefly of non-living intercellular substances which cannot be killed by immune bodies. That their intercellular substance may persist for a time, however, does not justify the conclusion that homogenous and heterogenous transplants are true grafts. It does, however, establish another. The fact that transplants of homogenous *compact* bone may seem to be almost as useful as transplants of autogenous *compact* bone, indicates that the chief utility of a *compact* bone transplant is not due to a survival of its cells but rather to the character of its intercellular substance. It should be pointed out that the utility of homogenous and heterogenous transplants of cancellous bone is not as great as that of autogenous bone for reasons which will be explained presently. The evidence already given does not prove that there is no cell survival in autogenous transplants and to investigate this matter we must depend on evidence obtained from histological studies. These will now be described.

1. AUTOGENOUS TRANSPLANTS OF COMPACT BONE

In considering whether or not cells of an autogenous bone transplant survive it is important to distinguish between (1) *osteocytes*, which, as has already been explained, are buried in intercellular substance, and meagerly nourished through canaliculi, and (2) the *osteogenic cells*, which cover and line the surfaces of bone.

It has already been explained that the osteocytes of compact bone near a fracture line always die, because circulation stops in the Haversian blood vessels on which they depend for nourishment. When a transplant is cut from adult compact bone circulation stops in all the Haversian vessels of the transplant, and it takes weeks to months for new vessels

to grow into the Haversian canals of the graft from the host tissue into which it is inserted. For any appreciable number of osteocytes of the transplant to survive would require that diffusion phenomena operate successfully in bone for relatively great distances and there is no evidence indicating that they do (Ham, 1953). There is, moreover, no histological evidence to indicate that an appreciable number of the osteocytes of a compact bone transplant survive because histological sections show that the lacunae of a transplanted piece of compact bone soon become empty, or contain pyknotic nuclei. A few osteocytes near surfaces may appear healthy, and may perhaps survive for a time, but for the most part, the lacunae of a compact bone transplant that has been in place for a few days resemble those of compact bone near the line of a fracture.

The osteogenic cells that cover and line the surfaces of a transplant of compact bone, however, may survive and give rise to new bone. They do not all, or always, do so, but instances of survival and growth, particularly of osteogenic cells of the periosteum of a compact bone transplant, are commonly seen, in sites where the periosteum of the transplant is adequately nourished by tissue fluid. The amounts of new callus tissue provided by survival and growth of cells that cover and line the transplant of compact bone, however, is not nearly so great as that provided by the cells that cover and line the surfaces of the bone into which the transplant is inserted. It should be appreciated that when a bed is cut in any bone for a transplant, the osteocytes along the line of the cut all die for the same reason that osteocytes near a fracture line all die. Furthermore, even if a transplant is not inserted into a newly-cut bed, the covering and lining cells of the recipient bone and the undifferentiated marrow cells that are available all react to the cutting of the bed just as they do to a fracture, by beginning to form callus tissue (Fig. 8). If a transplant is inserted into the bed the callus tissue spreads over to the transplant and fixes it in place (Fig. 8). As noted before the covering and lining cells of the transplant may act as a minor source of callus.

After, or even before, the callus tissue unites the transplant and the host bone, the Haversian canals of the transplant are slowly invaded by blood vessels and osteogenic cells from host. Two processes occur in these canals, resorption and new bone formation. Canals become widened in some sites and narrowed in others, depending on which process is more active. As these processes continue the transplant comes to consist of an interwoven mixture of dead bone and new bone, firmly cemented together (Fig. 8). Since new bone formation does generally occur in the transplant as rapidly as resorption, the transplant, for a time at least, may appear almost cancellous in type. Eventually, however, it is completely, or almost completely, replaced with new bone, and so it becomes compact in type.

From the foregoing it may be concluded that although some covering and lining cells of an autogenous transplant of compact bone may survive and even help to contribute to the callus which cements the transplant

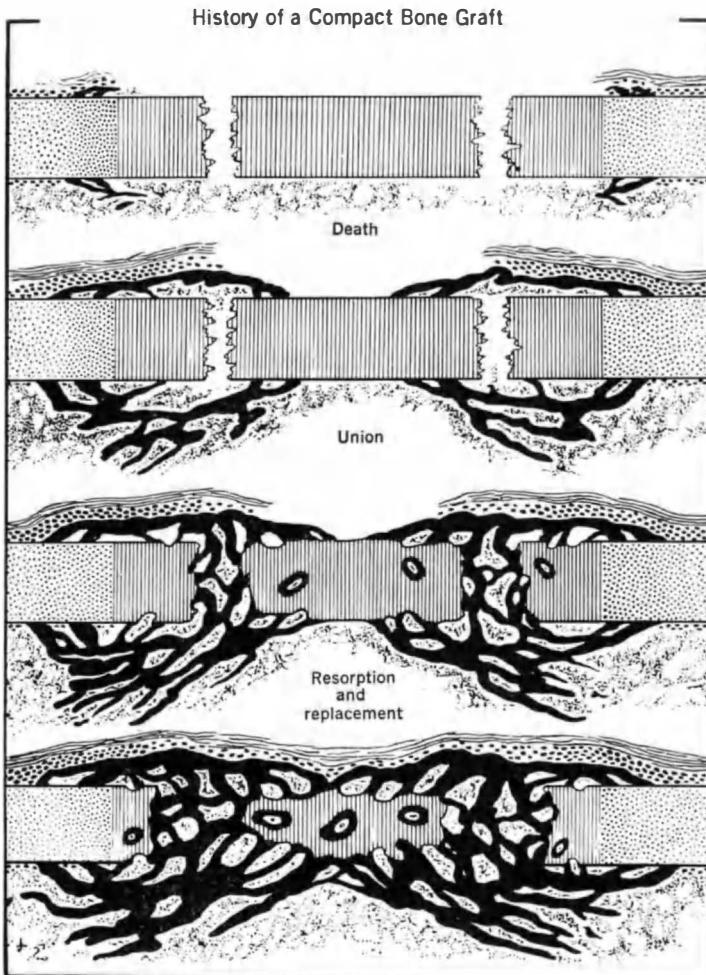


FIG. 8. Diagrams to show the steps in the history of a block of cortex that is cut free from its blood supply and then placed back in its bed. The periosteal surface is above and the endosteal surface below in each of the four pictures. Pre-existing bone that remains alive is shown in medium stipple; dead bone is lined, and new bone is black. (From Ham, A. W.: Some histophysiological problems peculiar to calcified tissues. *Journal of Bone and Joint Surgery*, 34A, 701-728, 1952.)

to the host bone, the chief functions of the transplant are (1) to serve as a structure to which callus tissue from the host can become firmly cemented, and (2) to thereafter serve as a medium which lends itself

to replacement by living bone derived chiefly from the host (Fig. 8). Since these latter two duties, which are generally the two most important ones a transplant of compact bone has to perform, do not depend on the survival of cells of the transplant, it can be understood why transplants of homogenous bone, or even dead bone, preserved for long periods of time, can be useful.

It should perhaps be noted here, that although homogenous bone, preserved or fresh, can, because of its content of calcified intercellular substances, perform very useful functions on transplantation, its use has two disadvantages as compared with fresh transplants of autogenous bone. In the first place, it contains foreign protein and hence it could be expected to incite some inflammatory reaction for this reason. It would, however, take very carefully controlled studies to determine whether this would be noticeable over the general reaction occasioned by the transplantation procedure. Certainly clinical usage of this type of graft suggests that any reaction incited because of the foreign nature of the transplant cannot be very great. One wonders, however, whether more reaction might not occur if a second homogenous transplant were ever used in the same individual—whether the first might not serve as an antigen and give rise to immune bodies which would produce a more violent reaction when the second homogenous transplant were used. Indeed, many clinicians have the impression that this does occur when homogenous transplants are used in multistage “grafting” operations and so they prefer to use autogenous transplants in this type of operation. This should be investigated experimentally.

Secondly, the use of an autogenous transplant of fresh compact bone provides the possibility of some foci of osteogenesis beginning from the covering and lining cells of the transplant, and these on occasion, might conceivably be a marginal factor in the success of the operation. It would seem, then, that if a choice is possible, autogenous transplants of compact bone would be preferable to homogenous transplants.

2. TRANSPLANTS OF CANCELLOUS BONE

The increasing use of cancellous bone transplants for certain purposes over the past decade again raised the question of whether or not transplanted bone lives and acts as a true graft. In many experiments that have been performed, and after many operations on man in which autogenous cancellous chips were transplanted, living cancellous bone has later been found in the sites to which the bone was originally transplanted; this has led to the development of a body of opinion to the effect that cancellous chips live after transplantation. The evidence for and against this view has been reviewed recently and extensively by Gordon and Ham (1950) who also performed experimental work which will now

be reviewed briefly.

Gordon and Ham (1950) found that it was very difficult, when cancellous chips were transplanted into bony defects, to determine the fate of the chips, unless animals in which identical operations were performed were sacrificed at short intervals. When this procedure was adopted it was found that the osteocytes of the chips mostly died and that the dead chips became cemented and bound into new bone and sooner or later replaced by new bone, so that little of the original intercellular substance of the chips remained. The source of the new bone that formed, however, posed a problem. In experiments in which bone defects are filled with cancellous chips it is almost impossible to determine whether the new bone that forms originates from the covering cells of the chips or from the covering and lining cells, and marrow cells of the bone in which the defect is created.

To settle this problem Gordon and Ham (1950) transplanted cancellous chips to muscle. They found that in this location the osteocytes of the chips, for all practical purposes, died. They found, moreover, that the covering and lining osteogenic cells of the chips also mostly died but that those of the chips that were close to capillaries of living muscle, survived, proliferated, and gave rise to new trabeculae of bone, which grew toward the capillaries of the muscle.

This work seemed to prove that the osteogenic cells of the endosteum that cover cancellous chips could survive transplantation provided they were placed in an environment in which there was abundant tissue fluid being liberated from living capillaries. This conclusion, however, could be criticized by those who believe that no special cells are needed for osteogenesis and that any fibroblast can form bone as readily as an osteogenic cell, for those who belong to this school could reason that the new bone which formed around the chips developed from fibroblasts and was induced to do so by the presence of the chips. To settle this problem Ham and Gordon (1952) performed further experiments in which thrice-frozen and thawed chips as well as fresh chips were transplanted to muscle and their behavior was compared. It should be noted a *single* freezing does not necessarily kill cells, but that three freezing and thawings generally accomplish this purpose.

No new bone was found in association with the transplanted thrice frozen and thawed chips and new bone was found in association with the fresh chips transplanted to muscle in each animal in which this was done. Accordingly Ham and Gordon concluded that the new bone that appears when cancellous chips are transplanted to muscle comes from the covering osteogenic cells of the chips, and that it is not induced to form from fibroblasts.

It should be pointed out that chips of compact bone, while they may

serve other useful purposes, could not generally be expected to serve as new foci of osteogenesis because they are not seldom covered with osteogenic cells as are cancellous chips. An exception to this rule is chips prepared from the growing compact bone of young individuals, for this is trabeculated (Ham and Gordon, 1952) and may be essentially cancellous rather than compact in type, and so be replete with covering osteogenic cells.

III. Bone Induction

The term *induction* is used in embryology for the phenomenon of one tissue, by exerting specific effects on an adjacent tissue, influencing its differentiation. Weiss (1950) should be read for a comprehensive discussion of the phenomenon. It is not the purpose of this brief section to deal with bone induction in embryonic life. Here we are concerned with bone induction in postnatal life.

Two factors affect cells, their heredity and their environment. The human body contains many different families of cells and the cells of these families in general tend to reproduce their own kind for reasons that are to be explained by the hereditary composition of the cells of the different families. On occasion, however, environmental influences seem to be able to induce some of the cells of one family to take on the characteristics of another. If, for example, bone develops anywhere in the body from cells other than those of the family of cells that ordinarily give rise to bone, bone induction is said to have occurred.

It has long been known that bone occasionally develops in association with areas of pathological calcification in arteries, tonsils, abdominal scars, and other sites (see Ham, 1930, 1932). Hence, there is reason to believe that calcium salts, under certain conditions, tend to induce cells that have the capacity to do so, to differentiate into cartilage and bone. Huggins, in 1931, provided a most interesting example of bone induction by discovering that bladder mucosa, transplanted into the abdominal wall, would induce bone formation from cells in the abdominal wall. Many experiments have been performed in an attempt to obtain cell-free extracts from young growing bone and other materials which would, on injection into animals, induce bone formation. The results of these experiments differ. Bertelsen (1944) reviews this subject.

Bone itself has been used in an attempt to induce bone formation. As has already been explained, bone transplanted, say to muscle, could act in two ways to cause further bone to form. The first way would be for cells associated with the bone to grow; such bone that develops in this manner would not be classed as having been induced to form. The second way would be for something in the bone to act on connective

tissue cells of the muscle and induce them to form bone. Many experiments have been performed to see if the latter result can be obtained. In these, it is, of course, important to destroy all the living cells of the transplanted bone. As noted before a single freezing does not achieve this result. Ham and Gordon (1952) thought the procedure of thrice freezing and thawing bone would be less likely to destroy any bone-inducing materials in bone than any other procedure that would kill the cells of the bone, but with such material they obtained negative results. A comprehensive study of bone induction, utilizing the anterior chamber of the eye for a testing ground, has recently been made by Urist and McLean (1952). They found evidence of certain devitalized tissues of the cartilage and bone group inducing bone formation in this location.

There is no question that bone can sometimes be induced to form in sites where bone is not normally present, and from cells which ordinarily would not have formed bone. There also seems to be no question that calcified material of some sort is often concerned in causing the phenomenon. It should be remembered, however, that most pathological calcifications in the body do not stimulate bone formation, it is only the occasional one that does so.

It is interesting to enquire into the type of cell that can respond to the bone-inducing stimulus or stimuli by forming bone. Many consider that the ordinary fibroblast can do so. It seems to us more probable, that when bone induction occurs, it is a relatively undifferentiated mesenchymal cell that responds rather than the more differentiated fibroblast.

It is our feeling that bone induction is an interesting field for further experimentation, but that it is rare for a metaplastic *center* of osteogenesis to develop in postnatal life because of this phenomenon. Once osteogenesis from pre-existing osteogenic cells is well under way, however, as has already been discussed in connection with the repair of fractures, we think it possible that other mesenchymal-derived cells from ordinary connective tissue, that intermingle with the growing osteogenic cells, may be led to take on osteogenic properties. Hence in the repair of fractures, or perhaps even in the callus that forms to bind transplants to their hosts, some contribution may be made by cells that do not originate from the covering and lining cells of bone. But if there were not enough osteogenic cells present to form a reasonably abundant callus in these conditions we do not think that mesenchymal-derived cells from other sources would become osteogenic in type and provide bony repair. So, for all practical purposes, the surgeon should, we think, depend on the cells that normally cover and line bone surfaces and on undifferentiated marrow cells for osteogenesis, for such bone induction as usually occurs in fractures, and in association with bone transplants, depends, we think, on these first growing abundantly.

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CHAPTER XVII

VITAMIN A AND BONE

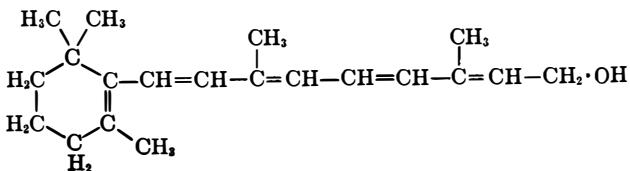
N. A. BARNICOT and S. P. DATTA

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I. The Chemistry of Vitamin A

The fat-soluble vitamins A_1 and A_2 appear to be entirely animal products, one or the other occurring in all vertebrates. They are obtained in the diet either pre-formed in foods of animal origin, or they are formed in the animal body by the conversion of the carotenoid pro-vitamins of which β -carotene is the most active. The efficiency of the conversion of the carotenoids into vitamin A, and the amount of carotenoids absorbed as such into the animal tissues, vary considerably in different species and between breeds in the same species.

Vitamin A₁ is a highly unsaturated primary alcohol of the following formula

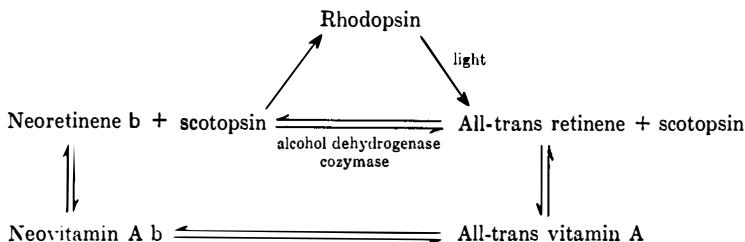


The free alcohol, its esters, the corresponding aldehyde (retinene), various vitamin A ethers, vitamin A acid and dimethyl amino vitamin A are all active biologically. The corresponding hydrocarbon, axerophthene, is also active though this has been disputed (Goodwin, 1951). According to Zechmeister (1944) vitamin A can exist in four possible stereoisomeric forms because bonds 3 and 5 are unhindered. This isomerism appears to have an important bearing on the action of vitamin A in the visual processes, but to date no indications have been given of any isomeric specificity in relation to other actions of the vitamin.

In mammals and birds the main stores of vitamin A are found in the liver with smaller amounts in the kidneys, lungs, adrenals and adipose tissue. In fishes apart from the liver considerable quantities of the vitamin are found in the intestinal tissues, which may even supersede the liver as the main storage site.

As it is only in relation to the visual processes that the precise metabolic function of vitamin A is known in any detail, a brief description of these processes is included here. This will serve to emphasize our complete lack of knowledge of the mode of action of vitamin A in bone and the complex nature of the isomeric changes which occur in at least one tissue.

In the retina of vertebrates there occur at least three well-defined light-sensitive, vitamin A-containing pigments: rhodopsin, and porphyropsin in the rods, and iodopsin in the cones. The metabolic changes undergone by these pigments under the influence of light and their subsequent resynthesis can be represented by the following diagram for rhodopsin,



the system being similar for other pigments (Wald, 1953).

With the pigment rhodopsin the protein is scotopsin and the carotenoid neoretinene b, with porphyropsin the protein is also scotopsin while the carotenoid is *cis*₁-retinene₂, and with iodopsin the protein is photopsin and the carotenoid again neoretinene b.

The stereoisomeric specificity of these systems is very high, and it appears probable that there is an enzyme system in the retina which isomerizes the all-*trans* retinene and Vitamin A produced by the photochemical decomposition of the visual pigments to the form required for the resynthesis of the pigment. While no evidence exists of any similar isomeric relationships occurring in other tissues, the possibility that they may be present and of importance must not be lost sight of.

II. Hypovitaminosis A

1. EFFECTS ON TISSUES OTHER THAN BONE

Apart from night blindness, which is caused by interference with the visual pigment systems detailed above and which is a well-marked and clear-cut symptom of vitamin A deficiency, the changes found are principally in the epithelial structures (see Bourne, 1953 for a general review). The various epithelia of the body undergo metaplastic change, the more differentiated epithelia regress to the simpler stratified type and those which are normally stratified become more cornified. The earliest changes are found in the respiratory system, marked desquamation occurring in the nares, the trachea, and the bronchi. Early changes are also found in various glands, particularly in the ducts of the salivary glands and the pancreas. The skin becomes rougher and hyperkeratinized, and sweating is interfered with by blockage of the ducts and other changes in the sweat glands. Changes in the sebaceous glands lead to degeneration of the hair follicles.

Classically the most obvious sign of vitamin A deficiency is xerophthalmia, which is characterized by keratinization of the surface layer of the corneal epithelium, the lining epithelium of the eyelids, and the conjunctiva. Atrophy of the tubules and keratinization of the ducts of the lachrymal glands leading to desiccation of the eye also occur. It is however only in severe deficiency that marked changes occur in the structure of the retina.

2. EFFECTS ON BONE

It is mainly in the dog that investigations have been made into the effects of vitamin A deprivation on the skeleton, though the rat and other animals have also been studied.

a. *Hypovitaminosis A in the Dog*

An extremely detailed and extensive description of the bony changes

in dogs due to vitamin A deficiency has been given by Mellanby as the result of a masterly and thorough investigation of the question over a number of years. The results have been summarized in a Croonian lecture to the Royal Society (Mellanby, 1944) and in a monograph (Mellanby, 1950). Reference should be made to these works for details of the numerous original publications.

The discovery of the effects of hypovitaminosis A on bone was due to the investigation of the extensive nervous lesions occurring in puppies reared on a vitamin A-deficient diet. These lesions included degeneration of both the cochlear and the vestibular divisions of the 8th cranial nerve; degenerations of the 2nd cranial nerve and the 1st and 2nd divisions of the 5th cranial nerve were also prominent. The changes in the cranial nerves were almost always confined to the afferent fibers. In the spinal nerves also the degenerations were mostly in the posterior roots; and in the cord itself changes were most marked in the ascending afferent fibers.

It was in relation to the lesions of the 8th nerve that the connection between the nervous degeneration and skeletal changes was first found. Histological preparations of the labyrinthine capsule of normal and vitamin A-deficient dogs showed masses of newly formed bone in the modiolus and the internal auditory meatus of the deficient animals. This new bone was interfering with and destroying both divisions of the 8th nerve (Plate IV, 1). It was soon found that abnormal bone growth and overgrowth was not confined to the internal auditory meatus, but that there was a great thickening of many of the skull bones and several other cranial nerves were being affected by these bony changes.

The 1st cranial nerve was affected where it passes through the cribriform plate. The plate itself was swollen, with enlargement of the marrow spaces between the limiting plates, which themselves were not always thickened. This enlargement of the plate did not greatly increase the distance to be traversed by the nerve fibers, but reduced the size of the nerve bundles, and these smaller bundles even showed signs of being pinched by the bone.

The optic nerve was very frequently degenerated in deficient animals and was found to suffer from stretching and compression by bone overgrowth. The optic canal was longer and instead of the nerve lying loosely in connective tissue, it was frequently gripped tightly and compressed. Similar changes (leading to blindness) (Moore, 1939) have been described in cattle deficient in vitamin A. Also where the nerve lies on the base of the skull, the groove was deeper and sharper than normal. The 3rd, 4th, and 6th nerves were seldom affected and the superior orbital fissure through which they leave the skull was found to be relatively unaffected by bony changes.

The 5th nerve was greatly affected by the considerable hypertrophy

of the bone about the petrous ridge. The Gasserian ganglion was sometimes squeezed by bone, and the nerve connecting the ganglion root to the central nervous system was compressed and twisted. The 7th nerve was often compressed both in the internal auditory meatus and in the facial canal, but being a motor nerve it appeared more resistant to the effects of pressure. Although the nerve was sometimes compressed to a thin ribbon it appeared little affected functionally.

The 9th, 10th, and 11th nerves showed no degeneration in vitamin A deficient animals. They all leave the skull through the jugular foramen the walls of which are formed by portions of the temporal and occipital bones. Both these bones were thickened in deficient animals, but nevertheless the jugular canal was only slightly narrowed, the main effect being a lengthening of the canal from the base of the brain to the wall of the tympanic cavity. Even where the canal passes over the tympanic cavity under the thickened basioccipital bone there was little or no constriction. The 12th nerve passes through the hypoglossal canal in the basioccipital bone and though this canal was lengthened because of the thickening of the bone the nerve was not interfered with.

The vertebrae, especially in the cervical region, were severely affected in deficient animals. The spinal canal was smaller than normal and the bones had lost their normal delicate appearance and had become coarse in outline; sharp edges were blunted and curved surfaces flattened. Though the overall size of the bones was about the same as in normal animals, the extra thickness of the bone made the canal smaller (Plate II, 3). This reduction in the size of the canal caused pressure on the cord, particularly on the dorsal columns. The spaces between the vertebrae were also frequently reduced with consequent squeezing of the posterior nerve roots.

In the skull as a whole the main effect of vitamin A deficiency was a reduction in the size of the posterior fossa; this was due to hypertrophy of the basioccipital, the supraoccipital, and the temporal bones. The basi-sphenoid was also thickened, and overgrowth of the posterior clinoid process caused the sella turcica to be deeper than normal. The parietal bones were slightly hypertrophied and the frontal bones but little affected. These changes in the shape of the skull distorted the brain stem in the posterior fossa. The fourth ventricle was squeezed between the cerebellum and the medulla oblongata. The pons and the medulla were misshapen, while the dorsal surface of the cerebellum was flattened and its posterior end was pushed through the foramen magnum.

Changes in the bones unrelated to the nervous system have also been described by Mellanby. The bones of the face, particularly the mandible, the malar bone, and the zygomatic process of the temporal bone were thickened and coarsened. The pelvic bones also were thicker and less

finely molded than in the normal. The wall of the femoral shaft was thickened and the marrow cavity reduced. The bony wall of the femur was for the most part cancellous instead of compact bone and the Haversian systems were incompletely developed. No major changes were found in the epiphyses or in growing cartilage generally.

Wolbach (1946) however has described extensive effects of vitamin A deficiency on epiphyseal growth. He states that all epiphyseal cartilage sequences are depressed, cells cease to divide and only those cells which have progressed to the vesicular stage continue to change. The cartilage trabeculae calcified before deficiency was established are reabsorbed and a densely calcified thin bony plate is formed across the face of the epiphyseal disc. This gives an appearance very like that seen in inanition, but in vitamin A deficiency this occurs before the rate of growth of other tissues is seriously affected.

b. *Hypovitaminosis A in the Rat*

The changes in the nervous system in rats on a vitamin A deficient diet have been described in detail by Wolbach and Bessey (1941) and their relation to disorders of bone growth discussed (Wolbach and Bessey, 1941; Wolbach, 1946). The nervous lesions were thought to be wholly of mechanical origin, being caused by a disproportionate growth of the central nervous system in relation to the surrounding bony case.

There was overcrowding of the cranial cavity, resulting in distortion of the brain, dislocation of the brain towards the foramen magnum with herniation of the cerebellum into the venous sinuses of the dura at the sites of the arachnoidal drainage structures. In the spinal canal there was also overcrowding and distortion of the spinal cord with herniations of the nerve roots into the intervertebral foramina and into the bodies of the vertebrae. Rats in which the growth had been stunted by a diet deficient in quantity but not in quality, or by a diet deficient in riboflavin or pyridoxine, did not show this disproportion between the central nervous system and its bony investment.

This general retardation of bone growth had previously been described (Hess, McCann, and Pappenheimer, 1921; Wolbach and Howe, 1925). The skeleton was thought however to be normal though bony overgrowth in relation to the bony capsule of the labyrinth was seen in rats and guinea pigs. In the inner table of the skull, immediately adjacent to the sutures, bone formation in excess of that found in normal rats of the same age was described, though this growth conformed in general to the normal pattern. The bony arch which forms the spinal canal was thinner than normal and evidence of the normal growth sequence by which the ventral side of the canal enlarges was absent.

Clovell (1940) has described thickening of the periosteal layer of

bone in the middle ear and exostoses in the internal auditory canal in rats kept for 52 days on a vitamin A-deficient diet.

c. *Hypovitaminosis A in Rabbits*

Perlman and Willard (1941) have tested cochlear function in vitamin A-deficient rabbits by determining the threshold curve for contraction of the tensor tympanic muscle over the range 128 to 4096 cycles per second. None of the depleted animals was deaf by this test and only slight differences in the threshold were observed before and after depletion of vitamin A. Such differences as were seen were within the experimental error.

Vestibular function was not tested but some unsteadiness in gait and some increase in extensor tone with occasional torsion of the neck was seen.

The bony changes observed were extensive hypertrophy of the cranial aspect of the otic capsule including the internal auditory meatus and the posterior fossa surface. The internal auditory canal was lengthened and narrowed with compression and stretching of the nerve and Scarpa's ganglion. In spite of the pressure on the 8th cranial nerve good cochlear function was retained.

Perlman (1949) has described the bony changes in vitamin A deficiency and recovery. After 5 months on the vitamin A free diet the deficiency was severe, the corneal changes were gross and the conditions of the animals very poor. In such animals new bone formation was uniformly found on the cerebral aspect of the temporal bone though changes in the 8th cranial nerve fibers and ganglion cells were variable. New bone also narrowed the cochlear aqueduct but none was found in relation to the endolymphatic duct or sac. No serious labyrinthitis was observed.

If vitamin A was fed to these deficient animals there was no reversal of new bone formation. The bone however became more compact and the large islands of vascular connective tissue in the bone were replaced by compact bone. Degenerative changes in the nerve fibers and ganglion cells were also not reversed.

In addition to the bony changes on the posterior fossa aspect of the temporal bone there were definite changes throughout the bone. Islands or buds of vascular connective tissue appeared to be invading the old bone of the capsule. These islands were not lined by multinucleate osteoclasts but rather by cells with a single nucleus resembling osteoblasts but no osteoid was seen. In the recovery experiments these islands of connective tissue were replaced by bone.

d. *Hypovitaminosis A in Man*

In spite of the extensive literature on vitamin A deficiency in man, which includes several series of reports of post-mortem examinations, very little information is available on skeletal changes.

Sweet and Kank (1935) reported the occurrence of headaches in their cases and found signs of rickets. Wilson and Du Bois (1923) reported that the line between cartilage and bone at the costochondral junctions was irregular and in one post-mortem examination reported that the middle ear was normal.

Blackfan and Wolbach (1933) reported a large series of cases which came to post mortem. Radiographic evidence of increased density of the metaphyseal margin of long bones was obtained in four cases. In one case of an infant of 8 months a histological examination of a costochondral junction and one vertebra showed complete arrest of bone growth with atrophy of proliferating cartilage.

Deafness has not been reported as being associated with vitamin A deficiency in man (Perlman and Willard, 1941).

Gerlings (1947) has described three cases of oxycephaly. In one case a histological and gross anatomical examination of the skull showed hyperostosis of the temporal bone with narrowing of the internal auditory canal. The 8th cranial nerve was compressed and the ganglion partially atrophied. Gerlings comments on the similarity of the findings to those of Mellanby (1944) in vitamin A-deficient dogs. Apart from the similarity in these findings there is no evidence of hypovitaminosis A being concerned in oxycephaly in man.

e. *Hypovitaminosis A in the Chick and the Duck*

Wolbach and Hegsted have described vitamin A deficiency in the chick (1952a) and in the duck (1952c). Evidence of great disproportion in the growth of the central nervous system and the axial skeleton were found in the cranial cavity and the spinal canal. There were obvious signs of compression and distortion of the cranial contents and herniations of the brain into the anterior confluence of the longitudinal sinus with the veins from the surface of the brain, the nose, and the orbits. The medulla was compressed and molded into the foramen magnum, and its dorsal surface showed the imprint of the first cervical vertebra. Evidence of compression of the spinal cord was seen at all levels. There was no gross evidence of injury to the spinal roots but it must be remembered that in the chick the spinal cord extends the entire length of the spinal canal and the nerve roots leave the cord at right angles.

The most obvious effect of vitamin A deficiency on the growth of long bones was on the epiphyseal cartilage sequences and endochondral bone growth. The tunneling of the epiphyseal cartilage was irregular and less extensive than normal. The zone of proliferating cartilage cells was less clearly demarcated and mitoses were absent. The intercellular matrix increased and there was formed a broad zone of moderately enlarged cells in non-calcified matrix. Cells of mature size surrounded by a calcified matrix constituted a narrow and irregular zone on the diaphyseal

side where tunneling was present.

Appositional bone growth was retarded earlier than in mammals on a deficient diet. Periosteal and endosteal osteoblasts were fewer and smaller than in the restricted diet controls. Much less cancellous bone was present in the metaphyseal region, and the adjacent cortical bone was thinner and less dense than normal. In all bones reabsorption where remodeling should occur ceased, as shown by a great scarcity of osteoclasts.

Retardation and suppression of endochondral ossification was very striking in the vertebrae. The bodies of the vertebrae were very deficient in cancellous bone, and the cortical bone was thin and wholly devoid of compact bone.

3. THE INFLUENCE OF THE DIET ON THE SEVERITY OF THE HYPOVITAMINOSIS A

In Mellanby's experiments with dogs the hypertrophied bone which distorted the normal shape of the brain was cancellous in structure and the interstices full of fatty marrow; frequently there was a diminution in compact bone. The microscopic structure of the bone appeared to be normal and there was no osteoid tissue or other abnormalities resembling those in rachitic bones. The diet which the dogs were receiving was thought to be adequate in phosphorus but the possibility existed that the hypertrophy of the cancellous bone might in some way be related to a relative lack of calcium in the diet. Experiments were therefore undertaken in which additional calcium, as CaCO_3 , was given to animals on both the normal and the vitamin A deficient diet. This addition of calcium was found to have the effect of making the bone more compact in both the deficient and the control animals, but the bone in the deficient dogs was still hypertrophied. Thus the part played by calcium in these effects was only secondary.

Wolbach (1946) regarded the inhibitory effect of vitamin A deficiency on endochondral bone growth of the bones of the base of the skull, with the consequent distortion and failure of the bony case to increase adequately in size, as the prime cause of the degenerative changes in the central nervous system. In Mellanby's experiments however the changes in the endochondral cranial bones were only slight and he did not consider them to be responsible for the changes in bone shape and size (Mellanby, 1950). Wolbach's rats were on a synthetic diet, completely devoid of vitamin A; they continued to grow until the 6th week of age, stationary weight or loss of weight being usual in the 9th week. There is thus some difficulty in deciding whether the cessation of endochondral bone growth, which is a common feature in young rats

in inanition, is in fact brought about by the specific vitamin A deficiency. Mellanby has repeated on a small scale Wolbach's experiments, with a diet and under conditions as nearly the same as possible, and has found that even when supplements of vitamin A were given, growth was still subnormal. The histological findings were similar to Wolbach's. Animals fed on Mellanby's deficient diet (diet No. 43) did not cease growth so soon, the epiphyseal changes were less severe, and they did not show the diaphyseal disc. This diet contained oatmeal and therefore traces of carotene, but nevertheless many of the changes associated with cessation of growth and vitamin A deficiency were seen; thus there is interference with endochondral ossification and slight overgrowth of periosteal bone.

Mellanby also reared rats on a normal diet to the age of 3-5 weeks and then placed them on the deficient diet (No. 43). In these animals endochondral ossification was almost normal, but there was a great overgrowth of periosteal bone. These results were similar to those of the dog experiments where conditions were similar.

It appears therefore that the main discrepancy between Wolbach's results and those of Mellanby may be attributed to differences in the age of the animals when deficiency starts, and the degree of inanition which develops. The earlier in life the vitamin A reserves are exhausted the more likely is the supervention of inanition and cessation of epiphyseal growth; periosteal growth however continues at most stages of deficiency. When deficiency develops at a later age the effects on epiphyseal growth are much less marked and periosteal bone overgrowth much more prominent. It is however clear that the one abnormal change in bone produced by vitamin A deficiency, whatever the age of the animals, is that affecting periosteal bone.

4. THE MECHANISM OF ACTION OF VITAMIN A DEFICIENCY ON BONE GROWTH

There are two different actions to be considered in the experiments of Mellanby with dogs. There is first the actual laying down of superfluous bone in some parts, such as in the internal auditory meatus (Plate IV, 1). Here bone is present in a position where it is not normally to be found at all. In these cases we may consider that there is an abnormal activity of the osteoblasts. In many cases however there is not so much a bone overgrowth but the failure, relative or complete, of absorption of previously formed bone. Thus in the overall dimensions of the skull there is not so much an increase in the outside measurements as a diminution in the inside measurements. Similar considerations apply to the spinal canal (Plate II, 3).

That these ideas are substantially correct has been demonstrated by

Mellanby who has shown that there is a difference in the number and activity of the osteoclasts on the inner side of the skull bones, that is, the part of the bone adjacent to the central nervous system, between normal and vitamin A-deficient animals. Sections of the basioccipital bones in normal and vitamin A-deficient animals (Plate IV, 2) show that in normal animals there are abundant osteoclasts on the surface of the bone nearest the brain, directly in contact with the bone under the periosteum; there are practically no osteoclasts in the same position in the sections from vitamin A-deficient animals. There are however many more osteoclasts on the marrow surface of the same area of bone in the section from the deficient animal than there are in the normal control. Thus the position of the osteoclasts seems to have been reversed. Similar changes and reversal of the position of the osteoclasts from the inner surface of the bone to the marrow surface are seen in the vertebrae. It may be pointed out that in the normal animals where the osteoclasts are on the inner surface of the bones they appear to be very active as judged from their lying in bone lacunae, which they have obviously hollowed out, in contrast to the relatively inactive state in which they are flattened against the bone surface.

Thus it would appear that in the normal animal the increase in the size of the central nervous system is being accommodated by removal of bone from the inner surface of the bony case, and its deposition on the outer surface, thus enlarging the capacity. In vitamin A deficiency this mechanism breaks down because of the changed position of the area of osteoclasia in the bone.

While these changes in osteoclastic activity in deficient dogs are undoubtedly responsible indirectly for many of the destructive changes in the central nervous system, they are not the whole cause. Thus in some bones there is obviously a great increase in osteoblastic activity at certain sites with the laying down of excessive amounts of new bone, as in the internal auditory meatus and in the petrous bone.

From these experiments of Mellanby it may be concluded that vitamin A acts as a specific chemical controller of the activities of osteoblasts and osteoclasts. The exact way in which this control is exercised is, however, unknown.

5. RECOVERY CHANGES ON THE RESTORATION OF VITAMIN A INTAKE

If vitamin A was added to the diets of dogs that had been on a deficient diet for some time but in whom growth had not ceased, recovery was initiated. The ataxia and incoordination of movement was reduced, and almost disappeared in early cases but not as completely in late cases. The pressure effects on the brain stem and cerebellum were relieved and their shape became more normal. Other effects on the

central nervous system were reversed, except for the deafness which persisted.

From histological studies it was seen that the administration of vitamin A caused a return of the osteoclastic and osteoblastic activity to the surfaces of the bone where they are normally found. The reaction was often very vigorous, with great numbers of very active cells to be seen, as if there was an attempt to restore the normal shape of the malformed bone as rapidly as possible. The changes in cellular activity were not anarchic but orderly, they occurred and attempted to restore the normal bone shape, even in regions adjacent to destroyed nerves or nerve tissue.

III. Hypervitaminosis A

1. HISTORICAL SURVEY

The earliest publication suggesting a toxic action of excessive amounts of vitamin A appears to be that of Takahashi *et al.* (1925) who found that if rats were given a vitamin A-rich fish-oil concentrate (Biosterin) in large doses, they died within a few days or weeks, showing emaciation, loss of hair, paralysis of the hind limbs, and internal hemorrhages. Drigalski (1933), using a different proprietary preparation, confirmed this result. The first investigators to report skeletal lesions, however, were Collazo and Rodriguez (1933); they administered approximately 60,000 I.U. of vitamin A daily to young rats, in the form of a fish-oil concentrate (Vogan), and noted that, among other symptoms, the animals suffered from fractures of the distal limb bones. Their observation was confirmed by Bomskov and Sievers (1933) and Davies and Moore (1934). The latter used a distillate rich in vitamin A and their work, therefore, marks some progress towards the identification of vitamin A itself as the causative agent; this was a point of some importance since preparations rich in vitamin A generally contained in addition considerable amounts of vitamin D, which might reasonably be suspected as a possible cause of skeletal changes. The work of Vedder and Rosenberg (1938), claiming that the severity of the bone damage was not closely correlated with the amount of vitamin A in the various preparations which they used, raised doubts concerning the importance of vitamin A in producing these effects. In 1945, however, Moore and Wang were able to demonstrate that the same skeletal lesions occurred if young rats (48–86 g initial weight) were daily given 50,000 I.U. of crystalline vitamin A acetate, dissolved in arachis oil; neither they nor previous authors succeeded in producing fractures in older animals. In the same year Pavcek, Herbst, and Elvehjem obtained fractures in rats given vitamin A in the alcohol form, and Rodahl (1950) confirmed the effect using oily solutions of the crystalline acetate.

2. EXPERIMENTAL HYPERVITAMINOSIS A IN THE RAT

The majority of workers have used the rat as an experimental animal and since information about the effects of hypervitaminosis A is greatest for this species, we shall deal with work on the rat at some length, reserving discussion of the results on other animals for a later section.

a. Dosage

The majority of workers have administered the vitamins by mouth, but as Rodahl (1950) showed, prolonged and repeated application to the skin can be effective. The dosage required to produce severe symptoms, including fractures, has generally been found to be about 25,000–50,000 I.U./day for a period of 1 to 3 weeks. Under these conditions, the animals cease to gain weight and ultimately die. Rodahl (1950) who has investigated the dosage relations most thoroughly, found that a dose of 50–100 I.U./g body weight was sufficient to interfere with the growth of young animals but did not produce skeletal lesions even after several weeks. He found, contrary to many other workers, that adult rats could be affected in the same way as young animals if the dosage was the same on a body weight basis, namely in the region of 500 I.U./g body weight.

The relative potency of different crystalline derivatives of vitamin A, from the point of view of the effects of excess on the bones, has not received much attention. Wolbach and Maddock (1951) state that vitamin A methyl ether is less potent than either the acetate or the alcohol and that, in relatively short experiments, vitamin A phenyl ether is without effect.

b. *Skeletal Lesions in the Rat*

(i) *Macroscopic.* A number of workers, for example, Moore and Wang (1945), Wolbach (1947), and Rodahl (1950) have published radiographs of the limbs of hypervitaminotic rats. In Plate II, 1 and 2, we have reproduced similar pictures from unpublished material of our own showing the hind limb of a young animal which had received 50,000 I.U. of vitamin A daily for a week in the form of a whale-oil concentrate (kindly supplied by Dr. Karen Rodahl); the corresponding region in a pair-fed control, matched in initial weight, is shown for comparison. The lengths of the femora and tibiae in the two animals are almost the same. In the hypervitaminotic animals, the antero-posterior diameter of the tibia is reduced at the proximal end, which is the most actively growing region, and the shadow of the cortical bone in the region of the tibial crest is more diffuse. The tibia is also more curved on its posterior aspect in the proximal half of the bone. The fibula is very much reduced in diameter and the marrow cavity is no longer discernable. The reason for this is well shown by Wolbach's (1947) diagrams of two superimposed

growth stages of the tibia and fibula (Fig. 1); the remodeling of the

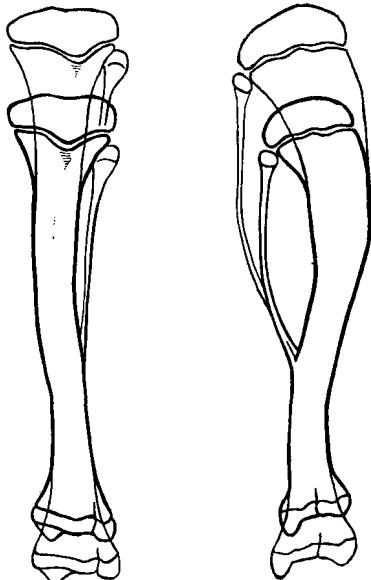


FIG. 1. Diagrams to illustrate the remodeling of the tibia and fibula of the rat during growth viewed from the anterior aspect (left) and in profile (right). The thick line shows the bone at a younger stage than that outlined by the thinner line. (Reproduced by courtesy of the late Dr. S. B. Wolbach and the editors of the *Journal of Bone and Joint Surgery*.)

bones as they increase in length involves removal of material from the posterior aspect of the tibia and the anterior aspect of the fibula, thus increasing the diameter of the region between the two bones. It is evidently an increase in the intensity of the resorptive processes at just these sites, without corresponding increase in the bone length and in the absence of normal rates of bone deposition on the opposite surfaces which leads to this characteristic change of shape. Turning to the femora, the most obvious difference is that in the hypervitaminotic animal, the shaft, due to narrowing at its distal end, becomes a parallel-sided cylinder; this again appears to be due to relatively greater resorption on the anterior aspect which is subject to resorptive remodeling in the normal growth sequence. The epiphyseal line is narrower in the hypervitaminotic animal, and, at the distal end of the femur, is only just discernable. In young animals given moderate excess of vitamin A for 6 weeks or more, the whole shaft of both tibia and femur may become much reduced in diameter so that the marrow cavity is, in places, almost obliterated as may be seen in Wolbach's (1947) photographs. When fractures occur they are most frequent in the tibia, generally near the level of the proximal one third of the bone, but they may also occur in the humerus and forearm.

Certain features of hypervitaminosis A can be studied to advantage in macerated skeletons; the general shape of the bones can be more easily appreciated, particularly in some regions of the skeleton where superposition of structures renders the interpretation difficult in X-rays. Details of surface texture under the binocular dissecting-microscope permit areas of resorption and active bone deposition to be located. We found in our own material that almost all regions of the skeleton are affected to a greater or lesser extent in severely hypervitaminotic animals. In some situations where resorption of thin sheets of bone is normally occurring, intensification of this process in the treated animals leads to perforations, which are very easily detected in macerated specimens. An example is given in Plate I, 1 and 2, which show the scapulae of an affected animal and its pair-fed control; both the supraspinous and infraspinous fossae are perforated in the former and the acromion also shows resorption. Pathological changes are also very evident in the mandible (Plate I, 4 and 5); an extensive area of resorption over the insertion of the masseter muscle leads to perforation of the tooth sockets and exposure of the roots of the molars. At the posterior end (Plate I, 5) resorption of the anterior aspect of the coronoid process is intensified, as may be seen by the more ragged contour, and because of this, together, in all probability, with decreased bone deposition on its posterior aspect, the shape is altered.* There is also evidence of resorption on the anterior aspect of the articular process, in the region of the incisor apex anterior to the inferior dental foramen and on the angular process, leading in both cases to perforation. In the macerated bones of rapidly growing animals it is often possible to distinguish areas of active bone deposition because of the more chalky, opaque appearance of the bone and the regular arrangement of its fine foramina; in hypervitaminotic animals such areas tend to be more translucent and the foramina fewer and less regular, suggesting, in agreement with Irving (1949), that osteoblastic activity is relatively inhibited. Inspection of macerated material suggests, in agreement with Wolbach, that resorption of bone in hypervitaminotic animals is most intense in regions where resorption is already occurring in normal remodeling; furthermore, the area of resorption is extended from these sites. This is clearly seen, for example, on the anterior aspect of the femur at its distal end; in the normal growing animal, resorption as indicated by bone texture is mainly confined to the immediate level of the metaphysis but in severely hypervitaminotic animals this area is extended up the shaft and the whole of the distal two thirds has a rough texture. In the fore limb intensification and extension of resorption is most evident at the actively growing ends of the bones, that is, the ends adjacent to the elbow. Resorption is not restricted to endochondral bones,

* See, however, M. J. Baer "Patterns of growth of the skull as revealed by vital staining." *Human Biology* 26, 80 (1954).

as the findings for the mandible show, and some membrane bones of the skull, particularly the interparietal, show evidence of thinning by endosteal resorption.

(ii) *Microscopic structure of the bones.* The histology of the bones in hypervitaminosis A has been dealt with by several workers, notably Strauss (1934), Wolbach (1947), and Rodahl (1950). A systematic account of the histological sequences at various levels of vitamin A intake and at various stages in the development of acute hypervitaminosis remains to be made, however; the present account is based on the work of the authors mentioned above, supplemented by a limited amount of material of our own.

The main changes which have been described are as follows: The epiphyseal line becomes reduced in thickness due to continued erosion of the hypertrophic zone from the diaphysial aspect without compensating multiplication of cells in the proliferative zone. In the guinea pig, a species in which the cartilage of the epiphyseal line is completely destroyed as part of the normal growth sequence, this process leads to premature closure of the epiphysis if the dosage of vitamin A is high enough. In the rat, the cartilage is not entirely removed either in normal growth or in hypervitaminosis A. Inhibition of the normal increase in weight accompanied by decreases in the longitudinal growth of the long bones (van Metre, 1947) is an early sign of excessive vitamin A overdosage, and is doubtless due in part to decreased food consumption. To this extent it is likely to be a non-specific effect. Essentially similar changes in the epiphyseal line can be induced by the administration of many other biologically active compounds in toxic quantities. Wolbach, 1947, and in other publications, believes that the skeletal lesions in hypervitaminosis A are essentially due to interference with the organizing activity of the cells of the epiphyseal cartilage. It is difficult to agree that a simple speeding up of the normal mechanisms controlling the shape of the bone is an adequate explanation, since this could only result in a more rapid differentiation, whereas the changes in shape which we have described indicate unbalanced resorptive and osteogenic processes. Evidence for the "organizer" function of the epiphyseal cartilage is reviewed by Lacroix (1947). Dissolution of the cartilage matrix comparable to the remarkable changes described by Fell and Mellanby (1950) in the embryonic bones of chicks cultured *in vitro* in media containing excess of Vitamin A, does not seem to have been observed in experiments on the intact animal, either birds or mammals.

We have already mentioned the intensification and extension of resorption in certain regions of the skeleton, which can be inferred from the shape changes of the bones and from close examination of the bone texture in macerated specimens; histologically, such areas show

numerous osteoclasts on the periostial aspect of the cortical bone. The cellular layer of the periosteum is unusually thick and mitoses are occasionally seen in the numerous spindle-shaped mesenchymal cells which occupy it. The capillary vessels are engorged and there is often evidence of blood extravasation even in the absence of fracture. These small



FIG. 2. Projection drawing of the upper end of the tibia of a pair-fed control rat to that shown in Fig. 3. Fully ossified bone indicated in black; the level of the junction between the zone of cell columns and of hypertrophic cells of the epiphyseal plate is indicated by a dotted line.

FIG. 3. Projection drawing of the upper end of the tibia from a hypervitaminotic rat which had received 50,000 I.U. vitamin A/day for a week, for comparison with pair-fed control in Fig. 2. The metaphyseal region is narrowed. The cortical bone of the shaft is reduced in thickness on both the anterior (left) and posterior (right) aspects and in places indicated by arrows there is deposition of osteoid (white).

hemorrhages, both in the periosteum and in other organs, have been remarked by many workers, and some have been led to compare the

lesions in hypervitaminosis A with those in scurvy on this account. In regions where osteoclasts are numerous, the layer of cortical bone may be very thin, as may be seen from Figs. 2 and 3 illustrating projection drawings of the upper half of the tibia in a severely hypervitaminotic animal and a pair-fed control. In regions where this thinning of the cortex is most severe, there is often a layer of pale-staining osteoid on the corresponding endosteal surface. This simultaneous periosteal resorption and endosteal bone deposition is well shown in Wolbach's (1947) publication, a figure from which is reproduced in Plate III, 1. The pale-staining, reticulate structure and the numerous osteoblasts and osteocytes of this new bone resemble the early stages of callus formation; insofar as the staining reaction can be taken as a guide, it may be supposed that calcification of this bone is defective. Abundant callus may be observed at sites of fracture in hypervitaminotic animals. In addition to resorption of bone periosteally there is some reduction in metaphysial trabeculae. No striking abnormalities are to be seen in the marrow tissue.

3. EXPERIMENTAL HYPERVITAMINOSIS A IN OTHER SPECIES

Ypsilanti (1935) failed to produce fractures in mice by administering various vitamin A concentrates by subcutaneous injection, but Rodahl (1950) obtained skeletal lesions and hemorrhages in this species when he gave 60–600 I.U./g body weight by mouth. Bone changes could also be produced by applying the concentrates to the skin. Rodahl also found that lesions essentially similar to those described in the rat could be produced in guinea-pigs and rabbits if the dose was of the same order of magnitude on a body weight basis. Maddock, Wolbach and Maddock (1949) have described the lesions in two littermate dogs, 2 months old, which were given 300,000 I.U./day/Kg body weight. Decline of the weight curve, compared with their control littermates did not occur until the 58th and 63rd day of the experiment. The long bones showed closure of the epiphysial line and periosteal resorption and hemorrhage; the fibula, as in the rat, was greatly reduced in diameter.

Some work has been done on hypervitaminosis A in birds. Rodahl (1950) administered 260 I.U./g body weight per day orally to young cockerels and although he noted decline in weight and a generally miserable appearance after a few days, he does not record any skeletal abnormalities. Wolbach and Hegsted (1952b), however, found skeletal changes in 7-day-old chicks which had been given 660–900 I.U./g body weight for 30 days or more. The long bones of the hind limbs showing narrowing of the epiphysial cartilage due to more advanced tunnelling of the cartilage from the diaphysial aspect; more osteoclasts were said to be present on the trabeculae and cortical bone adjacent to the epiphysial

cartilage, but fractures were not observed, and the cortical bone of the shaft, although reduced in total thickness in comparison with controls, was denser in structure in the sense that there were fewer Haversian systems. The same authors (1953) found similar but less pronounced changes in young ducks. It appears, therefore, that periostial bone resorption culminating in fracture is not a feature of hypervitaminosis A in birds as it is in mammals.

4. HYPERVITAMINOSIS A IN MAN

a. *Acute Vitamin A Poisoning*

It has been known for some time from the experiences of polar explorers and from the lore of the polar Eskimo, that toxic symptoms might develop as a result of eating the livers of certain arctic animals such as the polar bear and the bearded seal (*Erignathus barbatus*). Rodahl and Moore (1943) found that the livers of these animals contained very large amounts of vitamin A; the consumption of a pound would provide 7,500,000 I.U. of vitamin A which, from experience in animal experiments, might well be a toxic dose. The symptoms in man start within a few hours of the meal of liver, and include severe headache, vomiting, extreme lassitude, and irritability; in some cases, peeling of the skin of the face or of the entire body might occur after 24 hours. Subsequent work by Rodahl (1949) confirmed the suspicion that the toxic agent in the livers was vitamin A.

b. *Chronic Hypervitaminosis A*

The condition of acute vitamin A poisoning is less relevant from the present point of view than are the various reports of chronic vitamin A poisoning. This condition has been observed mainly in young children 2 to 3 years old, who have unwittingly been given doses of potent fish-oil preparations amounting to as much as 600,000 I.U. vitamin A daily for periods of a year or more. The first case of this kind was described by Josephs (1944), since then more than twenty cases have been described in the United States. Caffey (1951) has described and discussed seven cases, with radiographs of the bones, and the findings of a number of other authors seem to be essentially the same. The symptoms, which appear only some months after the start of the excessive vitamin A consumption, include anorexia, irritability, pruritus, and tender swellings over the limbs, with difficulty of movement. The X-rays reveal smooth hyperostoses on the middle region of the shafts of some of the long bones; the ulnae and metatarsals appear to be the most commonly affected sites, but Bair (1951) has described a case in which some of the skull bones were also involved. The serum calcium and phosphorus is generally normal but the serum alkaline phosphatase is somewhat

elevated. The plasma vitamin A has been found on several occasions to be raised from normal levels of 50–150 I.U./100 ml to 400–2000 I.U./100 ml. On cessation of the excessive vitamin intake the bone lesions disappeared within a few months. The reports of these cases do not suggest that skeletal resorption of the type seen in experimental hypervitaminosis A occurs; the dosage on a body weight basis, however, is in general considerably less than the level known to produce these resorptive changes in animals. On the other hand, hyperostoses resembling those in human cases have not, as far as we know, been described in animals, given more moderate excess of vitamin A over prolonged periods of time.

5. BIOCHEMICAL FINDINGS IN EXPERIMENTAL HYPERVITAMINOSIS A

a. *Calcium and Phosphorus*

The fractures which are such a striking feature of experimental hypervitaminosis A in the rat led several investigators to inquire whether the skeleton was imperfectly calcified in this condition. Bomskov and Sievers (1933), Moore and Wang (1945), and Rodahl (1949, 1950), found, however, no significant changes in the mineral content of the bones. The fractures presumably result, then, from the cortical bone in certain regions becoming very thin due to resorption, but the bone that remains is normally calcified; the osteoid which we have mentioned as occurring on some parts of the marrow surface of the cortex, if indeed it is deficient in mineralization, is presumably too small in amount to influence the overall analysis.

Major changes in the levels of blood calcium or phosphorus, such as occur in experimental hyperparathyroidism have not been found in hypervitaminotic rats, but Maddock, Wolbach and Maddock (1949) noted a terminal rise of blood calcium in the dog.

b. *Vitamin A Level in the Blood and Tissues*

Since the capacity of the liver to store vitamin A is very great, and most of the vitamin in the body is contained in this organ, it is interesting to consider whether in animals showing symptoms of hypervitaminosis A, the level of the vitamin in the blood is raised. The liver stores themselves are very much increased on large doses of vitamin A; Davies and Moore (1934) found, for example, that a rat which was given a total of 1,413,000 Blue Units (approximately 4,000,000 I.U.) of vitamin A over a period of 33 days and which died showing fractures, had 60,000 Blue Units/g in the liver as compared with a range of 500–100 Blue Units/g in animals on a maintenance diet; the vitamin A content of certain other organs such as the lung and kidney was also raised considerably. How-

ever, it did not appear that the level of the liver store and the severity of the symptoms were closely correlated; another animal which died with bone fractures had very much lower amounts of vitamin A in the liver. The authors suggested that symptoms of hypervitaminosis only occur when the ingestion and absorption of the vitamin exceeds the animal's capacity for elimination and storage. Walker, Eylenberg, and Moore (1947) found that in rats given large amounts of halibut-liver oil, the plasma vitamin A level was increased about ten times in some cases, and that animals with these high plasma levels showed bone fractures. In attempting to understand the control of the level of vitamin A in the blood, the particular chemical form in which the vitamin occurs appears to be important; according to Gray, Hickman, and Brown (1940), the liver vitamin is mainly in the form of esters, but about 10% of vitamin A alcohol is always present. Clausen *et al.* (1942) state that the alcohol is the chief form in the plasma, and Glover, Goodwin, and Morton (1947) suggest from their experiments on rats that the vitamin A level in the plasma is more closely related to the amount of the alcohol in the liver than to the ester content. The alcohol is said to be dispersed in the aqueous medium, perhaps carried by proteins, while the esters are in solution in fat droplets; such a difference in state might well be expected to affect the activity of the vitamin on the tissues, but as far as we are aware, studies on the composition and state of vitamin A in the plasma in experimental hypervitaminosis A, with concurrent examination of the pathology, have not been reported.

6. THE MECHANISM OF ACTION OF VITAMIN A EXCESS ON THE SKELETON

It has been suggested from time to time that the effects of excess of vitamin A is in some way related to the metabolism of other vitamins, but this has not in general been substantiated. The fact that the bone lesions and other manifestations can be produced with pure vitamin A, as already mentioned, rules out the simultaneous presence of large amounts of vitamin D as an essential factor. In spite of the apparent, but probably superficial resemblance of the bone lesions in hypervitaminosis A to those of scurvy, Moore and Wang (1945) and Walker, Eylenberg, and Moore (1947) were able to show that vitamin C supplements had no noticeable effects on the development of either these lesions or of hemorrhages. Light, Alscher, and Frey (1944) demonstrated the prothrombin level is low and the clotting time increased in hypervitaminotic rats, and that these abnormalities can be largely corrected by addition of vitamin K; the fractures and hemorrhages cannot, however, be prevented in this way, according to Walker, Eylenberg, and Moore (1947) and dosage with Dicumarol can produce extensive hemorrhages but no bone changes.

The possibility of an indirect action of vitamin A mediated by endocrine organs has also received consideration. Baumann and Moore (1939) found that young rats given excess of vitamin A together with thyroxine died earlier than rats given vitamin A alone. Wolbach and Maddock (1952) found that excess of vitamin A produced the characteristic bone lesions in hypophysectomized rats that have been described in intact animals. There are no reports of abnormalities in the parathyroid glands in hypervitaminosis A, and the bone lesions seem to differ in detail from those described in experimental hyperparathyroidism.

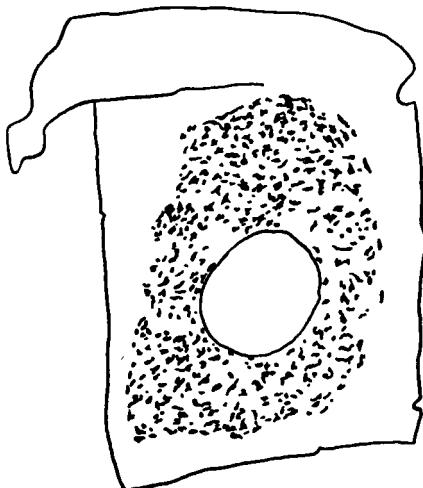
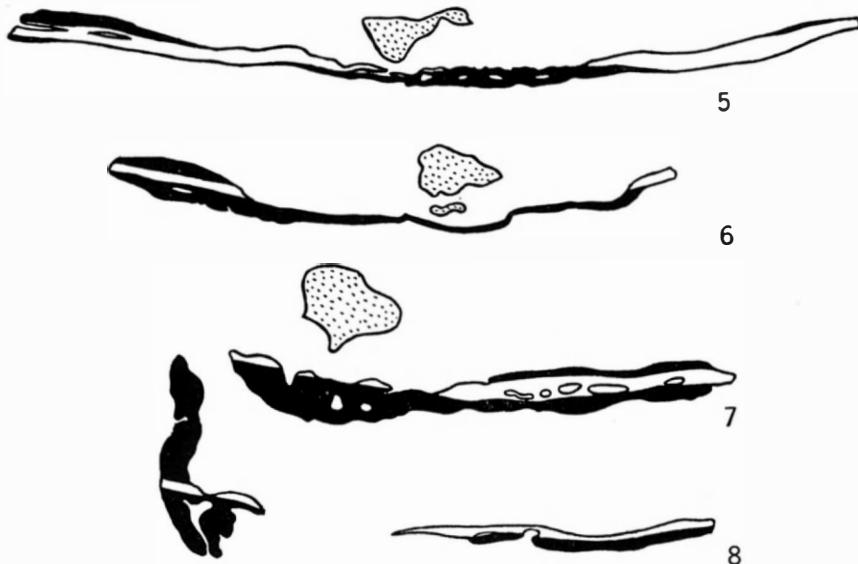


FIG. 4. Projection drawing of a fragment of parietal bone of a mouse which had been grafted for 14 days to the cerebral hemisphere of a littermate. There is perforation of the bone at the site of implantation of a fragment of vitamin A + estradiol. The position of osteoclasts, supravitally stained with neutral red on an area of new bone around this perforation is also shown. (Reproduced by courtesy of the editors of the *Journal of Anatomy*, London; Barnicot, 1950.)

Although there is no definite evidence that the effects of vitamin A excess on the skeleton are an indirect result of a primary action on some other tissue, nevertheless, histological and biochemical abnormalities in this syndrome are by no means confined to the skeleton; the kidney, liver, and skin, for example, show pathological changes. The effects of vitamin A overdosage in the intact animal are, therefore, no doubt complex. Even though the action of the vitamin might be a direct one on bone tissue, the response of the bone to the vitamin might perhaps be modified by concomitant changes in the cellular environment brought about by the actions of the vitamin elsewhere in the body. Some experiments by Barnicot (1950) in which an attempt was made to restrict the locus of action of the vitamin to bone tissue have some relevance here. Small fragments of crystalline vitamin A acetate were attached to the

endocranial surface of pieces of parietal bone cut from the freshly dissected skulls of mice about one week old, and the grafts were then thrust into the cerebral hemisphere of littermates. After a period of one to two weeks the grafts were examined either by maceration or by histological sectioning *in situ*. The macerated grafts (Plate I, 3) showed large perforations where the vitamin was attached, or, in other cases, an area of spongy new bone, which, as could be demonstrated from histological examination (Figs. 5-8), closed over a perforation produced in the original grafted bone. Using the technique of supravital staining with



Figs. 5-8. Projection drawings of histological sections of intracerebral grafts of pieces of mouse parietal bone bearing implants of vitamin A acetate. The residue of the vitamin is indicated by stippling, the original bone of the graft is white, and newly formed bone black. It will be observed that in all cases the original bone of the graft has been perforated adjacent to the vitamin implant and the hole closed at least partially by new bone. Figures 7 and 8 show different levels from the same specimen; in Fig. 8 the original bone is perforated, but in the area shown in Fig. 7 the hole is closed by new bone. (Reproduced from Barnicot, 1950, by courtesy of the editors of the *Journal of Anatomy*, London.)

neutral red, which as Barnicot (1947) showed, colors the osteoclasts an intense red, it was possible to demonstrate that after a few days of grafting there were no osteoclasts remaining on the surface of the grafted bone, but that after about a week these cells began to appear in the immediate vicinity of the vitamin implant. In later stages, as illustrated in the drawing reproduced in Fig. 4 they became extremely numerous. It was argued that with the vitamin closely applied to the bone in this way, the likelihood of such localized resorption being other than a direct

action of the vitamin was remote, and the conclusion was drawn that vitamin A acetate, or perhaps some derivative formed from it, is capable of causing differentiation of osteoclasts and the resorption of bone. The tissue culture experiments of Fell and Mellanby (1950), which achieve an even more complete isolation of the skeletal tissue from systemic influences tend to the same conclusion. The writers were doubtful in this case, however, whether osteoclasts were numerous enough to account for the degree of bone destruction observed. It does not necessarily follow from either of the researches just mentioned that in the intact animal the effect of vitamin A on the skeleton is a direct one, but they certainly indicate that this is a possibility worthy of serious consideration.

PLATE I

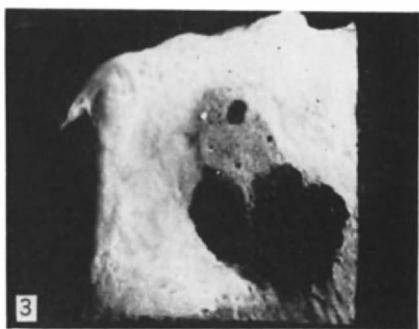
The effects of hypervitaminosis A.

1. Scapula of normal rat. Pair-fed control to that in 2.
2. Scapula of hypervitaminotic A rat for comparison with pair-fed control, 1. 50,000 I.U./day given as whale-oil concentrate for one week. Perforation of subscapular and suprascapular fossae and resorption on the acromion process.
3. Fragment of parietal bone of a mouse after 14-days grafting to the cerebral hemisphere of a normal littermate, showing perforation at the site of implantation of a fragment of crystalline vitamin A acetate. (Reproduced from Barnicot 1950, by courtesy of the *Journal of Anatomy, London*.)
4. Articular end of the mandible of a normal rat; pair-fed control to that shown in 5.
5. Articular end of the mandible of a hypervitaminotic rat for comparison with its pair-fed control, 4. Resorption at the anterior aspect of the coronoid process, the upper border of the articular process; perforation in the fossa of the angular process and in the region of the incisor root anterior to the inferior alveolar foramen. (Photographs by the late F. J. Pittock.)

PLATE I



1



3



4



2



5

PLATE II

1. Radiograph of the femur, tibia, and fibula of a normal rat; pair-fed control to that in 2.
2. Radiograph of the femur, tibia, and fibula of a hypervitaminotic rat for comparison with pair-fed control in 1; 50,000 I.U. vitamin A/day for one week. Note narrowing of the lower end of the shaft of the femur and upper end of the tibia and reduction in diameter of the fibula.
3. Hypovitaminosis A. Photographs of atlas vertebrae of two littermate dogs of the same age.

a, +A diet; *b*, -A diet.

- Note:* (1) The overall sizes of the comparable vertebrae are not greatly dissimilar.
- (2) The vertebra of the -A animal is coarse and blunted and has lost its delicate outline.
 - (3) The spinal canal in the vertebra of the -A animal is smaller than that of the +A.

(Reproduced by courtesy of the late Sir Edward Mellanby, and the editors of the *Proceedings of the Royal Society*, London.)

PLATE II

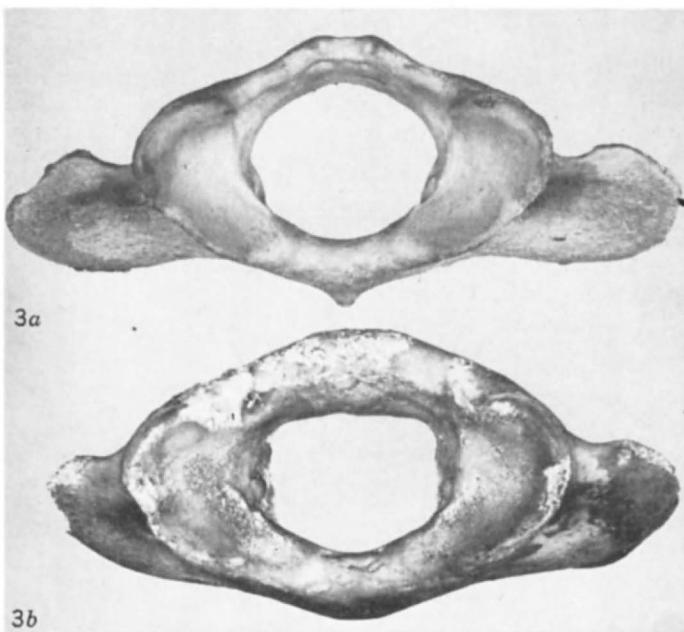


PLATE III

Histology of the bones in experimental hypervitaminosis A.

1. Transverse section of the humerus of a hypervitaminotic rat which had received 1250 I.U. of vitamin A daily from the twenty-first to the twenty-eighth day of age. Note thick layer of endosteal osteoid on the left hand side where the original shaft bone is much reduced in thickness. (By courtesy of the late Dr. S. B. Wolbach and the *Journal of Bone and Joint Surgery*.)

2. Photomicrograph of a portion of the cortex of the shaft of the tibia on its posterior aspect approximately one third of the length from the upper end; pair-fed control to animal shown in 3. The marrow tissue is seen on the left-hand side; there are large osteoblasts on this surface of the bone. Some osteoclasts and an irregular contour are noted on the periosteal surface and the periosteum is thin. (Magn. 220.)

3. Photomicrograph of the same region of the tibia as in 2, but from a hypervitaminotic animal which had received 20,000 I.U. vitamin A/day as whale-oil concentrate for one week. There is a layer of osteoid on the endosteal surface (left); the original cortical bone of the shaft is only about one quarter the thickness of the control. Osteoclasts are seen in the periosteum, which is unusually thick and contains spindle-shaped cells and enlarged capillaries ($\times 220$). (Photomicrographs by Mr. J. Armstrong, Anatomy Dept., University College).

PLATE III

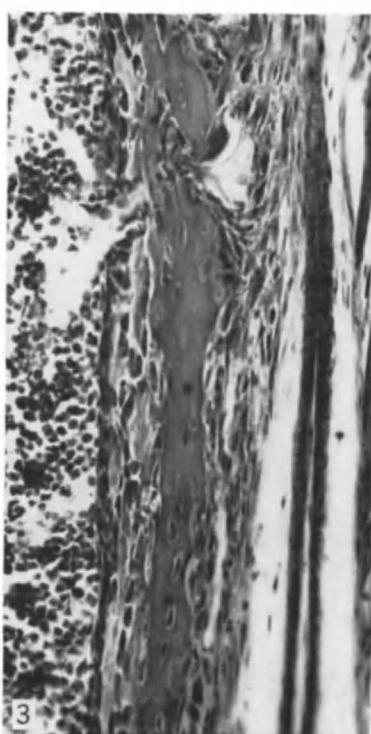
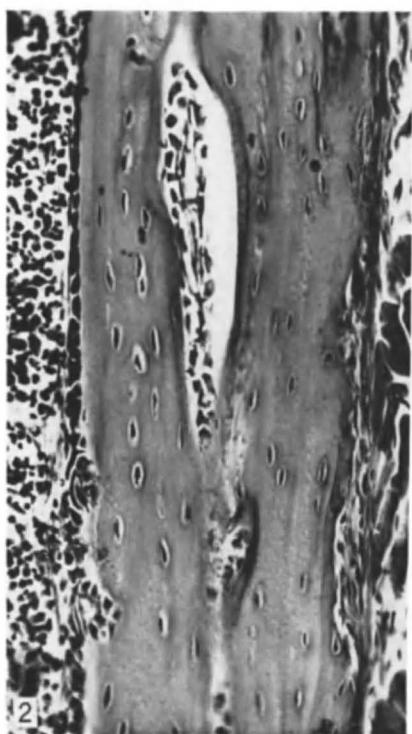


PLATE IV

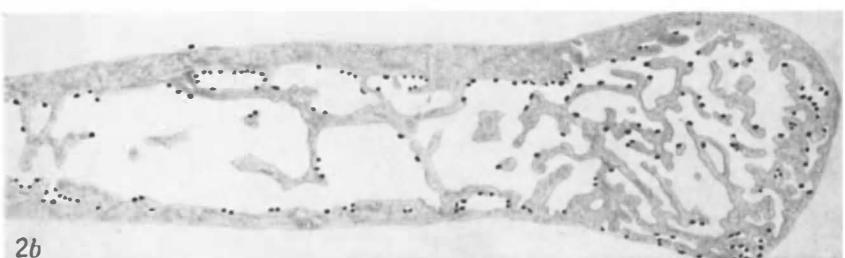
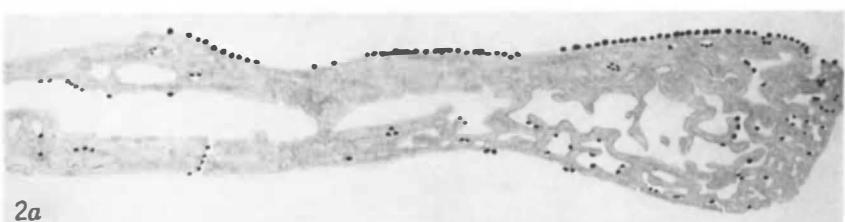
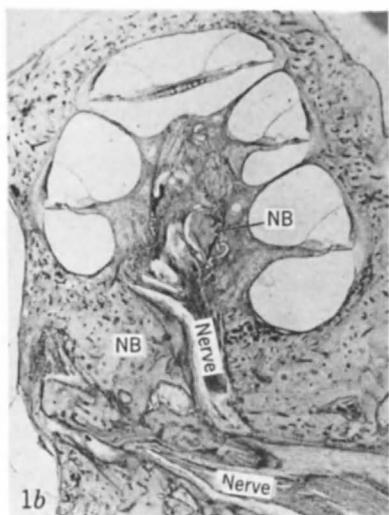
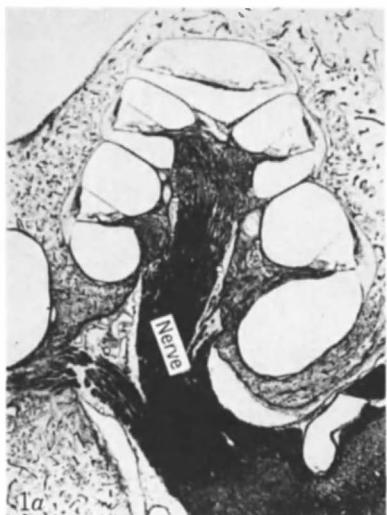


PLATE IV

Hypovitaminosis A in the dog.

1. Photomicrographs ($\times 12.5$) of sections of cochlea and surrounding bone of two littermate dogs of the same age.

a, +A diet; *b*, -A diet.

Note: Great narrowing of internal auditory meatus and compression of nerve by bone overgrowth (NB) in -A animal *b*.

2. Semi-diagrammatic drawings of the basioccipital bones of two littermate dogs of the same age.

a, +A diet; *b*, -A diet.

Osteoclasts are indicated by black dots.

Note: (1) Thickening of bone in *b* as compared with *a*.

- (2) In *a* there is a large number of osteoclasts on the surface of the bone adjacent to the brain (upper surface in diagram). In *b* they are absent from this region, but are abundant on the marrow surface of the same portion of bone. There seems to have been a reversal of the position of the osteoclasts.

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CHAPTER XVIII

VITAMIN C AND BONE

GEOFFREY H. BOURNE

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I. Introduction

The distinguished pathologist, S. Burt Wolbach, had agreed to write this chapter. Despite the fact that his health was failing and that major surgical interference seemed necessary, he devoted considerable thought to the subject and prepared a table of contents which promised to give us the considered judgment of one who had for 25 years been working on and thinking about the pathology of vitamin C deficiency. His death robbed us of this privilege and the scientific world of a distinguished pathologist. The burden of writing this chapter has fallen on my shoulders and I have tried to follow the line which Dr. Wolbach might have taken; my efforts, however, will inevitably seem but a feeble glim by comparison with the light that would have been shed by the master.

II. Vitamin C Deficiency and Intercellular Substances in General

1. COLLAGEN AND WOUNDS

In 1748 Richard Walter, Chaplain to Lord Anson's expedition round the world (1740-1744) described how old wounds broke open under the influence of scurvy.

"At other times the whole body, but more especially the legs, were subject to ulcers of the worst kind, attended with rotten bones, and such a luxuriance of fungous

flesh, as yielded to no remedy. But a most extra-ordinary circumstance, and what would be scarcely credible upon any single evidence, is that the scars of wounds which had for many years healed, were forced open again by this violent distemper: of this, there was a remarkable instance in one of the invalids upon the *Centurion* who had been wounded above fifty years before at the battle of the Boyne (1690); for although he was cured soon after, and had continued well for a great number of years past, yet on his being attacked by the scurvy, his wounds, in the progress of his disease, broke out afresh, and appeared as if they had never been healed. Nay, what is still more astonishing, the callous of the broken bone, which had been completely formed for a long time, was found to be hereby dissolved, and the fracture seemed as if it had never been consolidated."

This account constitutes the earliest record of the effects of scurvy on wounds and since the tensile strength of wounds is a function of the fascial scar, it becomes the first indication that vitamin C is associated with the maintenance of collagen. Lind, in 1753, refers to a letter from Mr. Ives describing a seaman on *H.M.S. Dragon* who had a "shattering of the humerus from a Spanish musket ball," the healing of which was delayed by the onset of scurvy.

Many other references exist in the older literature which show not only that healing of wounds was delayed but that they were likely to break open again in scurvy (see, for example, Major Charles Smart in the "Medical and Surgical History of the War of the Rebellion"; and Eve, 1866).

That vitamin C was essential for the maintenance of collagen was suggested by the work of Höjer (1923), and more recently Hunt (1941) showed a reversion of newly formed collagen in a wound to an argyrophilic precollagenous state when his animals were made scorbutic. Pirani and Levenson (1953) showed that ascorbic acid was necessary for the maintenance of scar tissue many weeks old which had formed during the healing of experimental wounds. Although there are relatively few references to the necessity of vitamin C for the maintenance of formed collagen there are many references to the effect of vitamin C deficiency on delaying the healing of wounds, thus indicating an inhibition of collagen production and/or maturation.

Most of the early literature already quoted makes reference to this and there is a record that even during the 1914-1918 war Turkish soldiers suffered severely from scurvy and that in those who developed the disease both skin and flesh wounds and fractures healed poorly.

Höjer showed in 1923-1924 that in experimental guinea-pig tuberculosis there was deficient scar production by the lung tissues due to the lack of ability to produce collagen. Ishido (1923) and later Saitta (1929) showed that there was considerable delay in the healing of wounds in scorbutic animals. Lauber (1933) found that application of vitamin C to skin wounds in mice stimulated healing but that injections of vitamin

C into normal guinea pigs had no effect on the speed at which wounds healed (see also Proto, 1936). This is not surprising since the guinea pigs were already receiving their optimum dose of vitamin C and it has been shown (Bourne, 1942b) that there is an optimum requirement of vitamin C for healing wounds. It may be possible that the increased local concentration of the vitamin as a result of its direct application to the lesion in animals such as mice (which synthesize their own vitamin C) may have enabled the repair processes to proceed faster than if the injured tissues had been dependent upon the slower diffusion from the blood. However, rabbits are also able to synthesize their own vitamin C and it is possibly surprising therefore that Mann and Pullinger (1940) were unable to find that local applications of vitamin C had any stimulatory effect on the regeneration of a cornea which had been damaged with mustard gas. If an increased local concentration of vitamin C was important in securing rapid regeneration one would have thought that an avascular tissue such as the cornea would have been ideal for demonstrating such an effect. The positive result obtained by Saitta with mice might therefore have been due to lack of adequate statistical means of measuring regeneration. Lauber and Rosenfeld (1938) showed that if animals on a low vitamin C intake are wounded most of the organs and tissues lose their vitamin C which appears to become mobilized in the injured region. So there seems to be some local concentration of the vitamin in a lesion but presumably under normal circumstances an optimum concentration is secured fairly rapidly. Bartlett, Jones, and Ryan (1942) found in fact that the tensile strength of a scar depended directly upon its vitamin C content, e.g. scars with 7.65 mg of vitamin C per 100 g of tissue broke at 258 mm Hg and those having 0.31 mg vitamin C per 100 g tissue burst at 127 mm Hg.

Wolbach (1953) has pointed out that the rate of production of inter-cellular material has a quantitative relationship to the amount of ascorbic acid administered. The first quantitative experiment which demonstrated this was described by Lanman and Ingalls (1937) who carried out direct measurements of the force required to break wounds in normal and scorbutic animals. The wounds were made in the abdominal wall and measurements of their strength was obtained by distending the abdomen with air and noting the pressure on a mercury manometer at which the wounds burst. Similar observations were made on wounds in the stomach wall. The results are shown below.

	2 mg vitamin C	0.5 m. vitamin C
Abdominal wounds	160 mm Hg	65 mm Hg
Gastric wounds	70 mm Hg	30 mm Hg

Similar results were obtained on the stomachs of guinea pigs by Taffel

and Harvey (1938), Hunt (1941), and Hartzell and Stone (1942). Wolfer and Hoebel (1940) demonstrated that patients in whom the plasma vitamin C was lowest showed the slowest healing of surgical wounds. Wolfer *et al.* (1947) working with human volunteers concluded that up to 50% reduction of tensile strength in a wound could be produced by a diet deficient in vitamin C. Crandon, Lund, and Dill (1940) recorded their results on experimental human scurvy and showed that only after a subject had been on a diet completely deficient in vitamin C for about 6 months, and after the plasma vitamin C had been nil for over 2 months, did a wound in the back refuse to heal. The subject, however, was adult; a very different result may have been obtained with a child. In growing guinea pigs, for example, it was shown (Bourne, 1944a) that the curve of tensile strength of skin wounds against plasma vitamin C ran roughly parallel (see Fig. 1). It was further shown that there was a close cor-

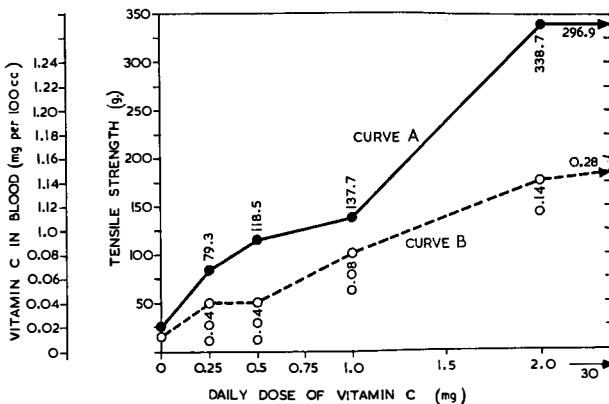


FIG. 1. Curves showing relationship of blood vitamin C to tensile strength of wounds in guinea pigs. Curve A: Tensile strength of wounds in 52 guinea pigs on graded doses of vitamin C after healing for one week. Curve B: Blood vitamin C in 14 guinea pigs on varying doses of vitamin C. (See Bourne, 1944a.)

relation between tensile strength of these wounds and the intake of vitamin C by the animal (see Table I).

This work also showed that in the animals receiving the least amount of vitamin C there was a greater amount of reticular (precollagen) fibers than in those receiving the greatest amount, and this was correlated with the tensile strength of the wounds. Carney (1946) was not able to find a relationship between wound healing and the level of vitamin C in the plasma in military patients; however, he too was dealing with adults.

The relation between wound healing and vitamin C intake is now amply confirmed: see, for example, the more recent work of Campbell, Ferguson, and Garry (1950); Campbell and Ferguson (1950); Galloway, Garry, and Hitchin (1948); Pirani, Stepto, and Consolazio (1952). Hines

et al. (1944) have shown that the vitamin is essential for the healing of nerves.

TABLE I
TENSILE STRENGTH IN GRAMS OF WOUNDS IN GUINEA PIGS
GIVEN GRADED DOSES OF VITAMIN C

Each figure is the average tensile strength of two wounds

	Dose of vitamin C per day (mg)				
None	0.25	0.5	1.0	2.0	30
0 gms.	58 gms.	156 gms.	111 gms.	343 gms.	255 gms.
0	56	86	143	331	255
46	65	220	231	185	270
21	91	150	120	480	310
55	125	123	95	400	394
0	101	123	150	295	313
21	34	62	96	320	281
34	120	40	135	316	..
50	64	206	158	378	..
Means {	25.3 ± 7.4	79.3 ± 10.4	118.5 ± 18.0	137.7 ± 13.9	338.7 ± 26.8
					296.9 ± 18.5

That this failure of wounds to heal normally is due primarily to failure of collagen production is also unequivocal. The relation between vitamin C and collagen production is demonstrated by the work of various authors, for example, Jeney and Törö (1936) found that if vitamin C is added to the culture medium in which fibroblasts were being grown, fibers were produced more rapidly. Mazoué (1937) injected kieslgühr intraperitoneally into a guinea pig and demonstrated a close association between the dose of vitamin C and the time of the first appearance of fibroblasts and fibers, he also showed that vitamin C deficiency had a profound effect on the organization of a clot produced by severing the limb muscles of guinea pigs. Querido and Gaillard (1939) demonstrated the relation between the vitamin and the production of collagen fibers by culturing osteogenetic cells from the chick in three types of plasma, (1) from scorbutic guinea pigs, (2) from scorbutic guinea pigs with crystalline vitamin C added (3) from scorbutic guinea pigs which had been treated with large doses of vitamin C for a few days before the experiment. Hardly any fibers were formed by the cells in the first plasma, but in the second and third, fibers were formed as rapidly as in the normal plasma. The cells in the scorbutic plasma showed fatty degeneration. However, Hass and McDonald (1940) found that vitamin C did not promote the formation of fibers in guinea pig tissue cultures. Hunt (1941), Bourne (1942b), and Danielli, Fell, and Kodicek (1943) also showed that there was a delay in the change from precollagen to mature collagen in scorbutic guinea pigs. The latter authors also showed

a lack of phosphatase in wounds in scorbutic guinea pigs, a finding which has been confirmed by Bunting and White (1948). The present author (1946) found also a deficiency of phosphatase in healing bones but this appeared to be due not so much to the fact that there was less phosphatase in the osteoid that did form but that less osteoid was formed. That which was present appeared to contain as much as normal osteoid. It is of interest that Danielli and his coworkers found that in the healing of wounds the precollagen fibers formed (as in healing bone injuries) contained phosphatase and that as they matured into collagen the phosphatase disappeared.

Although the relation between collagen production and vitamin C seems to be well established the relation between the *maintenance* of the collagen fiber (as distinct from the intercellular substance) is perhaps not so certain in view of the fact that Neuberger, Perrone, and Slack (1951) have shown that, at least in the rat, once collagen is formed its metabolic turnover is virtually negligible. It is of interest too that Ham and Elliott (1938) have claimed that the basic defect in scurvy is a failure to produce new fibers, not a weakening or destruction of those already formed. This suggestion is supported by the work of Elster (1950), by Robertson (1950), and by Burns, Burch, and King (1951). This may be the explanation why the development of scurvy is much more rapid in the growing animal than in the adult. Wolbach (1953) has drawn attention to the necessity that "Biochemists, interested in quantitative studies of collagen under conditions of dietary deficiency or in the estimation of collagen turnover by the use of heavy nitrogen or amino acids containing radioactive carbon, should not fail to take into account the growth rate of their experimental animals."

2. GROUND SUBSTANCE

Although vitamin C deficiency is known to affect collagen production there is no certainty as to how this is done. Any explanation is affected by the fact that the method of formation of collagen fibers is in dispute. Wolbach and Howe (1926) claimed that collagen fibers were produced in the intercellular ground substance by a sort of jelling process and that in scurvy it was this which was affected, whereas the cells remained more or less normal. This view has been supported by a number of other workers including Dalldorf (1939). However, others including Höjer (1923), Fish and Harris (1934), and Ham and Elliott (1938) considered that it is the cells which are primarily affected. Other authors, e.g. Follis (1948) claimed that the jellation theory is not proved. Evidence is accumulating that in fact, as Margaret Lewis claimed in 1917, the collagen fiber is secreted in the cell and excreted to the exterior. It may be of

interest that Barnett and Bourne (1942) found that many mesenchyme cells in a 5-day-old chick embryo contained numerous vitamin C granules (demonstrated by the acid silver nitrate technique). The presence of these granules may have been related to the production of fibers. It is of interest too, that mesenchyme cells in certain sites become chondroblastic and the first morphological indication that they are about to do this is the withdrawal of their processes and the appearance of a "mucoid matrix": the resulting tissue being called precartilage. Before this happened an increased deposit of vitamin C appeared, mainly in the processes of the cells. A similar process was observed, not only in the early embryo, but later when some cartilage was already formed and was undergoing accretionary growth: such cartilage showed a well-defined outer zone of mesenchyme, which was precartilage; both layers contained ascorbic acid and the amount diminished with increasing distance from the surface. Perhaps this accumulation of ascorbic acid is associated with the formation of intercellular matrix of ground substance. It may be significant that the majority of differentiated cartilage cells showed little or no reaction. In the earlier stages although most of the vitamin C granules were present in the cells some granules could be seen in the ground substance of the precartilage. Although there was not much evidence that cartilage was affected in scurvy, Wolbach and Maddock (1952) had recently produced proof that it is. This was particularly apparent in the epiphyseal cartilage plates of the long bones of young guinea pigs; it is not so obvious in costal cartilages which grow more slowly.

In scurvy, Wolbach & Maddock found cartilage cells failed to produce matrix, developed an abnormal appearance, and became shrunken and irregular; the nuclei stained very deeply with basic dyes; and the cells were separated from each other by small quantities of material which did not give the normal staining reaction for matrix. Reddi and Norström (1954) have shown, using radioactive sulfur, that the incorporation of sulfate into chondroitin sulfate is reduced to about one third in scurvy. This had also been demonstrated autoradiographically in the present author's laboratory by Miss C. R. Hill (see Plate II, 9 and 10). Friberg and Ringertz (1954) find reduction of uptake of radioactive sulfur in scurvy all over the body but not particularly in cartilage. It is of interest too that Follis (1951) has shown that in cartilage cells in scorbutic guinea pigs there is no cytochrome oxidase, no mucopolysaccharide and a reduction in ribonucleic acid and glycogen.

Gersh and Catchpole (1949) have shown that fibroblasts contain a glycoprotein to which a CHO-radical is attached. This, they believe, may be the precursor of the ground substance. They speculated that the fibroblast might secrete a glycoprotein of high molecular weight which would

then become precipitated in the extracellular fluid. Continued precipitation would result in fiber formation which would eventually crystallize into the highly organized cross-striated elements which compose mature collagen. This is fundamentally a modified view of the jellation theory of Wolbach, but whether such precipitations occur or not this substance presumably represents the non-fibrillar ground substance of connective tissue which must be a product of cell secretion, and which is believed to be affected by vitamin C deficiency.

In healing wounds of scorbutic guinea pigs, Penney and Balfour (1949) found that a considerable amount of metachromatic substance (acid mucopolysaccharide) was produced. An increased concentration of periodic acid-Schiff (PAS) positive material was also found associated with the edges of the trabeculae in costochondral junctions (Hill and Bourne, 1954) (see Plate II, 7 and 8) although there was a decrease of this material in the inner parts of the trabeculae. Increased mucopolysaccharides were also demonstrated in scurvy by Bunting and White (1948). Thus one effect of vitamin C deficiency appears to be an increase of intercellular material, but this material is abnormal in nature according to Bradfield and Kodicek (1951). It appeared to be non-sulfated and not capable of removal by amylase or hyaluronidase.

Wolbach (1953) has drawn attention to the fact that the first material which has staining properties similar to collagen appears as a homogeneous substance surrounding the fibroblast and its processes and that fibers subsequently appear in this material. However, as Wolbach mentions, these first formed fibers are argentophilic and do not have the staining properties of collagen. The fact that this amorphous material has a similar reaction to collagen is therefore not evidence that it represents the first stage of the cycle of collagen production. It seems more likely to represent matrix, perhaps eventually associated with the mature fibers but not necessarily forming an integral part of its structure. Wolbach, in referring to the glycoprotein granules of fibroblasts described by Gersh and Catchpole noted that the vacuoles which apparently contain these granules were described by him in 1933. He suggests that the extracellular liquid, frequently described in different parts of the body in both experimental and clinical scurvy, may be due to the failure of polymerization of this glycoprotein after excretion from the cells and that normally it becomes polymerized to form part of the connective tissue matrix.

The tendency to hemorrhage in scurvy has suggested some alteration in the permeability of the capillary vessels and this indicates some alteration in the intercellular cement substance binding the endothelial cells together. In this respect one should mention that Chambers and Cameron (1943) found that vitamin C was not necessary in tissue culture of

epithelial cells to enable the cells to cohere into sheets. Findlay (1921) observed "diapedesis" of red cells between the endothelial cells of the capillaries of scorbutic guinea pigs (see also the findings of Hess, 1920) but it has been found that various dyes passed through the capillary walls of scorbutic animals no faster than through those of normal animals (see Elster and Schack, 1950). Göthlin, Frisell, and Rundquist (1937), however, have produced substantial evidence based on human experiments that the capillary resistance was lowered in scurvy and could be restored with crystalline vitamin C, but Lazarus, Munro, and Bell (1948), showed that many scorbutic patients show no signs of increased capillary fragility. Wolbach and Bessey (1942) stated that the capillary bleedings in scurvy may have been due either to the changes in the cement substance binding the endothelial cells together or in the collagen fibrils supporting the capillary. They pointed out that new capillary formation was prevented by a severe deficiency of vitamin C.

In interpreting the effects of vitamin C deficiency we are hindered by the absence of the knowledge as to what biochemical function the vitamin serves in the body. King (1950) has shown biochemically and Hill and Bourne (1954) histochemically, that there is a decrease in activity of oxidative enzymes, e.g. cytochrome oxidase and succinic dehydrogenase. Furthermore, King showed a decrease in phosphatase and esterase activities and a decreased ability to metabolize tyrosine and phenylalanine. These reactions suggest, as King has stressed, that there is a general lowering of normal cellular metabolic activities in scurvy and none of these decreased reactions appear to be associated specifically with the failure of formation of intercellular substances. One can only assume that since the secretion of these substances is a normal metabolic activity of these cells the lowered metabolism of the cells in scurvy results in lowered production of matrix. According to Lloyd and Sinclair (1953) ascorbic acid or its oxidation products may play a part in the removal of polysaccharides in the final stages of collagen formation, or they may have a catalytic function in the organization of cross linkages such as —S—S— bonds between the protein chains of collagen. Lloyd and Sinclair also suggest that ascorbic acid and its oxidation products may be associated with the oxidation of organic sulfur to sulfate in the production of chondroitin sulfate. Support for this is seen in the work of Reddi and Nörstrom (1954). According to Barrenscheen and Valyi-Nagy (1948) there may be also an association between ascorbic acid and the oxidation of methionine sulfate.

3. CORTISONE AND WOUND HEALING

Since 1928 it has been known that the adrenal gland contains a considerable amount of vitamin C, usually estimated at about 10 times

that found in any other organ. Similar high concentrations are, however, found in the anterior pituitary gland, the corpus luteum and the interstitial cells of the testes. Because of the association of vitamin C with the adrenal cortex it is of interest that cortisone injections delay or suppress wound healing. This was first described by Ragan *et al.* (1949). They found that in the course of treating patients with various complaints there was a delay in the healing of previously incised wounds and an inhibition of the growth of granulation tissue in open wounds. They confirmed this with experimental wounds in rabbits (Ragan *et al.* 1949 and 1950; Plotz *et al.* 1950). In these animals histological study of the wounds showed that there was a suppression of fibroblast migration and proliferation, new capillary vessels did not form and there was less ground substance than normal. A decrease in skin thickness was also noted. The authors also found a suppression of callus formation of a broken bone by cortisone. Similar results were obtained by the use of ACTH. Ragan's work was repeated and confirmed by Bangham (1951). Spain, Molomut, and Haber (1950a and b) repeated the work in mice, and Plotz *et al.* (1950) found delay in the healing of incised wounds of both skin and stomach as well as with fractures. Howes *et al.* (1950) also showed that the bursting strength of sutured wounds was reduced in cortisone-treated rats and that fracture healing was delayed; they noted also that cortisone delayed the absorption of extravasated blood. Further work confirming the failure of wound healing and of collagen production in cortisone-treated human beings and animals was published by Baxter, Schiller, and Whiteside (1951); Aldrich, Carter, and Lehman (1951); and Margarey and Gough (1952). It is also of interest that Tauberhaus and Amromin (1950) found that desoxycorticosterone acetate stimulated fibroblasts and fibrous tissue development (supported by Selye, 1949; Pirani, Stepto and Sutherland, 1951; Bourne, Parkes and Wrigley, 1952) and that cortisone exerted a suppressive effect. This suggested a balance of cortical hormones controlling fibroblast activity and fiber production. Baker and Whitaker (1950) and Castor and Baker (1950) found that hog adrenal extract applied locally had a striking inhibitory effect on the closing of experimental wounds in animals and that when cortisone, or compound F, were applied a similar result was obtained. Baldridge *et al.* (1951) and Steen (1951), however, found no effect of cortisone on fibroblast proliferation and migration. Nevertheless, in embryos cortisone was found to disturb growth in chick, mouse, and rat (Karnovsky, Ridgeway, and Stock, 1951; Karnovsky, Ridgeway and Patterson, 1951). Follis (1951) found that the growth of long bones was retarded by cortisone, in fact he found the growing ends of such bones to be composed of spindles of calcified cartilaginous matrix enclosed in bone.

The effects of cortisone on wound healing were virtually identical with those produced by scurvy. This led both the present author (Bourne, 1952) and Wolbach and Maddock (1952) to endeavor to prevent the effects of cortisone on connective tissue by treatment with vitamin C. The present author worked with experimental wounds in rabbits and guinea pigs and studied the effects of cortisone and vitamin C upon their tensile strength and their histological nature.

In control rabbits the average tensile strength of three wounds on the left flank of each of five rabbits was 340 g; in the same rabbits injected with cortisone daily for a week the mean of three wounds on the right flank of each was 192 g. The tensile strength of these wounds was therefore only about half that of the controls.

The similarity between the effects of cortisone and scurvy had impressed other authors and was commented upon by Plotz *et al.* (1950), and Spain *et al.* (1950a and b). The first of these groups of authors were so impressed by this similarity that they considered the possibility of cortisone inducing a scurvy-like condition in the connective tissue. Accordingly, they made determinations of the vitamin C levels in animals treated with cortisone but found them to be normal. They also tried flooding patients who were receiving either cortisone or ACTH with vitamin C, but this had no effect on the action of the hormones. This is of interest in view of the fact that ACTH causes a pronounced loss of vitamin C from the adrenal cortex.

To study this problem further the present author used a second series of rabbits: control incisions on the left flank gave a mean tensile strength of 237 g; then each animal was given daily injections of cortisone together with 1 g of sodium ascorbate injected parenterally at the same time. The mean tensile strength of wounds on the right flank was then found to be 134 g, still only a little more than half the tensile strength of the controls. It was obvious therefore that cortisone did not exert its effect on healing wounds by depriving them of vitamin C. Histologically, scars of the cortisone treated animals showed less fibrous tissue and there were few fibroblasts and other tissues present. There were also unabsorbed blood clots. These characteristics were the same as those found in scurvy. In animals treated with vitamin C as well as cortisone the wounds showed exactly the same changes, without any signs of mitigation.

It is of interest that Schaffenberg, Masson, and Corcoran (1950) put forward the theory that vitamin C deficiency (in view of the association of the vitamin with the adrenal cortex) might lead to inadequate production of glucocorticoids. This is supported by the fact (Persson, 1953) that adrenalectomy aggravates the effects of scurvy. This seems a strange denial of the work of the various authors which has just been discussed. However, Schaffenberg *et al.* (1950) and Hyman, Ragan, and

Turner (1950) found that both cortisone and ACTH ameliorated some of the effects of scurvy. As against this Clayton and Prunty (1951) found that cortisone did not affect the weight loss in scorbutic guinea pigs, and Upton and Coon (1951) and Bourne (1952) found that it had no beneficial effects on wound healing in such animals. It may be of interest at this point to mention that cortisone in general seems to retard wound healing in those species of animals which synthesize their own vitamin C.

Wolbach and Maddock in their investigation into the problem asked three questions. (1) Will cortisone administered after the development of the lesions of scurvy produce a demonstrable histologic effect? (2) Will the daily concurrent administration of cortisone modify the progress of events in guinea pigs fed a diet deficient in vitamin C? (3) Will cortisone modify the reparative sequences resulting from ascorbic acid treatment of scorbutic guinea pigs? These questions, they stated, were all decisively answered in the negative after examining a number of organs and tissues including epiphyseal cartilage, bone, adrenal, and other soft tissues.

Persson (1953) has made a detailed study of the changes in scurvy and compared them with those produced by cortisone. The summary of his results is reproduced below.

(1) An increased amount of ground substance mucopolysaccharides is found in scurvy; the concentration of hexosamine within the skin is significantly increased in comparison with normal animals.

(2) The ground substance complex is present in an abnormal state of organization in scurvy, showing an increased solubility in water and a significantly lowered resistance towards penetrating agents; the dermal spread of Evans blue is increased similar to the velocity of flow of Ringer's solution through isolated muscle fascia.

(3) The water binding capacity of the skin is increased in scurvy.

(4) In repair after wounds in scurvy a depolymerized water-soluble ground substance is found within the granulation tissue. This ground substance does not show any metachromatic potency but reacts with Schiff's reagent after periodic acid and with Hale's colloidal iron. The fibroblasts contain large amounts of Schiff-reactive material.

(5) Adrenalectomy aggravates the symptoms of scurvy, and seems to inhibit the cellular activity in granulation tissue.

(6) Cortisone induces a marked gelation of the matrix. The dermal spreading of Evans blue shows a tendency to be depressed; the amount of hexosamine in the skin decreases and the dermal content of water is lowered as compared to untreated scorbutic animals. The histochemical properties of the ground substance change at treatment with cortisone in scurvy, the matrix becomes metachromatic and its reactivity to the periodic acid-Schiff staining seems to be diminished.

(7) Local treatment of skin wounds with ascorbic acid and dehydroascorbic acid were effective when the treatment is extended over several days.

From these results it is obvious that the changes brought about by cortisone treatment are not directly comparable with those due to scurvy.

It seems fairly certain that, as Persson points out, the ground substance is present in scurvy in a disaggregated and depolymerized state and shows no metachromatic activity although it gives a positive reaction with the periodic acid-Schiff technique for polysaccharides. It is impossible to say whether this ground substance is secreted by the fibroblasts in this depolymerized state or whether it is subjected to depolymerization after secretion possibly due to excess activity of hyaluronidase. Persson refers to the work of Bensley (1934) who characterized all healing processes by the three stages of: (1) edema, (2) gelation, (3) fiber production—and points out that in scurvy the process stops at the first stage, namely edema. In cortisone-treated animals, however, it stops at the second stage, i.e. gelation. The setting of the intercellular matrix is believed by some workers to be associated with the production of chondroitin sulfuric acid, but there is evidence that cortisone inhibits the formation of this substance. In fact Layton (1951) claims that the hormone prevents the incorporation of labelled inorganic sulfate *in vivo* or *in vitro* into chondroitin sulfate in connective tissue ground substance. However, the gelling is probably not a property dependent entirely upon chondroitin or mucoidin sulfate and is undoubtedly also concerned with the state and amount of the hyaluronic acid present.

It therefore appears to be soundly established that vitamin C deficiency is associated with profound changes in connective tissue, and since the principal elements of connective tissue are also present in bone it might be expected that bone would also be affected. There is ample evidence also for this.

III. Bone

1. VITAMIN C IN CHICK EMBRYO

In the chick embryo, mesenchyme that is to form bone contains vitamin C distributed in the cells in the same way as in precartilage. When the differentiation into osteoblasts begins the cells lose their vitamin C; the early osteoblasts themselves generally containing few granules; in well-developed bone, such as is seen in the femur two days after hatching, a few cells are heavily impregnated. The deposit is always granular and diffuse (Barnett and Bourne, 1942). Klein (1938) using AuCl_3 instead of AgNO_3 for the demonstration of ascorbic acid had observed a similar impregnation in periosteal mesenchyme in pig

embryos; he found that the osteoblasts also give a strong reaction, either local or diffuse, both when on the surface and when embedded in the calcified matrix. In regions where the collagenous (osteogenic) fibers have appeared, extracellular granules of what appears to be vitamin C are present. These granules can often be observed to be arranged in rows along the collagen fibers. Very fine granules are also present, rather unevenly distributed, in freshly calcified bone laminae. In the development of cartilage bone, whether endochondral or perichondral, the osteoblasts do not differ from those of membrane bone. The special phenomenon associated with this process is the appearance of a deposit in the cartilaginous matrix around the hypertrophied cartilage cells; the latter do not contain vitamin C. There is a possibility that in this case the granules may be of bone salt. Before cartilage cells enlarge and degenerate they align themselves in a characteristic fashion; at this stage they may contain ascorbic acid in small amounts, generally localized at one pole of the nucleus. This association of the differentiation of the osteoblast with the disappearance (the using up?) of the vitamin C they contain is of interest in view of the claim by MacLean, Sheppard, and McHenry (1939) that in young scorbutic guinea pigs the differentiation of mesenchymal cells to osteoblasts is impaired in scurvy.

It is of interest that in most developing cartilage groups of strongly vitamin C positive cartilage cells occur particularly in the regions of active cell division (Plate II, 11). Prior to cell division the vitamin C was found to be collected round the nucleus, which was consequently obscured; when the spindle elongated it, too, was surrounded by granules containing the vitamin. In what appeared to be the telophase the reaction was confined to the regions of the two daughter nuclei. It is of interest that other dividing cells in the chick embryo do not seem to contain this high concentration of vitamin C. Cartilage cells seem to be unique in accumulating the vitamin before division: as a rule in animal cells the presence of vitamin C is not a correlate of cellular proliferation (Barnett and Bourne, 1942).

2. SCURVY AND BONE REPAIR. HISTORICAL

The association of scurvy with the failure of injured bone to undergo repair has been well known for some two hundred years. It is however, a little difficult to accept, in the account by Richard Walter (1748) of Lord Anson's voyage round the world, the claim that the callus in the leg of an old soldier which had been broken at the battle of the Boyne 50 years before, softened and refractured.

Mead in 1762 quoted the case of a sailor who had suffered from a fractured clavicle which had apparently healed normally and which broke again 4 months later when the sailor was suffering from scurvy.

Six months after this when the sailor had been on a diet of green vegetables for some time the fracture re-united.

Marrigues (1783), Bell (1788), Callisen (1798), and Budd (1840) all found softening of the callus of old fractures of bones in scurvy, sometimes with a separation of the ends of the bones. Hammick (1830) reported a number of cases of spontaneous refracture of bones and pointed out that it was impossible to secure the uniting of a fracture so long as the patient had scurvy. Dr. Linton (quoted in the "Medical and Surgical History of the British Army which served in Turkey and the Crimea," 1858) described the case of a grenadier aged 23 who broke his humerus while carrying a log of wood across some frozen snow. After he had been put in hospital, old ulcers on his leg opened up and his gums became spongy. The callus which formed at the site of the fracture in the humerus was unusually small (suggesting a reduced inflammatory reaction to injury). Only when this man was placed on a non-scorbutogenic diet did his fracture unite and his ulcers heal.

Moore (1859) has recorded two cases of fractures of the forearm in which healing was prevented because the patients also had scurvy. Moore stated that this disease has a "powerful effect in retarding the consolidation of fractures."

Even during the first World War, Lobmayer (1918) claimed that Turkish soldiers suffered severely from scurvy and that in those who developed the disease both skin and flesh wounds and fractures healed poorly. In many cases the fractures showed not the slightest sign of formation of a callus even after several months. In confirmation of the fact that it was lack of the antiscorbutic substance which was responsible for this failure of fractures to unite he quoted two cases who suffered from pseudoarthrosis of the humerus and who recovered rapidly and completely as soon as they were put on a diet which was rich in antiscorbutic material.

Since these original clinical observations, various authors have called attention to their importance in the treatment of fractures (notably Bier, 1923, 1925, and Kappis, 1927).

Experimental work with animals has supported these clinical observations. It might be of interest at this point to mention various re-fracture experiments which have been carried out in order to test the old and oft-repeated clinical observations that an old fracture would soften and break again when a person developed scurvy. The first of these experiments were carried out by Israel and Frankel (1926) and Israel (1925 and 1926). They claimed to confirm the clinical observations on human scurvy by their experiments on guinea pigs. Roegholt (1932) has also supported this fact. Hertz (1936), however, believes that their experiments were not critically carried out and that what really occurred was

a new fracture resulting from the fragility of bones which ordinarily accompanies scurvy. Murray and Kodicek (1949a) when repeating Israel and Frankel's experiments, were unable to support them.

The earliest experimental observations on the importance of vitamin C in the healing of bone was that of Shinya (1922). He concluded that when bones from scorbutic guinea pigs are transplanted into normal animals they do not take. He attempted also to transplant sound bones into scorbutic animals but the latter died of scurvy before results could be obtained.

Ferraris and Lewi (1923) found that in scorbutic animals there was not only a delay in the reformation of bone after a fracture but there appeared to be inhibition of the normal process of the removal of cellular debris, e.g. the hematoma which formed as a result of the fracture persisted for a very long time.

Watanabe in 1924 sawed furrows in the skulls of guinea pigs suffering from acute scurvy and as each animal died he examined the injury histologically. He found that the scorbutic animal showed practically no power of bone regeneration. Wolbach and Howe (1925, 1926) investigated the effect of vitamin C deficiency on the regeneration of bones in small saw cuts made in the femora of guinea pigs. In the scorbutic animals there was complete lack of formation of osteoid trabeculae and although there was some fibrous organization of the clot resulting from the injury there was no penetration into it of capillaries and blood vessels.

Schilozew (1928) repeated the work of many other authors, including that of Watanabe and of Israel and Frankel. His findings were in agreement with theirs. He also showed that a diet containing adequate vitamin C resulted in the formation of massive well-ossified calluses and found that if he added plenty of fresh vegetables to the diets of patients suffering from fractures the healing was accelerated. Similar results, derived from wounds made in the heads of scorbutic animals were obtained by Jeney and Korpassy (1934).

Hanke (1935, 1936) found that a vitamin C-free diet retarded fracture healing not only in guinea pigs but also in rabbits, which are thought not to require this vitamin in their diet. Lauber (1936), and Lauber, Nafziger, and Bersin (1937) stated that once the normal requirements of a rabbit were satisfied extra vitamin C had no further effect on the rate of regeneration of injured bone. It is difficult to appreciate that a rabbit has a normal dietary requirement for vitamin C if it synthesizes the vitamin. Giangrasso (1939a and b) and Giangrasso and Gangitano (1939) were also able to secure more rapid regeneration of fractured bones in rabbits by giving vitamin C. These facts might possibly be explained by assuming that the healing of a fractured bone calls for more vitamin C than the animal is able to manufacture for itself. It has been

shown (Bourne, 1942b) that in a relatively small injury of the bone in rats, which also synthesize vitamin C (e.g. a 1-mm hole bored in the femur) the administration of vitamin C has no effect on the healing process. It should be noted, however, that when calcium ascorbate was injected subcutaneously into rats the amount of bone healing was statistically increased over that of animals injected with calcium gluconate. However, this may be associated with the availability of the calcium in calcium ascorbate rather than with the effect of the ascorbate fraction (see later).

These experiments were also repeated with rabbits in which the calcium ascorbate was injected intravenously in the marginal ear vein. A similar increased rate of bone healing was observed.

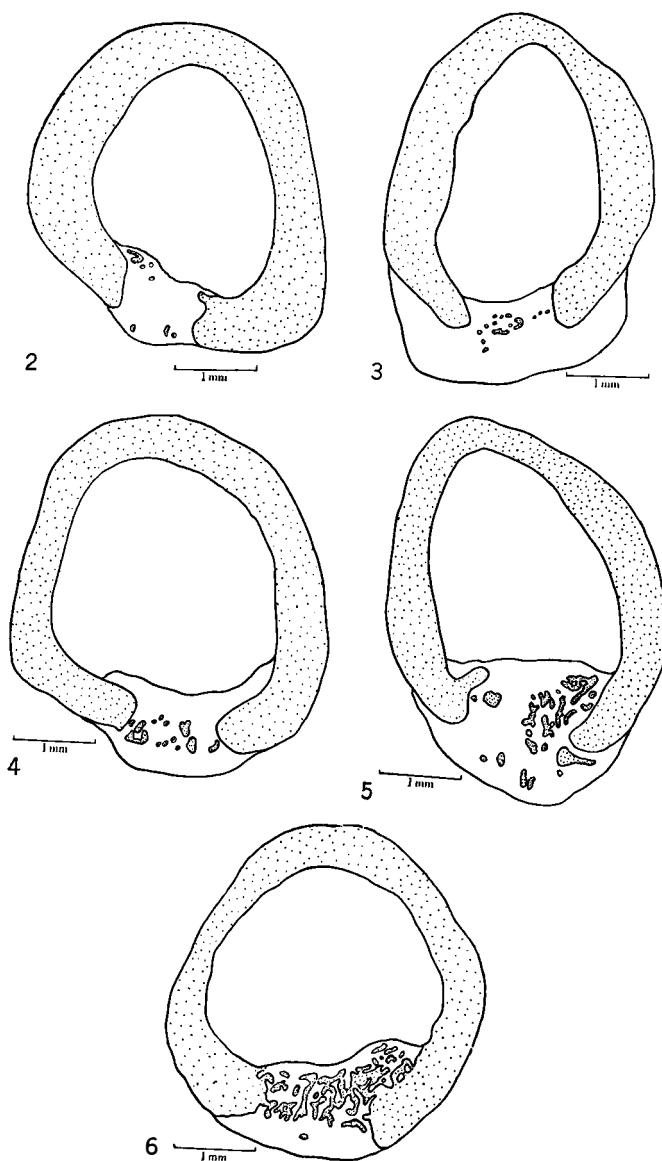
One of the most detailed of the earlier investigations of the effect of vitamin C in bone healing was that of Hertz (1936). He studied the formation of a callus in a fractured fibula of a guinea pig, choosing this bone because the tibia acted as a splint. He found delayed absorption of the fracture hematoma, deficient production of osteoid trabeculae and increased necrosis of the broken ends.

Lexer (1939a and b) also found a failure of fracture healing in scorbutic guinea pigs. He studied the process in a fractured femur by means of X-rays. The failure to heal in scorbutic animals was ascribed by Lexer to collapse of blood vessels and failure of proper blood supply to develop around the fracture. Subsequent histological examination showed that, according to the degree of vitamin C deficiency (starting on the day of the fracture) there was a regression of the callus and excess formation of connective tissue. In animals on a normal diet, or with extra vitamin C, there was a rich blood supply at the fracture site and a good callus formation. The majority of the previous papers had stressed deficient fiber or osteoid production in scorbutic animals, Lexer is the only one to lay special stress on the deficiency of blood supply in causing failure of healing of the tissues.

In the experimental work just recorded the results were obtained by depriving animals of foods containing vitamin C and claiming that the results obtained were due to deficiency of this vitamin. Supplements of vitamin C were administered as natural foods and in fact Mazoué, writing even as late as 1939, used lemon juice as his source of vitamin C. The present author (1942b) used graded doses of synthetic crystalline vitamin C.

3. QUANTITATIVE ESTIMATION OF BONE REPAIR IN SCURVY

A quantitative estimate of the rate of bone healing has been devised (Bourne, 1942a) for measuring the amount of repair taking place in a period of one week in a 1-mm hole bored in the femur. Transverse



FIGS. 2, 3, 4, 5 and 6. Projection drawings of sections through healing holes of femurs of guinea pigs on graded doses of vitamin C. One week after injury. The drawings show the development of bony trabeculae in the area of injury to be greater as the dose of vitamin C increases.

- FIG. 2. No vitamin C.
FIG. 3. 0.25 mg vitamin C daily.
FIG. 4. 0.5 mg vitamin C daily.
FIG. 5. 1.0 mg. vitamin C daily.
FIG. 6. 2.0 mg vitamin C daily.
(See Bourne, 1942b.)

sections were made through the femur in the region of the hole. The outline of the femur and the trabeculae in the section passing through the center of the hole were projected onto paper and drawn. Then the area representing the hole was cut out and weighed and so were the trabeculae. Division of the second by the first figure gave an index of density of bone formation, and if the final figure was multiplied by 100, whole numbers were obtained. The result was described as a trabecular index. This technique was applied to the femurs of guinea pigs placed on a scorbutic diet supplemented by 0, 0.25, 0.5, 1.0, or 4.0 mg of vitamin C daily.

The results were obtained as follows:

Vitamin C, mg	Trabecular index ($\times 100$)
0.00	7.73 \pm 2.25
0.25	6.70 \pm 2.30
0.50	9.74 \pm 2.48
1.00	19.41 \pm 3.53
2.00	23.73 \pm 6.75
4.00	18.09 \pm 4.48

It is obvious that the critical level for vitamin C in healing of bone injury lies somewhere between 0.5 and 1.0 mg (see Figs. 2, 3, 4, 5 and 6).

In 1936 Boyle, Wolbach, and Bessey investigated the rate of dentine formation in the persistently growing incisor teeth of guinea pigs. By administering alizarin to the animals, which stains the dentin pink as it is formed, they were able to measure the amount of dentin formed in

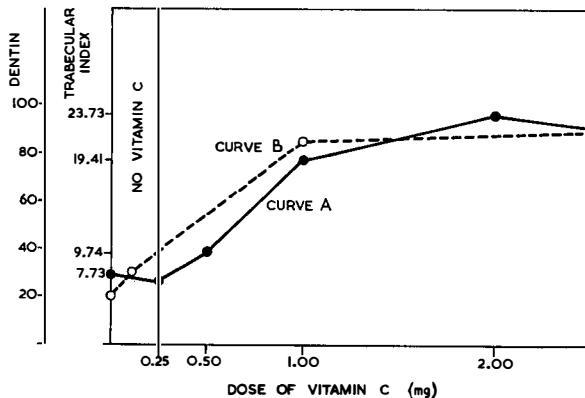


FIG. 7. Curves showing rate of formation of dentin compared with rate of formation of bony repair tissue in healing holes in femurs of guinea pigs on graded doses of vitamin C. Curve A shows rate of formation of trabeculae. Curve B shows rate of formation of dentin. The two curves are seen to be similar.

response to the administration of various amounts of vitamin C. Their results have been graphed and the curve compared with that obtained by the present author for bone regeneration. It can be seen that the two sets of results give virtually identical curves (Fig. 7).

The effects of complete deficiency of vitamin C on bone injury obtained in this work were similar to those obtained by other workers (e.g. Wolbach and Howe, 1926; Hertz, 1936). Hertz pointed out that in a completely scorbutic animal following bone injury there was no periosteal hyperemia, that in general it is difficult for a scorbutic animal to produce an inflammatory reaction in response to injury, and that in the absence of such a reaction the normal process of healing could not be induced. The enlargement of the cambial layer of the periosteum is of course an essential stage in the reaction. Hertz, like Watanabe, noticed a delayed absorption of the hematoma at the fracture site in scorbutic animals. The same phenomenon has been observed by the present author (1942b). For example, in guinea pigs receiving no vitamin C and in those receiving only 0.25 mg of the vitamin, by the end of the experimental period the whole of the connective tissue supporting the muscles associated with the femur was permeated with blood which had not been absorbed even as long as 7 days after the injury. It appeared that this blood had not clotted very readily and had continued to ooze from the hole for some time after the operation. Finally, it had extended through practically the whole of the soft tissues of the upper part of the leg. The connective tissue (as has been found by other workers) was edematous and had a soft jelly-like appearance. Animals receiving 0.5 mg of vitamin C showed a small hematoma at the site of injury but in animals receiving 1, 2, or 4 mg it had disappeared by the time the animals were killed.

There is no agreement (as already shown) that extra vitamin C given to animals which normally synthesize their own supplies of this vitamin accelerates healing processes in bone. Halasz and Marx (1932) found that by administering amounts of orange juice much greater than that needed to prevent guinea pigs from getting scurvy they obtained no acceleration of the normal time of fracture healing. None of the authors working in this field subjected their results to statistical analysis or indeed used methods which lent themselves to such an analysis, although Hanke (1935), who used rabbits, published some rather convincing X-ray photographs. The present author, using rats and the technique for estimating bone regeneration already described and subjecting the results to statistical analysis, was unable to find any significant difference between controls and vitamin C-treated animals.

The weight of evidence suggests there is an optimum level of vitamin C intake for bone regeneration and there is no detectable advantage in administering more than this amount.

4. VITAMIN C AND THE PHOSPHATASE REACTION

Although techniques are, and have been for some time, available for

demonstrating phosphatase in decalcified sections of bone (see Chapter IX), the present author preferred to study the effect of vitamin C on the phosphatase reaction in healing bone injury by undecalcified sections of 1-mm drill holes in the skull and of costochondral junctions.

In such preparations it was found (Bourne, 1948) that one of the effects of injury to the bone is to stimulate the absorption or synthesis of phosphatase by the periosteal cells. Also 24 hours after injury a large number of phosphatase positive cells were found to be present in the blood clot (a similar result was found by Danielli, Fell, and Kodicek, 1946, in skin wounds). The phosphatase in such cells appeared to be present mainly in the nucleus and the cells appeared to be mainly polymorph leucocytes. Further experiments using trypan blue showed that there was simultaneously a large number of macrophages in the injured area but they showed no signs of phosphatase activity. After 3 days the numbers of both these types of cell were greatly reduced but phosphatase-positive fibroblasts and fibers were present. Later these cells and fibers lost their phosphatase and a second cycle of phosphatase production developed with the appearance of calcified trabeculae (see Chapter IX).

In the injured areas in the scorbutic animals there were fewer of any kind of cell. However, such cells as were present, mainly fibroblasts, appeared to contain as much phosphatase (histochemically) as those present in the normal animal. Although the deficiency delayed the migration of these cells into the injured area it did not completely inhibit them and by 4 days there were as many present (and with a phosphatase reaction apparently equal to normal) as could be found in 24 hours in the normal animal. However, in the scorbutic animal no fibers could be seen. The vitamin C deficiency therefore delays cell migration to the injury area (polymorphs, macrophages, fibroblasts), does not affect profoundly the synthesis of phosphatase by the fibroblasts, but tends to prevent completely (if it is severe enough) the formation of phosphatase impregnated fibers.

Experiments in which Gomori phosphatase preparations were first made on whole femurs containing a healing hole and which were subsequently decalcified and sectioned, showed a reduced amount of phosphatase positive trabeculae compared with the normal control (Plate II, 12 and 13). Also there was less phosphatase activity in the periosteum of the scorbutic femur (Bourne, 1943). See also Plates I, 5 and 6.

If alizarin is added to Gomori's substrate mixture for demonstrating alkaline phosphatase it forms a "lake" with calcium ions and when these are deposited due to the activity of phosphatase they produce a pink-stained calcium phosphate. If a formalin-fixed whole-guinea pig femur and an alcohol fixed one are incubated in such a mixture the alizarin

stains the recently formed bone salt on the surface of the bones a light pink but in the alcohol fixed bone (in which the phosphatase is still active) there is intense red staining in regions of greatest phosphatase activity. Femurs from scorbutic guinea pigs showed greatly reduced activity, indicating a decrease in phosphatase activity in this condition (Bourne, unpublished).

Sections of costochondral junctions also showed a histochemical reduction of phosphatase activity in scorbutic guinea pigs (Plate I, 3 and 4). The activity was reduced in periosteum, in both fibrous and cellular parts, in the osteoblasts at the junction and in the osteocytes in the trabeculae. There was also a reduction in phosphatase activity in the endosteum. At the junction in normal animals there was a line of intense phosphatase activity which was continuous at the periphery with the periosteum. In this line the enzyme appeared both extra- and intracellular. The line was greatly reduced in width in scorbutic animals (Bourne, 1943). It is of interest that Gould and Schwachman (1942) and Zorzoli and Nadel (1950) have shown reduction of bone phosphatase in scurvy.

Van Wersch (1954) has recorded the results of an extensive investigation into chemical, histochemical and X-ray changes in experimental scurvy in guinea pigs.

After 3–4 weeks on a scorbutic diet he found histochemically a decrease of phosphatase activity in bones, particularly in osteoblasts and in soft tissues such as the kidney. He confirmed this biochemically by showing that the alkaline phosphatase activity of bone and kidney was reduced to about one fifth of the normal. However, in hemopoietic islands in the bone marrow he found that there was an accumulation of phosphatase-positive cells. In addition he found histochemically by the use of the periodic-acid-Schiff method and by chemical analysis that there was an accumulation of glycogen in scorbutic bones; in the histochemical preparations it was noted that there was considerable accumulation of glycogen in the osteocytes and that there was also an increase in extracellular glycogen. Also, in the marrow there was an accumulation of granulocytes loaded with glycogen.

Although van Wersch found a general increase in PAS-positive substances, in addition to glycogen he recorded a decrease in toluidine blue metachromasia, suggesting a loss of chondroitin or mucoitin sulfate.

5. VITAMIN C AND CALCIFICATION

Vitamin C obviously plays an important part in the production of an inflammatory reaction to injury of bone—in the organization of the blood clot resulting therefrom and in the formation of the ground substance of trabeculae. It remains to be considered whether it plays any part in the

process of calcification, particularly in view of its apparent association with phosphatase production, either directly or through its association with phosphatase activity. Calcium has been found to be deposited in various tissues in scurvy but it is deposited in an amorphous form. This is probably due to the absence of an adequate fibrous matrix. Fish and Harris (1934) have pointed out that in scurvy calcium salts may be deposited in teeth but that there was no matrix available for their reception. For the relation of matrix to calcification see Chapter IX. It is of interest, however, that as long ago as 1934 Schmidt suggested that when collagen fibers are first formed they adsorb precipitated inorganic salts and he has pointed out that such fibers possess the ability to orientate particles precipitated in their presence.

The calcium balance appears to be disturbed in scurvy (see Hess's summary of the work of Baumann and Howard, 1917). This has been shown also for scurvy in adult human beings (Lust and Klocman, 1912; Moll, 1919), infantile scurvy (Moll, 1919) and monkey scurvy (Howard and Ingvaldsen, 1917). Lust and Klocman found a positive calcium balance in the scurvy stage and a negative balance in the healing stage. Bahrt and Edelstein (1913) found a diminished calcium content of the bones of a scorbutic infant but Kapp and Schetty (1937) found the mineral content of bones remained normal in scurvy. Höjer (1923) stated that his histological observations gave no reason to suppose that the calcium metabolism is primarily disarranged in scurvy. He stated that dying tissue generally has a greater affinity for calcium and that this "explains" the calcification of late scurvy.

Humphreys and Zilva (1931) found that calcium and phosphorus retention by guinea pigs was lowered in scurvy but only in the last stages when the whole metabolism was affected. Matricardi (1938) found that blood phosphate fell in scurvy and Lucké and Wolf (1938) claimed that extra vitamin C administered to an animal led to a greater retention of calcium and phosphorus. If one adrenal of an animal was removed there was an upset of the calcium/phosphorus balance which was restored by the administration of vitamin C (Lucké and Heckman, 1938).

Lanford (1939) claimed that orange juice added to the diet of rats caused increased calcium retention. Against this observation Henry and Kon (1939) found that the addition of 2 mg daily of vitamin C to rats had no influence on the retention of calcium. Ruskin (1938) and Ruskin and Jonnard (1938a and b) held that there was an intimate association between vitamin C and calcium, believing that calcium was absorbed from the intestine as calcium ascorbate. This compound they found to be adsorbed onto the blood proteins much more strongly than other salts of calcium.

If, in fact, calcium is absorbed from the intestine as ascorbate and if this ascorbate is more efficiently adsorbed on to the blood proteins than other salts of calcium, one might expect that by artificially increasing the amount of calcium ascorbate available it might be possible to secure a higher concentration of ionic calcium in the blood (since McLean and Hastings, 1934 and 1935, have shown that $\frac{\text{Ca}^{++} + \text{protein}^-}{\text{Ca proteinate}} = K$). It is possible that such a higher ionic calcium (see Logan, 1940) might stimulate the formation of bone in an injured area.



FIG. 8. Projection drawing of section through the femur of a rabbit one week after drilling a hole and daily injection of calcium glucono-galacto-gluconate. Formation of trabeculae just beginning.

FIG. 9. Projection drawing of section through the femur of a rabbit one week after drilling a hole and daily injections of calcium ascorbate. Note great increase in formation of bony trabeculae.

The present author (Bourne 1942a) found that in fact subcutaneous injection of calcium ascorbate produced statistically more bone in drill holes in the femora of rats than animals receiving normal saline, or equivalent amounts of vitamin C, or equivalent amounts of calcium as calcium glucono-galacto-gluconate, or equivalent amounts of vitamin C and calcium glucono-galacto-gluconate injected together. In fact, the last of these appeared to depress the rate of bone formation. It was also shown that calcium ascorbate did not affect the earlier healing processes at 3 and 4 days and an effect could only be demonstrated when active formation of osteoid trabeculae was taking place. Further investigation

was carried out on rabbits by injecting the calcium ascorbate into the marginal veins of the ears. Increased bone formation over the calcium glucono-galacto-glucoside injected animals was obtained (Figs. 8 and 9). Further confirmation was also obtained from experiments on guinea pigs and in these animals the calcium ascorbate was found to be superior also to calcium lactate in stimulating bone regeneration.

Possibly if more bone is formed by injecting calcium as the ascorbate, this substance may cause an increase in the calcium ion concentration in the serum, a result which is difficult to achieve by feeding. According to Shohl (1939) calcium is present in serum in a concentration of only 10.0 to 10.5 mg per 100 ml of serum, and that unless an animal has been on a calcium-deficient diet the ingestion of calcium salts produces only a minute rise in serum calcium. This rise is so small that for some time it was thought not to be significant. Whether calcium ascorbate does cause a rise in ionic calcium in the blood can of course only be determined for certain by direct estimation. On the other hand, Bell, Cuthbertson, and Orr (1941) stated that a daily consumption by growing rats of dietary calcium up to 0.36 g of calcium per 100 g dry weight of food, increased the weight, the calcium content, the bending and twisting strength, and the thickness of the cortices of their femora.

The promotion of bone formation by calcium ascorbate even if one assumes that it works by increasing the ionic calcium is not easy to explain. The calcium cannot be deposited unless there is an organic matrix to receive it and the results described imply, therefore, that calcium ascorbate either by increasing the ionic calcium or by some other means stimulates the more rapid formation of bone matrix even in animals saturated with vitamin C.

If there is failure of matrix formation, calcification is not likely to take place, except for the type of calcification represented by the amorphous deposits of calcium which sometimes occur in acute scurvy. In the long bones of animals bone salt is constantly being deposited on the outside and withdrawn from the inside. The brittleness and thinning of the cortex of long bones in scurvy seems likely to be due to the fact that the process of resorption of bone continues during scurvy while the process of deposition of bone on the outside ceases. This would explain why Kapp and Schetty (1937) found the mineral content of the bones of scorbutic guinea pigs to be normal. That bone salt deposition does cease in scurvy is shown by the work of Salter and Aub (1931). See also Plate I, 1 and 2. These authors found that in normal animals injections of sodium alizarin sulfate stained the long bones of young guinea pigs a bright pink. In scorbutic animals the bones were unstained. In the normal animals the trabeculae in the cancellous tissue of the ends of the long bones were also stained (this indicates that bone salt is being

deposited in this region). Externally, the heads of the bones near the epiphysis stained more intensely than the shaft. None of these parts stained in the scorbutic animal.

If vitamin C promotes the absorption of calcium from gut then presumably the greater the amount of vitamin C given by mouth the greater the amount of calcium absorbed and presumably available for deposition on bone. On the other hand, if no vitamin C is given by mouth and the animal receives all its vitamin C by injection, minimal absorption of calcium should occur and less calcification of bones should take place—assuming of course that more calcium absorbed will result in more calcification. However, we cannot neglect the possibility that in a state of emergency calcium will be taken from the trabeculae and laid down on the surface of the bones. If, however, we use alizarin to stain freshly deposited bone salt the trabeculae would be unstained should this occur and the superficial part of the shaft stained. But another source of calcium is the inner surface of the shaft and alizarin experiments would not tell us whether or not calcium was being removed from this region.

In experiments described by the present author (Bourne, 1943) vitamin C was administered by mouth or by injection to guinea pigs and the animals were also injected with alizarin, the staining of the bones obtained was compared with another group of animals injected with equivalent amounts of calcium ascorbate. The results obtained were not entirely unequivocal but indicated that there might be more bone laid down in animals receiving vitamin C by mouth or injected as ascorbate than in animals receiving vitamin C by injection.

Despite the work of Salter and Aub (1931) already mentioned, Boyle (1938), and Boyle, Bessey, and Howe (1940) state that the process of calcification continues in the incisor teeth of guinea pigs even when they have been for some time on a scorbutic diet, but Friberg and Ringertz (1954) find a lowered uptake of radiophosphorus in bone in scurvy. Wolbach and Bessey (1942) stated that it was a generally accepted fact that vitamin C played no part in the process of calcification. The present author (Bourne, 1943) using 1:2:5:8:tetrahydroxyanthraquinone (alizarin Bordeaux) and alizarin itself (dihydroxyanthroquinone) was able to stain recently-formed bone *in vivo* and showed that the degree of staining (and therefore the amount of calcification) varied directly with the amount of vitamin C in the diet. This suggests that the vitamin does play a part in the process of calcification. However, calcification does not in normal animals take place in the absence of the appropriate matrix to receive the mineral salts so this result may still only mean that vitamin C deficiency affected the matrix formation.

Further investigations were made on the effect of vitamin C on the calcification of bone regenerating in a 1-mm hole in the guinea pig

femur. Alizarin staining again was greatest in the repair tissue in the holes in the animals receiving the largest amount of vitamin C (5 mg). Further experiments showed that this difference was due not to progressive differences in calcification but to variation in the *amount* of osteoid trabeculae present. In other words, if the amount of vitamin C given to the animals permitted any trabeculae to form at all then these trabeculae were as heavily calcified, as far as can be shown, by the technique used, as those formed in an animal receiving optimum amounts of the vitamin.

The result to be deduced from this work is therefore that the significance of vitamin C in bone formation and bone healing lies in its ability to promote the growth of the matrix.

It is of interest that vitamin P and sodium citrate had no effect in promoting bone healing or in increasing the alizarin staining of normal growing bones. On the other hand, an interesting result was obtained by the present author (1949b) by administering certain sugars and other substances to guinea pigs on a scorbutic diet.

The substances used were D-arabinose, D-glucose, D-mannose, sorbitol, sorbose, ketogulonic acid, L-rhamnose, rhamnolactone, sodium D-isoascorbate, and galactouronic acid. Animals receiving rhamnose and rhamnolactone gave almost as intense an *in vivo* stain in the skeleton with alizarin as those receiving ascorbic acid. The animals receiving the other substances showed little or no staining. This work suggests that rhamnose and rhamnolactone can permit matrix formation in the absence of vitamin C. Collagen and presumably ossein appear to be made of long-chain protein molecules with carbohydrate and probably vitamin C linkages. If the latter is correct then the deficiency of collagen in scurvy is due to the absence of sufficient vitamin C to maintain these linkages. It would appear that rhamnose and rhamnolactone might be able to replace vitamin C in this respect. However, in view of the fact that McManus and Saunders (1950) found that collagen is attacked by a polygalacturonidase suggests that galacturonic acid may be involved in these intrafibrillar linkages in the collagen fiber in which case it is surprising that this acid did not have the same effect as rhamnose. Although the possibility exists that the carbohydrate in ossein may be different from that of collagen, nevertheless, as Eastoe shows in Chapter IV, galactose and galactosamine have been separated from ossein.

6. HISTOLOGICAL CHANGES IN SCORBUTIC BONES AND TEETH

In intact bones characteristic changes develop in scurvy. In human scurvy the epiphyses in young persons were often found to be separated from the shafts of the bones (Aschoff and Koch, 1919; and Hess, 1920) and beading of the ribs may occur both in scorbutic infants and monkeys (Hart and Lessing, 1913).

Histologically, in scorbutic human bones one of the most obvious signs was subperiosteal hemorrhage. Marked thinning of the cortex of the bone (which was also brittle) was also present and the marrow had become yellowish and developed a gelatinous consistency (*Gerüstmark*). It is of interest that in experimental animal scurvy the *Gerüstmark* does not form in an artificially immobilized limb (Follis, 1951). At the junction of bone and cartilage in the heads of bones or at costochondral junctions the line becomes wavy instead of straight. At the junction of a Trümmerfeld zone develops in which irregular and unorientated trabeculae are scattered, and in which irregularly shaped and scattered cells are also present. Cell and bone detritus is also present. According to Hess the "picture is that of weakened bone having been crushed by the pressure of the more compact cartilage." The cells which are present are mainly spindle or stellate connective tissue cells with relatively few osteoblasts. Those which are present in association with the reduced trabeculae become spindle-shaped and shrivelled. It is of interest that there is no increase of osteoclasts associated with those trabeculae or with the walls of the medullary cavity so that the rarefaction observed is not obviously due to increased osteoclastic activity. Hess in fact suggested that the thinning of the trabeculae is due to the combination of normal bone resorption and the failure of normal bone regeneration. Aschoff and Koch showed that the cells of the framework "marrow" (*Gerüstmark*) are normally able to make bone trabeculae but that in scurvy there is a lack of the material required to build the osteoid; however, since the cells presumably make this material it is difficult to follow their reasoning.

Follis (1948), describing bone changes in scurvy, based his report on a study of the experimentally induced disease in guinea pigs and on a hundred cases of human scurvy.

As the scorbutic state developed, he found that the cartilage cells of the epiphyseal plate continued to multiply and orientate themselves and calcium salts continued to be deposited between the rows—however, beyond that point trouble occurred: the osteoblasts failed to lay down osteoid, and the calcified matrix was not absorbed; the result was an accumulation of calcified matrix under the cartilaginous plate of the epiphysis which continued to grow rapidly. Park described this arrangement as a "scorbutic lattice." Breaking the spindles of bone in the matrix produced the "crushing" effect described by Hess. Finally, constant fracturing of the spicules resulted in the building up of an area completely lacking in orientation which is the "Trummerfeld" zone of the older authors.

Follis (1948) however, showed that if he immobilized one leg by placing it in a plaster cast thus protecting it from normal stresses and

strains, it did not exhibit the characteristic changes of scurvy while the opposite leg did. The absence of fractures, hemorrhage, pink-staining material and fibroblastic proliferation from such a femur, i.e. the absence of a "Trümmerfeld" zone indicated that these were all secondary to the stresses and strains and that the fundamental lesion caused by the deficiency was simply an unabsorbed calcified lattice and absence of osteoid formation.

Although a good deal of experimental work has been carried out on the effects of acute deficiency of vitamin C on experimental animals, only Murray and Kodicek (1949a, b and c) appear to have studied the effects of prolonged partial deficiencies. In guinea pigs subjected to such treatment they found that stiffness of the knee joint was a characteristic early symptom and that this was due to degeneration and edematous changes in muscles and the formation of hyperplastic connective tissue at and around the joint. Later, the shafts of some of the long bones showed a considerable amount of spongy subperiosteal bone. Associated with this was a typical osteoporosis. It was of interest that this osteoporosis was restricted to the old bone of the shaft, the new bone which formed simultaneously showed no signs of it. Höjer had also shown in 1924 that in scurvy it was possible to obtain osteoporosis of the jaw bone simultaneously with the production of spongy bone on the surface. The diaphyseal thickenings of scurvy had also been demonstrated by Mouriquand and his colleagues by radiographical means (1937, 1938, 1940).

In the production of this new subperiosteal bone Murray and Kodicek stated that it appeared to be preceded by subperiosteal absorption of old bone. In the space so formed between the periosteum and the shaft of the bone a diffuse mesenchyme-like tissue developed and in this appeared a number of stellate osteoblasts which bore many resemblances to fibroblasts. These cells deposited a granular bone which subsequently became covered with a fibrillar bone. If the bone was fractured the amount of periosteal bone formed was greatly increased. The summary of the changes found by these authors in a fractured bone (fibula) in the chronically scorbutic guinea pig are as follows. There was more absorption from the broken ends of the bones, no cartilage formed (unlike the normals) and the subperiosteal bone (a small amount of which forms in all fractured bones some distance back from the injury, "distant periosteal reaction") increased enormously and formed a great thickening which extended the whole length of the diaphysis. Eventually this newly formed subperiosteal bone moved towards the fracture and in some cases covered it—the callus formed inside, however, was never complete and remained of light trabecular construction.

As already mentioned there are characteristic changes in the teeth as well as in bone of guinea pigs on a scorbutic diet. The teeth which

are particularly susceptible to such changes are the persistently growing incisors.

Jackson and Moore (1916) and Zilva and Wells (1919) were the first to show that when guinea pigs are placed on a scorbutic diet they suffer from characteristic changes in the incisor teeth. Howe (1920, 1921, 1923) was able to produce alveolar resorption, spongy gums, irregularities in the teeth and even caries with a scorbutic diet. Histological studies of scorbutic teeth were made by Höjer and Westin (1925), Toverud (1923), and Key and Elphick (1931), and in 1934 Fish and Harris published a very detailed work on this subject. They found that in full scurvy the odontoblasts lost their fibrils and died. Even in sub-scurvy the odontoblasts degenerated before their normal time, casting off their fibrils in the process. The normal process of scar formation in the pulp was also interfered with. The ameloblasts were also affected though rather later in the disease than the odontoblasts. In sub-scurvy some enamel continued to be formed but in full scurvy there was a complete failure of formation—the ameloblasts either disappeared altogether or became completely keratinized. Normal cementum was also found to be absent in scorbutic teeth, being replaced by an amorphous deposit of calcific scar tissue which enclosed occasional cementoblasts.

Harris and Fish concluded that deficiency of vitamin C in guinea pig diets affected the viability of all cells associated with the laying down of the three hard tissue components of teeth. This was associated with general degeneration of the connective tissue of the dental pulp and congestion of its vessels. Other changes present in the teeth were obviously secondary to the degeneration of the cells. Particularly was this so in the case of the dentin where any changes in this tissue naturally followed the degeneration of the odontoblastic fibrils. One of the results of this process was the deposition of calcific scar tissue, which had been described by a number of earlier workers as "pulp bone," inferring that the odontoblasts changed to osteoblasts and produced bone instead of dentin. There is no real evidence in the literature that such a metaplasia of the odontoblasts occurs and the explanation is much more likely to be, as Fish and Harris assert, that the so-called "pulp bone" is simply calcific scar tissue. Other workers on this problem include Wolbach and Howe (1926); Dalldorf and Zall (1930); Wolbach (1933); Boyle *et al.* (1936); Glasunow (1937); Boyle (1938); Ham and Elliott 1938; Dalldorf 1939; McLean, *et al.* (1939); Boyle, Bessey, and Howe (1940); and Wolbach (1947). All have established the essential nature of vitamin C for the development of incisor teeth in guinea pigs and in particular that it is necessary for the production of predentin, which can be regarded as the equivalent in the tooth of the osteoid of bone. Boyle *et al.* (1936) claimed that vitamin C was not essential for the calcification of teeth and

Wolbach and Howe that the pulp became shrunken, completely detached from the dentin and appeared to float in a fluid material. It seems that this latter appearance may have been due to an artefact. They claimed (Wolbach and Bessey, 1942) that the prompt appearance of bone matrix and of dentin when vitamin C was administered to a scorbutic animal suggested "that the failure of cells to produce an inter-cellular matrix in scurbutus is the result of the absence of an agent common to all supporting tissues which is responsible for the setting, fibrillation, or jelling of a product which would otherwise remain liquid."

7. CONCLUSIONS

Follis (1951) in summing up the effect of vitamin C deficiency said,

"It would seem best to designate scurvy as that part of the overall picture of ascorbic acid deficiency which is characterized by a failure of certain specialized cells, i.e., fibroblasts, osteoblasts and odontoblasts to promote the deposition of their respective fibrous proteins: collagen, osteoid and dentin."

That the effects of the disease are due primarily to the failure of specific cells is also born out by Follis's observations that osteocytes, osteoblasts, and the cells of the Gerüstmark showed a lowered oxidative activity (Nadi reaction for cytochrome oxidase). Administration of the vitamin brought about simultaneous return to normal of cytochrome oxidase activity and of fiber production. The Gerüstmark cells also showed very little periodic acid-Schiff (PAS) positive material (see Plate II, Figs. 7 and 8 for altered distribution of PAS positive material in scorbutic trabeculae). When the scurvy was cured the spindle-shaped cells of this tissue changed into a plumper shape and their cytoplasm became metachromatic and gave a stronger PAS reaction. A return of basophilia in these cells and in osteoblasts and osteocytes was shown to be due to ribonucleic acid, which in the scorbutic condition had been depressed.

We are faced with the established fact, enunciated by Wolbach some twenty years ago, that there is a failure of the production of inter-cellular materials in scurvy. Although a relation has been shown between vitamin C and tyrosine metabolism and an ascorbic/ascorbate/glutathione redox system is likely, it is difficult to see exactly why the deficiency of the vitamin so specifically effects the formation of connective tissues. Any attempt to explain its action can be little more than speculation. In addition we have the information that the respiratory activity of most cells in the body (Bourne and Hill, 1954; King, 1950), particularly those associated with the connective tissues (Follis, 1951), is depressed in scurvy, which might itself explain the decrease in synthetic activity of these cells. Although there appears to be loss of phosphatase activity in scorbutic bones this appears to be due mainly to

decrease of phosphatase-impregnated matrix. Histochemically, the matrix-producing cells appear to give as intense a histochemical reaction for phosphatase as the normal and any matrix that is produced seems to contain at least a near normal amount of phosphatase (however, see King's results). It appears to be the synthesis of both fibers and structureless matrix which are inhibited by lack of vitamin C and the decreased incorporation of radioactive sulfur into chondroitin sulfate appears to be an indication of one type of breakdown in the synthetic mechanism. It is of interest that a decreased synthesis of chondroitin sulfate also occurs under the influence of cortisone and in this case it seems unlikely to be due to decreased respiratory activity of the cells since it has been shown (Malaty and Bourne, 1953) that cortisone appears to increase the respiratory activity of the cells of many organs. Further work on these lines, however, should ultimately reveal the secret of the role of vitamin C in the maintenance and repair of the connective tissues.

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PLATE I

1. Von Kossa preparation of costochondral junction of normal guinea pig to show number of trabeculae. *Ca* = cartilage; *T* = trabeculae; *CR* = cortex of bone; *M* = marrow. See Bourne, 1943.
2. Von Kossa preparation of costochondral junction of scorbustic guinea pig to show greatly reduced number of trabeculae. *B* = deposit of cartilaginous bone salt in portion of rib. See Bourne, 1943.
3. Phosphatase preparation of undecalcified section of costochondral junction of normal guinea pig. Moderately dark reaction given by cartilage is artifact. Note thick line of phosphatase activity at *A* which is continuous on right with phosphatase-positive periosteum (*P*). (Note: periosteum has been cut away on the left side of the shaft.) See Bourne, 1943.
4. Phosphatase preparation of undecalcified section of costochondral junction of scorbustic guinea pig. Note great reduction of line of phosphatase (*A*) at junction. See Bourne, 1943.
5. Longitudinal section of an area of the epiphyseal plate at the proximal end of the tibia of a control guinea pig. Phosphatase is present in the columnar cells and hypertrophic cells. Less activity occurs in the zone of calcification. The dark grey appearance of the trabeculae in the metaphysis is due to bone salts which were not completely removed in this section.
6. Scorbustic animal. Area comparable to that of 5. Some indication of columnar arrangement of cells is present. The cells contain the enzyme but the amount is less than in the controls.

(5 and 6 and descriptions are reproduced by permission of Drs. A. Zorzoli and E. M. Nadel, and the *Journal of Histochemistry and Cytochemistry*.)

PLATE I

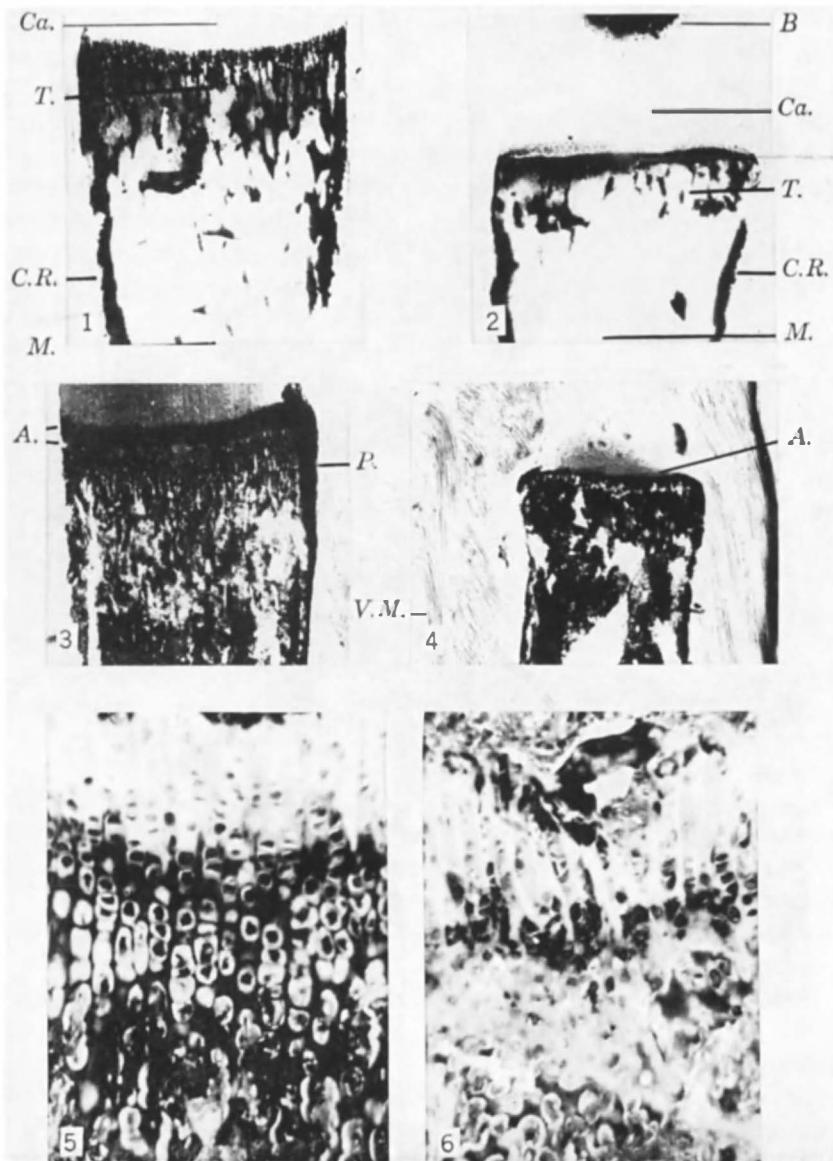


PLATE II

7. Periodic acid Schiff reaction in costochondral junction of normal guinea pig. Note even distribution of reaction through developing trabeculae and in cartilage matrix.

8. Periodic acid Schiff reaction in costochondral junction of scorbutic guinea pig. Note general reduction of reaction but greatly increased intensity along the edges of developing trabeculae.

9. Autoradiograph of costochondral junction of normal guinea pig showing incorporation of radiosulfur (S^{35}) into cartilage (below) and at junction.

10. Autoradiograph of costochondral junction of scorbutic guinea pig showing greatly reduced uptake of radiosulfur in cartilage and at junction.

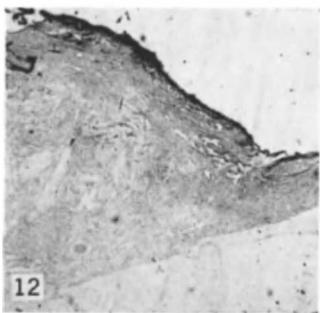
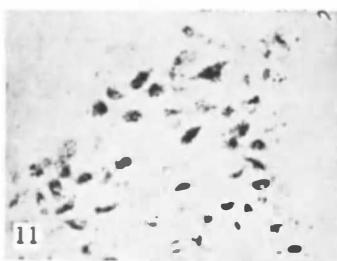
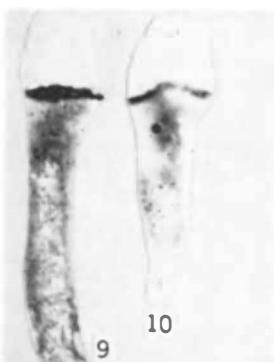
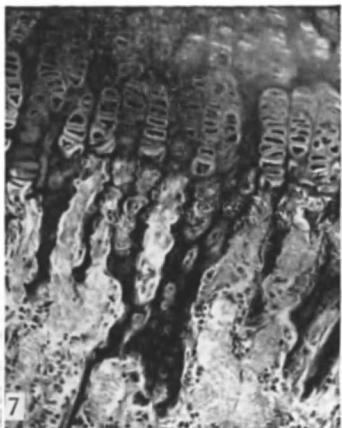
(7, 8, 9, 10 are from preparations by Miss C. R. Hill made in the author's laboratory.)

11. Group of cartilage cells in 12-day chick embryo stained to demonstrate vitamin C. They show a strong positive reaction. See Barnett and Bourne, 1942.

12. Phosphatase preparation of repair tissue in hole bored in femur of scorbutic guinea pig. One week after operation. Virtually no phosphatase is present in this area with the exception of a line of activity on the surface of the repair tissue. (See Bourne 1942a.)

13. Phosphatase preparation repair tissue in hole bored in femur of normal guinea pig. One week after operation. Note phosphatase-positive fibers and a number of phosphatase-positive trabeculae. (See Bourne 1942a.)

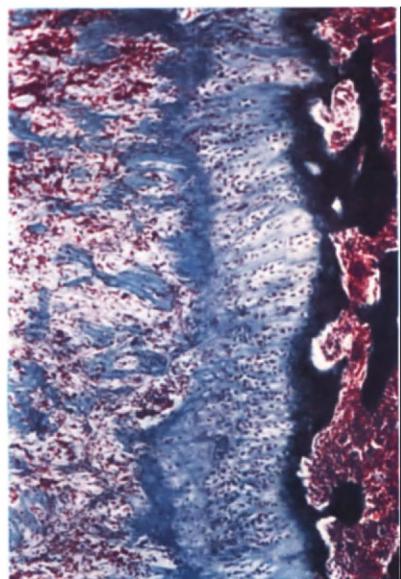
PLATE II



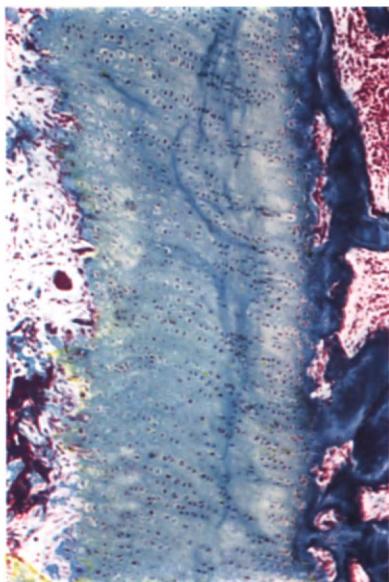
COLOR PLATE

1. Proximal epiphyseal cartilage of tibia. Scorbatic guinea pig showing failure of matrix formation. Mallory's connective-tissue stain.
2. Repair after 72 hours following ascorbic acid administration. Proximal epiphyseal cartilage, tibia.
3. Repair after 96 hours, proximal epiphyseal cartilage, tibia, guinea pig. To show approximately normal cartilage cells and matrix.
4. *Upper left.* To show collagen deposition in relation to an isolated cell deep within a blood clot. Repair after 72 hours following excision of muscle.
5. *Middle upper.* Same preparation to show collagen deposition of cells which have penetrated fibrin strands. Repair after 72 hours.
6. *Upper right.* A corresponding field, reticulum stain, repair of a blood clot after 72 hours.
7. *Lower left.* Repair of a blood clot after 96 hours. To show collagen deposition in a group of fibroblasts which have not yet assumed their normal morphology.
8. *Lower right.* Resumption of deposition of matrix in endochondral bone formation. Reticulum stain. Recovery period of 40 hours.
9. Fibroblasts in relation to fibrin strands in organization of a blood clot in absolute scorbatus. Note prominence of fibroglia fibrils and cytoplasmic vacuoles in fibroblasts. No stainable collagen or argyrophilic fibrils present. Guinea pig operated on the 23rd day of deficient diet, killed on the 33rd day.
10. To illustrate collagen formed in organization deep within a blood clot, following 42 hours of ascorbic acid therapy. Note the homogeneous appearance of the collagen.

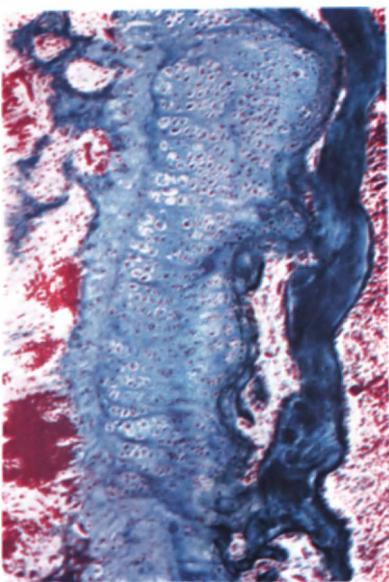
(From Wolbach, 1953, by permission of the *British Journal of Nutrition* and *Proceedings of the Nutrition Society*.)



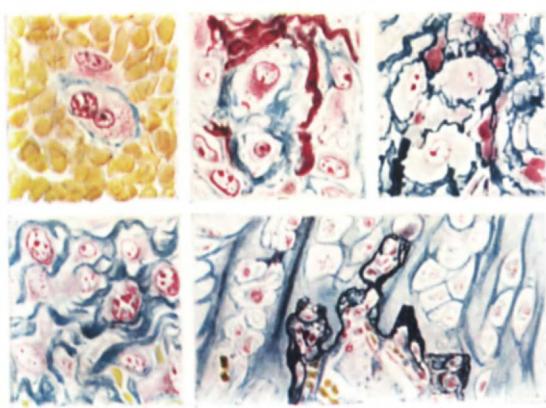
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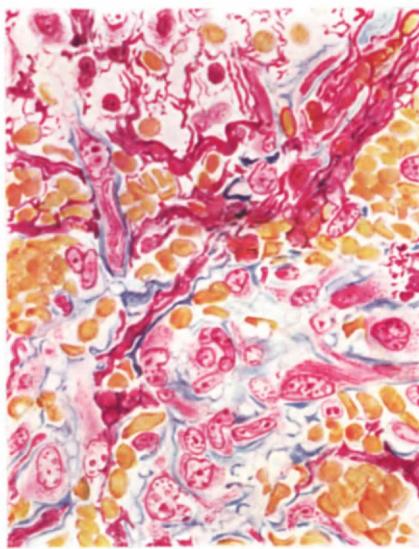
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CHAPTER XIX

VITAMIN D AND BONE

LESLIE J. HARRIS

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I. Introduction

Vitamin D is the anti-rachitic factor. That is to say, it is the substance, or to be more correct the group of closely related substances, whose most remarkable property is in relation to the calcification of newly forming bone—in other words, in preventing the deficiency disease, infantile rickets.

II. Chronological Survey

1. EARLY ACCOUNTS OF RICKETS

The first detailed description of rickets is to be found in the celebrated book "*De Rachitide*," published in 1650 by F. Glisson of Gonville and Caius College, Cambridge. Five years earlier, Daniel Whistler, a young medical student from Merton College, Oxford, published at Leyden, in the Low Countries, for his M.D. thesis, a work "*De morbo puerili Anglorum quem patrio idiomate indigenae vocant*, The Rickets."

2. "DIE ENGLISCHE KRANKHEIT"

Possibly because of these investigations of Glisson and other English authors, rickets soon came to be known in Central Europe as *die englische Krankheit*—or it may be it was because of its special prevalence in England.

3. COD-LIVER OIL

The use of cod-liver oil as a cure for rickets dates from about 1782, and from then on gained ground steadily. By 1865, the eminent French clinician, Trousseau, referred to cod-liver oil as "the well-known and perfect cure for rickets."

4. RICKETS IN ANIMALS; RECOGNITION OF A DIETETIC FACTOR

Perhaps the first to produce rickets experimentally in puppies had been Jules Guerin in 1838, and he, like many others who followed him, was able to substantiate the theory that it arose from a faulty diet. The same conclusion followed from the observations of Bland Sutton, an English surgeon, who in 1889 described how he had cured rickets in lion cubs and other animals at the London Zoological Gardens by giving them cod-liver oil, milk, and crushed bones.

Nevertheless, clinical opinion was divided, and many rival theories were propounded.

5. AN ANTI-RACHITIC VITAMIN POSTULATED

Perhaps the first definite suggestion that rickets was due to deficiency of what we should now call a vitamin was due to F. G. Hopkins, who in 1912 said: "In diseases such as rickets, and particularly in scurvy, we have had for long years knowledge of a dietetic factor; but though we know how to benefit these conditions empirically, the real errors in the diet are to this day quite obscure. They are, however, certainly of the kind which comprises these minimal qualitative factors that I am considering."

Still more definitely Funk, in 1912, propounded what he termed the "Vitamine Hypothesis," according to which the existence was postulated of separate anti-beriberi, anti-scurvy, anti-rickets, and anti-pellagra "vitamines."

There followed the recognition of various separate vitamins: first called "Fat-soluble A," "Water-soluble B" (McCollum and Davis, 1915), but then more definitely renamed vitamins A, B, and C—A being present in cod-liver oil and curing xerophthalmia and restoring normal growth in rats (Osborne and Mendel, 1913a, b; McCollum and Davis, 1913), B in rice bran and wholemeal cereals and curing beriberi (Eijkman, 1890) and C in fresh fruit and vegetables and curing scurvy (Holst and Frölich, 1907, 1912).

6. EXPERIMENTAL RICKETS; DIFFERENTIATION OF VITAMIN D

In 1918, E. Mellanby published an account of his detailed observations on the various factors that, as he found, conducted to experimental rickets in dogs. He concluded that the disease could be prevented by

the inclusion in the diet of certain fats such as cod-liver oil or butter, but not by various vegetable oils, such as linseed or olive oil. At this time, cod-liver oil was already recognized as the source *par excellence* of vitamin A, which had not yet been differentiated from vitamin D; and it was natural, therefore, to ascribe the anti-rachitic action of cod-liver oil to its vitamin A. A little later, workers in America (McCollum *et al.*, 1921; Sherman and Pappenheimer, 1921) found for the first time how to produce experimental rickets in rats (namely, by an imbalance of the mineral elements in addition to a simple deficiency of vitamin D); and shortly afterwards followed the differentiation of the anti-rachitic factor, then renamed vitamin D, from vitamin A proper (McCollum *et al.*, 1922). The main points of distinction were: differences in distribution, and differences in stability to heat.

7. RICKETS AND SUNLIGHT

The chemical identification of vitamin D, or rather of the form of vitamin D now called D₂, followed, historically viewed, from a study of the anti-rachitic action of sunlight. In 1890, Palm had pointed out that rickets was most prevalent in the temperate zone or wherever the sunlight was deficient, e.g. in humid climates, in smoky or overcrowded industrial areas, or where the rite of *purdah* (seclusion) was observed. (This influence of sunlight is apparent also in the seasonal variation and in the greater susceptibility of more deeply pigmented peoples, the so-called "racial factor.")

Another important step forward came in 1919, when Huldschinsky in Berlin showed how rickets could be cured by artificial light therapy no less than by direct sunlight—namely, by the rays from a quartz mercury-vapor lamp. The explanation of what at first seemed a strange phenomenon remained a puzzle until 1924, when it was discovered that to cure rickets it was equally effective to irradiate either the animal or the food that the animal consumed (Hess and Weinstock, 1924; Steenbock and Black, 1924). The deduction correctly drawn was that the ultraviolet rays reacting on the skin of the body, or on the foodstuffs, activated some vitamin precursor in them, and thereby converted it into the vitamin itself.

8. IDENTIFICATION OF VITAMIN D₂

It remained to isolate the components in foodstuffs which became active on irradiation. For this purpose, experimental animals had to be used to test the potency of the irradiated products. The first step was to find that the provitamin resided in the unsaponifiable residue of the cod-liver oil after the fat had been removed, namely, in the crude

cholesterol fraction (Rosenheim and Webster, 1925; Hess and Weinstock, 1925; Steenbock and Black, 1925). Repurified cholesterol was, however, inactive, and various other sterols were tested, and of these ergosterol was identified as a provitamin, that is to say it is converted into the vitamin by irradiation (Rosenheim and Webster, 1927a, b, c, and d; Windaus and Hess, 1927). At first it was thought to be the one and only provitamin, but this supposition was subsequently to be disproved.

Finally, the vitamin was itself isolated in a pure state from the irradiation products of ergosterol (Askew *et al.*, 1931; Windaus, 1931a, b). It was first named D₂* or calciferol, but the official name, by international agreement, is ergocalciferol.

9. VITAMIN D₃; OTHER D VITAMINS

Not until 1933 was it shown that the form of vitamin D present, in preponderating proportion, in cod-liver oil (i.e. vitamin D₃) is distinct from that obtained by irradiating ergosterol. It differs but slightly in chemical structure (see p. 616), and can be obtained artificially by irradiation of 7-dehydrocholesterol (cholestadien-3-ol). Its new name is cholecalciferol. This is the "natural" form of vitamin D, produced in the animal body under the action of ultraviolet rays.

Other forms of vitamin D also have been found to occur in traces in some products, and are sometimes referred to as D₄, D₅, etc., but they are of little practical importance.

10. OTHER DEVELOPMENTS

Perhaps the most notable feature in the field of vitamin D is the almost complete elimination, in living memory, of severe rickets, which at the beginning of the present century was well nigh universal in various industrial areas in the temperate zone. Mild rickets, however, still remains.

Public health measures have been taken by government authorities in many countries to guard against risks of widespread deficiency of vitamin D, especially among infants and expectant or nursing mothers (pp. 599, 605, etc.).

No generally applicable chemical methods are yet available for estimating vitamin D, the standard procedure being the biological tests on rats or chicks which have now been in use for upwards of thirty years. Because of its limited distribution in foods, this is perhaps less of a handicap than it would be for various other vitamins.

Vitamin D was the first of the vitamins with which was demon-

* For explanation of the now obsolete term "vitamin D₁," see below, p. 617.

strated the possibility of producing toxic effects by giving an excess (Kreitmair and Moll, 1928; Harris, 1932). The effects of excess can be interpreted as essentially an intensification of its normal mode of action (Harris, 1932). Less detailed knowledge is as yet available about vitamin D than about some other vitamins as to the biochemical mechanism of its mode of action: the essential features (see also p. 606) are a diminution in the loss of phosphate or calcium (or both) in the feces, and a corresponding rise in the blood level, with a consequent increased deposition of calcium salts in the sites to be calcified (Harris, 1932).

One of the most interesting of recent developments has been the use of vitamin D for purposes other than that of simple replacement therapy, for example in parathyroprivic tetany, and in lupus vulgaris.

III. Vitamin D as Anti-Rachitic Vitamin

Vitamins have been defined (Harris, 1951) as "substances that (a) are distributed in foodstuffs in relatively minute quantities, that (b) are distinct from the main components of food (i.e. proteins, carbohydrates, fats, mineral salts, and water), that (c) are needed for the normal nutrition of the animal organism, and (d) the absence of any one of which causes a corresponding specific deficiency disease."

Vitamin D satisfies these criteria: (a) The amount present in foods being almost incredibly small: less than one in 400,000 in cod-liver oil or one in 50,000,000 in summer butter; apart from fish-liver oils, egg yolk, and milk, vitamin D is not present in measurable amounts in other known foods. (b) Vitamin D, in all its forms, possesses a distinctive chemical character, with a specialized steroid structure. (c) Calcium and phosphate metabolism become deranged in absence of vitamin D. (d) Its deficiency is the major cause of rickets, as well as of infantile tetany and of osteomalacia (adult rickets).

IV. Definition of Rickets

Rickets may be defined as a disease seen principally (but not exclusively) in early childhood, and characterized by an inadequate calcification of the bony structures, especially of the newly forming bone, with a liability to consequent bony abnormalities and deformities, such as enlarged epiphyses, knock knees or bow legs, pigeon chest, etc. The characteristic biochemical abnormalities are the low levels of blood phosphate (specifically the inorganic phosphate fraction), and sometimes of the blood calcium; the inadequate mineralization of the bones; and (in active rickets) a raised blood phosphatase level. The hypocalcemia sometimes associated with rickets is the cause of tetany. The inadequate

mineralization of the bone, especially at the growing end of the long bones, gives rise to the characteristic X-ray appearance, which is so useful in diagnosis, and in following the effect of treatment.

V. Abnormality of Bone Structure and Composition in Rickets

1. PATHOLOGY

From what has been said above, it is clear that the underlying lesion in rickets is a deficient calcification of the bones, particularly manifested in the newly forming bone at the epiphysis. The zone of provisional calcification becomes increased in breadth and inadequately mineralized. An irregular growth of the soft osteoid tissue follows, and a consequent swelling of the epiphyseal junction ("rachitic metaphysis"). The bending of the inadequately calcified bones and the overgrowth of osteoid tissue causes the well recognized deformities—bow legs, knock knees, rachitic pelvis, "green-stick" fractures, the enlargement of the ends of the bones, and the beading of the ribs (p. 599).

The characteristic changes occurring progressively in rickets, at the epiphyseal end of the bone, have been listed by Park (1938-9) as being essentially as follows:

- (1) Calcium salts fail to be deposited in the cartilage matrix.
- (2) The cells consequently fail to mature, hence are impervious to invasion by the blood vessels, and therefore accumulate instead of becoming destroyed.
- (3) The proliferating cartilage cells suffer compression.
- (4) The proliferating cartilage becomes swollen, elongated, and degenerate.
- (5) The cartilage is subject to irregular invasion by tufts of capillaries.

For further information concerning the histopathology of the bone in rickets, the legends accompanying the illustrations may be consulted (Figs. 1 to 5).

2. PATHOGENESIS

In the view of the present writer (Harris, 1932) the immediate cause of the deficient calcification of the bone in rickets is the low level of the phosphate in the blood, or more correctly the low calcium phosphorus product (Iversen and Lenstrup, 1919; Howland and Kramer, 1921, 1922, 1923). Ultimately this chemical defect in the blood is related to an inadequate "net absorption" (see p. 606) of the phosphorus and/or calcium from the intestine. This will be considered later (See Section VII, "Mode of action of vitamin D").

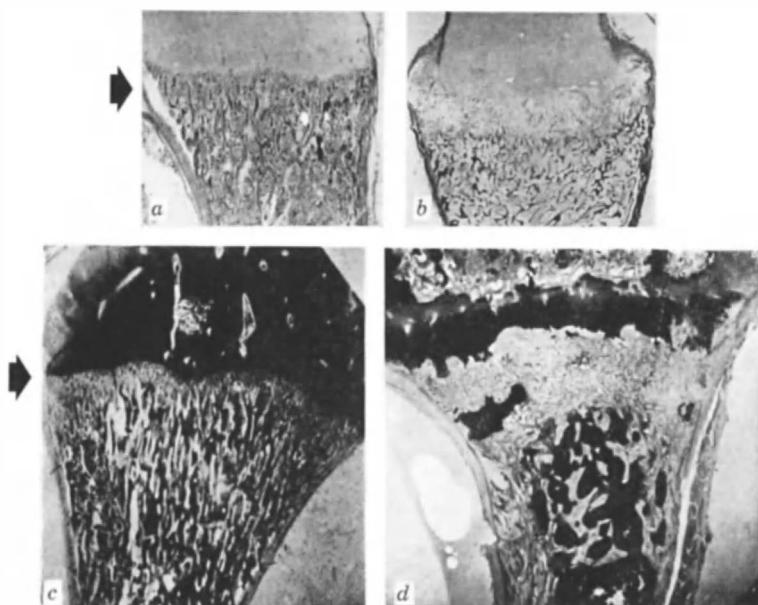


FIG. 1. Abnormal bone structure in rickets (from Harris 1951, after Boyd and Kihm).

1a and 1c. Normal bone.

1b and 1d. Rachitic bone.

The normal bone has a regular line of ossification (at arrow), whereas in rickets there is a wide irregular band of imperfectly calcified cartilage and osteoid tissue. The end of the bone is abnormally widened.

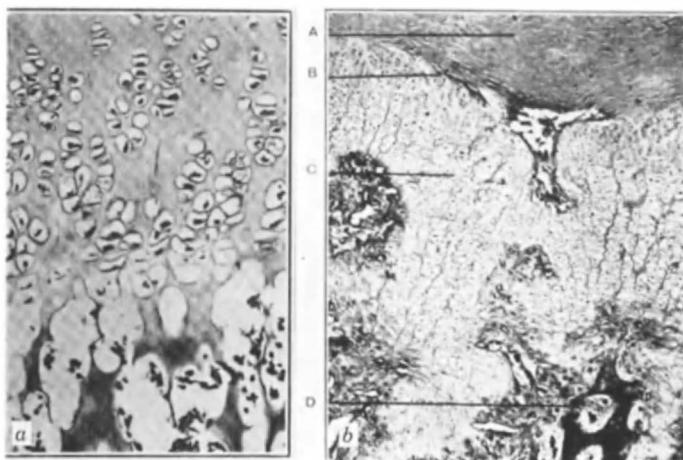


FIG. 2. Abnormal ossification in rickets (after Vines, 1940).

2a. Normal ($\times 110$). Note the orderly process of ossification.

2b. Rickets ($\times 30$). Cartilaginous proliferation is apparent (at B), with production of hyperplastic cartilage cell columns (C), and osteoid tissue (D). Note the irregular and excessive vascularization throughout.

3. CLINICAL PICTURE

The symptomatology of rickets is discussed below, p. 597.

4. DIAGNOSIS

A low blood phosphate (or $\text{Ca} \times \text{P}$ product) is characteristic of active rickets. In fact an analysis of the blood either for phosphorus and calcium or for blood phosphatase (see Smith, 1933) is the only certain method of detecting incipient rickets or mild rickets. The radiographic abnormalities at the epiphyses do not become apparent until a later stage, but they form the most useful guide in following the course of healing (Figs. 6 and 7).

5. CHEMICAL COMPOSITION OF BONE IN RICKETS

The reduction in the calcium and phosphate content of the bones in rickets is well shown in the accompanying diagram (Fig. 8, p. 596) drawn from the data of Schabad (1909), or in Table I, based on the data of Czerny and Keller (1928).

TABLE I
MINERAL CONTENT OF BONE IN RICKETS

Group	Ash in bones, %
Normal children	56-60
Rachitic children	22-32

VI. RICKETS AS A DEFICIENCY DISEASE

Our knowledge of the nature of rickets, and of the various inter-related factors concerned in its production, is derived largely from the study of rickets produced experimentally in laboratory animals: it will, therefore, be convenient to consider this aspect before turning to rickets in the human being.

1. EXPERIMENTAL RICKETS

The pioneer investigations on experimental rickets in dogs and rats have already been briefly mentioned above (Section II, 4 and II, 6); the other species most frequently used have been chicks (e.g. Hart *et al.*, 1923-24; Methods of Analysis, 1950).

The characteristic appearance of rickets in dogs and chicks is indicated in the accompanying illustrations (Figs. 9 and 10, p. 598). In various other species, including pigs, lambs, kids, calves, and poultry, rickets has also been noted, occurring either experimentally or spontaneously. Two of the diets commonly used for the experimental production of rickets are given in Tables II and III.

TABLE II
RACHITOGENIC DIET FOR RATS
(McCollum Diet No. 3143)

Ingredient	Parts by weight
Whole yellow maize, ground	33
Whole wheat, ground	33
Gluten, ground	15
Gelatin	15
Calcium carbonate	3
Sodium chloride	1

TABLE III
RACHITOGENIC RATION FOR CHICKS
(United States Association of Official Agricultural Chemists, Diet No. 156)

Ingredient	Parts by weight
Yellow maize, ground	58
Wheat flour middlings	25
Casein, crude and precipitated	12
Calcium phosphate, precipitated	2
Iodized salt (0.02% KI)	1
MnSO ₄ • 4H ₂ O	0.02
Yeast, non-irradiated	2
Vegetable oil	Variable

2. LIST OF ETIOLOGICAL FACTORS IN EXPERIMENTAL RICKETS

In animals, as in human beings, the two major rachitogenic factors are:

- (1) Absence of vitamin D.
- (2) Lack of exposure to ultraviolet irradiation.

Other important rachitogenic factors, in the diet, include

- (a) insufficiency of calcium and phosphate;
- (b) an imbalance between the calcium and phosphate intake (particularly a relative excess of Ca);
- (c) phosphorus in non-available form, including,
- (d) phytic-acid phosphorus (functioning as an anti-calcifying factor);
- (e) an increased base-acid ratio, or an increased pH value;

FIG. 3. Rickets. Early changes at chondro-osseus junction (from Kramer and Kanof, after Park).

3a. Focal defects in calcification in the zone of preliminary calcification (vertical section of proximal end of tibia).

3b. Same, higher power. Note cartilage cells at first maturation, with some of them disappearing at chondro-osseus junction; irregular vascularization beginning; inadequate calcification in zone of provisional calcification.

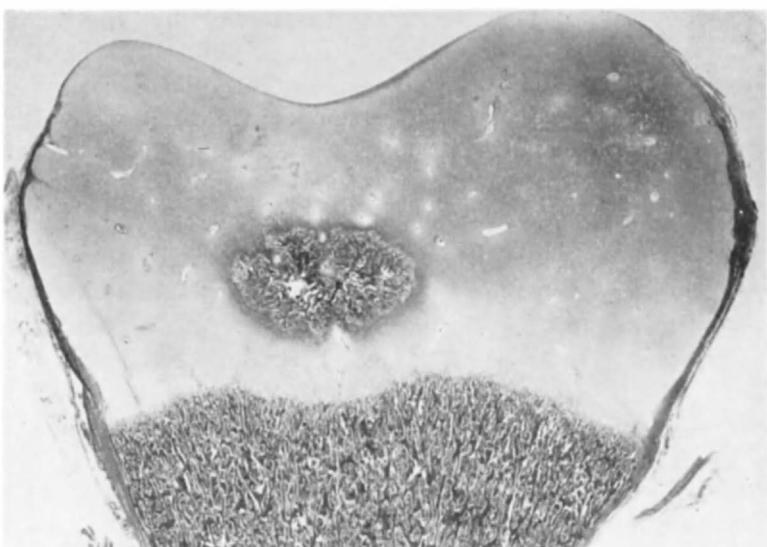


FIG. 3a

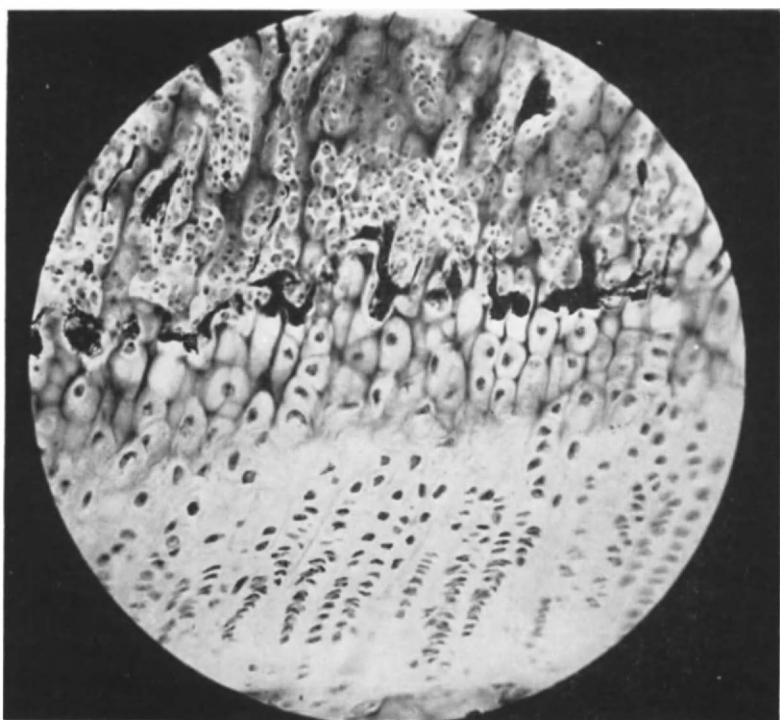


FIG. 3b

- (f) various other factors making for insolubility, or diminished absorbability, of the phosphate and calcium salts, e.g. (i) presence of beryllium salts, (ii) excess of fat.

In the reverse direction, certain components in the diet can diminish the severity of rickets: for example, lactose. Its action is thought to consist of the promotion of an acid reaction in the gut.

3. SIGNIFICANCE OF THE VARIOUS ETIOLOGICAL FACTORS

a. *Deficiency of Vitamin D and Ultraviolet Irradiation*

As already emphasized, the two most potent practical influences in the production of rickets, certainly in the medical field, are (1) the consumption of a diet low in vitamin D, and (2) the absence of ultraviolet irradiation (which can generate vitamin D from its precursor, or precursors in the body). The studies that first most clearly established this dual causation of infantile rickets were those carried out for the Medical Research Council at Vienna during the famine at the close of World War I (Chick *et al.*, 1923).

b. *Phytic Acid in Cereals*

Phytic acid (inositol-phosphoric acid) is also mentioned in the above list. The work of Bruce and Callow (1934) first demonstrated that the well-known rachitogenic action of cereals could be attributed to their containing this particular form of combined phosphorus. Not only is the phytic acid phosphorus poorly utilized, in addition, it can actively interfere in the absorption of calcium by forming insoluble salts with it. Thus, phytic acid can be regarded as an anti-vitamin D, or a "toxamin" (Mellanby, 1937).

There has been some discussion about the practical consequence of phytic acid in human nutrition. It happens that there is more of this substance in wholemeal flour, or "wheatmeal," than there is in white flour. On the assumption that this fact was a possible objection to the abandonment of white bread in World War II, the government in the United Kingdom decided on the addition of calcium carbonate to the National Wheatmeal Loaf, the object being thereby to balance any relative deficiency of calcium produced by the phytic acid. It was perhaps not sufficiently realized at the time that, during the process of bread

FIG. 4. Advanced rickets (from Kramer and Kanof, after Park).

4a. The epiphyseal line has become very wide and irregular, calcification and ossification are defective at chondro-osseus junction, and there is some appearance of osteophorosis in the rachitic metaphysis.

4b. Note the capillary tuft invading the cartilage, and the large masses of uncalcified osteoid.

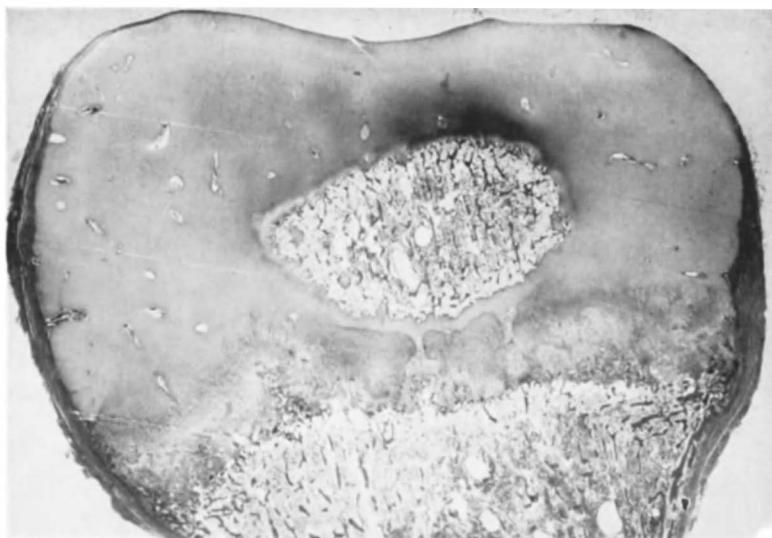


FIG. 4a

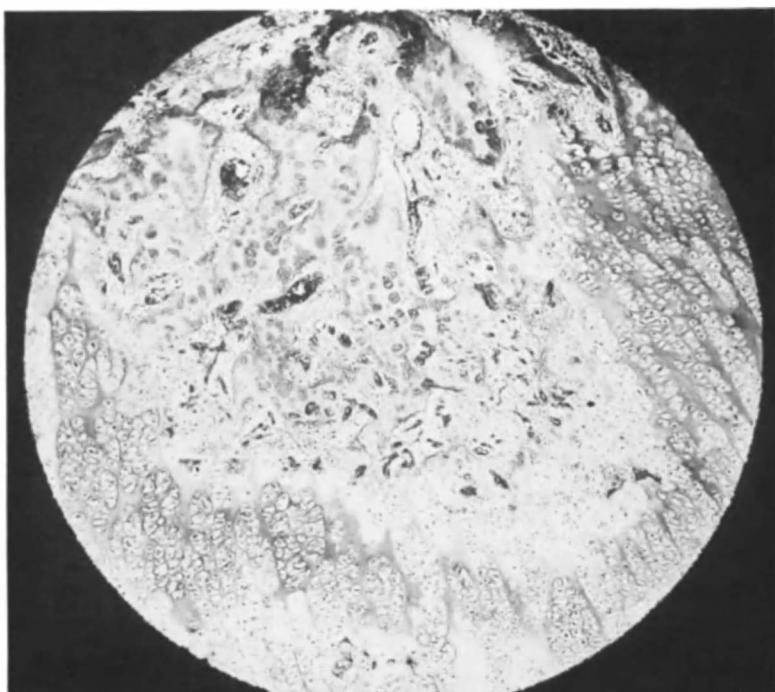


FIG. 4b

making, in the raising of the dough, much of the insoluble inositol-phosphoric acid is rendered innocuous by hydrolysis into readily assimilable phosphate, by the action of the enzyme phytase present in the flour.

c. Other Anti-Vitamin D Factors

As already indicated, in the opinion of the present writer (Harris, 1932), the various factors antagonizing the action of vitamin D may be considered to exert their effect essentially by reducing the absorbability of the calcium and/or phosphate salts from the intestine. Thus, the two factors combined under "e" in the list above, an increased alkalinity or increased base-acid ratio in the diet, both serve to diminish the solubility of Ca and P, and hence their availability.

Likewise, the addition of beryllium salts to the rat's diet precipitates a highly insoluble beryllium phosphate, and thereby produces a type of rickets that is distinguished by being incurable with vitamin D.

4. RICKETS IN MAN: INCIDENCE IN THE PAST

Some of the most detailed estimates of the prevalence of rickets relate to the British Isles. About one third of the school children were said to show evidence of severe rickets in London in 1868 (Gee, 1868), or in Manchester in 1871 (Ritchie, 1871). At that time, the more modern diagnostic methods were not yet available, and mild rickets therefore generally escaped detection. Some fifty years later, evidence of "some degree of rickets" was found in 80 to 87% of school children examined in the London County Council area and in Durham. In 1926, the British Ministry of Health found a 50% incidence among 1,000 children examined throughout England and Wales. Further details will be found in Table IV.

Similarly, in North America, statistics emphasize that after the winter months, in the absence of preventive treatment, some degree of rickets may be almost inevitable (Gebhart, 1924; Hess *et al.*, 1930).

Too often, preventive treatment has been neglected. In consequence, surveys in which the more sensitive methods of modern diagnosis have been employed have demonstrated a continued high incidence of *mild rickets*. At the same time, it is true that the severe cases formerly so common have now become rare, and for this we must give credit to the

FIG. 5. Severe rickets. Abnormalities in rachitic metaphysis (from Kramer and Kanof, after Park).

5a. Irregular masses of cartilage cells, swollen or compressed; compressed trabeculae of uncalcified osteoid tissue; irregular vascular invasion of cartilage; degenerated cartilage cells; osteoid surrounding the invading marrow.

5b. Mass of compressed cartilage projected into the metaphysis.

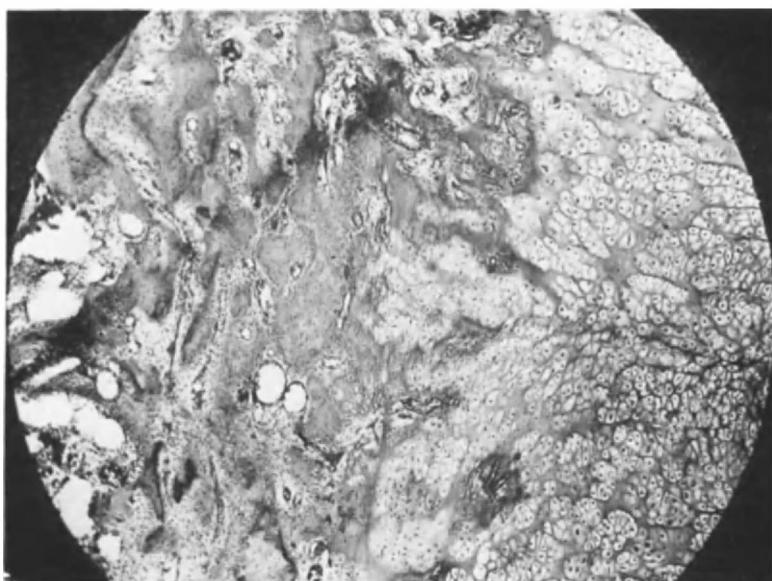


FIG. 5a



FIG. 5b

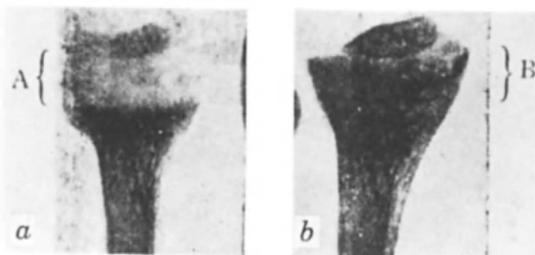


FIG. 6. Radiological appearance of epiphysis in rickets (from Harris, 1951, after Huldschinsky). Distal end of radius.

- 6a. Active rickets, showing the rachitic metaphysis at A.
6b. Healing rickets, indicating recalcification in progress.



FIG. 7. Radiological diagnosis of rickets (after Hutchison, 1952).

- 7a. Active rickets.
7b. Rickets healing after 3 weeks.
7c. Rickets healed, after 8 months.

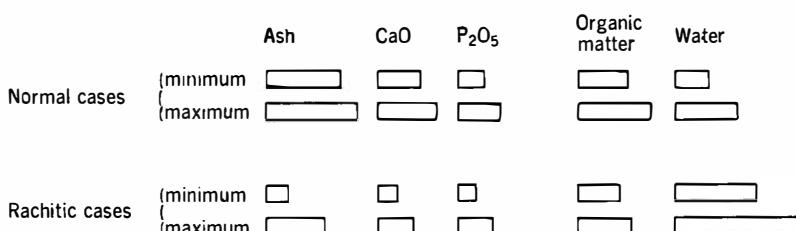


FIG. 8. Composition of rachitic and normal rib bone (from data of Schabab, 1909).

TABLE IV
PREVALENCE OF RICKETS AMONG ENGLISH SCHOOL CHILDREN
(After Harris, 1955)

Date	Place	Incidence of rickets	Reference
1868	London	33% with severe rickets	Gee
1871	Manchester	33% with severe rickets	Ritchie
1915	L.C.C.	80% with some degree of rickets	Dick (1600 children examined)
1920	Durham	82% with some degree of rickets	McIntosh (1300 children examined)
1928	L.C.C.	87% with some degree of rickets	Newman (1600 children examined)
1926	England and Wales	50% with some degree of rickets	Ministry of Health (1000 children examined)
1933	Woolwich	30% with some degree of rickets	Medical Officer of Health (1440 children examined)
1933	Manchester	20-43% with some degree of rickets	Chisholm
1935	Leeds	30% with some degree of rickets	School Medical Officer

admirable work of the infant welfare movement, as well as to the general spread of knowledge and to public enlightenment.

One of the most recent large-scale surveys on the incidence of rickets was that carried out by the British Paediatric Association, for the British Ministry of Health in 1944. Its object was to ascertain whether there had been an increase in rickets as a result of the war. Over 5,000 children from 23 areas in Great Britain and Ireland were examined for clinical signs, as well as for radiological evidence of rickets. The conclusion finally reached was that "the incidence of rickets diagnosed radiologically in children between 3 and 18 months of age is 2½% before 6 months, 4% in the first year, and negligible after that period." Although this may give the impression of being a low rate of incidence, it should nevertheless be borne in mind that it meant that about 24,000 children in Great Britain suffered every year from an avoidable disease. The rate of incidence of active rickets varied from 61% in Sheffield, a smoky, working class, industrial area, to nil in St. Albans, a well-to-do, suburban district.

5. SYMPTOMATOLOGY OF RICKETS IN INFANTS

The most usual age at which rickets is seen in infants is between 8 to 15 months. For obvious reasons, it is most common in early spring or late winter.

Among the earliest clinical indications of developing rickets may be included restlessness and night sweats. The rickety child is liable to be pale and flabby, and is frequently fat. Mention has previously been

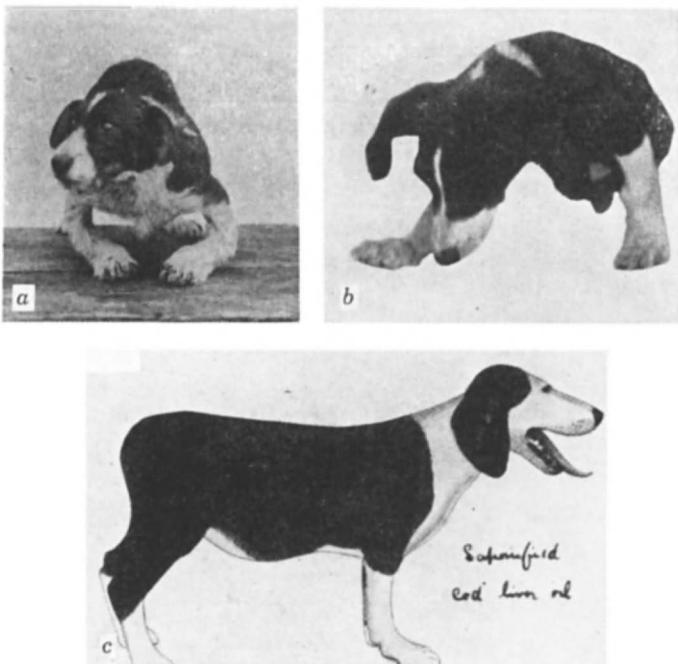


FIG. 9. 9a. Rickets in a dog (from Harris, 1955, after Mellanby, and Steenbock).

9b and 9c. Littermates: b, with no vitamin D in diet (rickets), c, with cod-liver oil (protected).

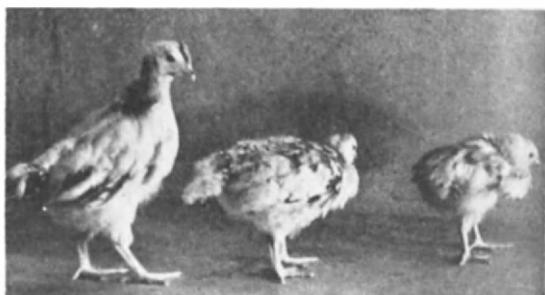


FIG. 10. Rickets in chicks (from Harris, 1951, after MacLennan). The chick on the left received an adequate dose of vitamin D₃ and is normal.

The chick in the middle was given vitamin D₂ in the same amount, but is not fully protected, because, for birds, D₂ is less potent than D₃.

The chick on the right received no vitamin D, and has severe rickets.

made (Section IV) to the underlying biochemical abnormalities, and to the consequent enlarged epiphyses, bony deformities, green-stick fractures, as the pathological features of the disease (p. 587). Translated into clinical terms, these constitute the external signs (see Figs. 11 to 14), which may include craniotabes (the earliest sign, involving soften-

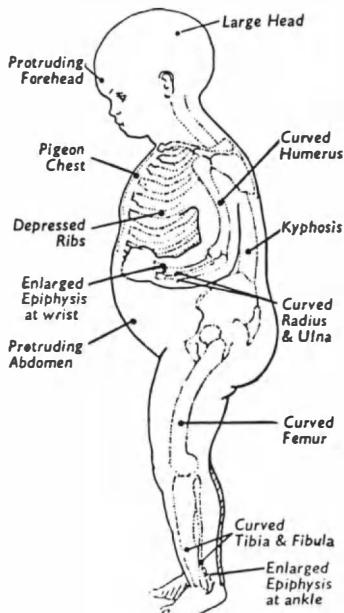


FIG. 11. Diagrammatic representation of some of the notable external manifestations of rickets (after Harris, 1955).

ing of the occipital bones and sometimes of the posterior parts of the parietal bones), caput quadratum or "hot-cross bun head," delayed closure of the anterior fontanelle, "rachitic rosary" with beading of the ribs (i.e. enlargement of the costochondral junctions), other deformities in the chest wall such as grooves or fiddle chest, etc., spinal deformities (thoraco-lumbar kyphosis), enlarged epiphyses, especially recognizable at the wrists and ankles, curvature of the long bones, contracted pelvis.

Accompanying the bony changes, there is generally a loss of muscular tone, and the pot belly may be a prominent feature. Tetany may occur as a concomitant (see below, Section VI, 7).

The diagnosis of rickets has been dealt with above (Section V, 4).

6. PROPHYLAXIS AND CURE OF RICKETS

There is only one certain way of preventing the development of rickets in northern climates (and other areas deficient in sunlight) during the winter months, and that is to supplement the diet with vitamin D (Table V). Cod-liver oil, or, better, halibut-liver oil, or vitamin D₂

(or D₃) concentrates may be given. For artificially fed infants, the most convenient practice is to use a milk substitute already enriched with a standardized amount of vitamin D. Other measures that have sometimes

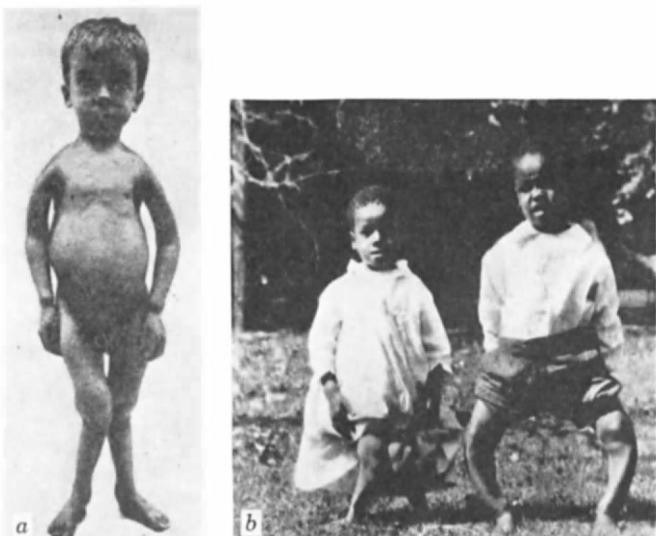


FIG. 12. Cases of severe rickets, showing knock knees, bow legs, and other bony deformities (from Harris, 1951, after (a) Mellanby and (b) Major).

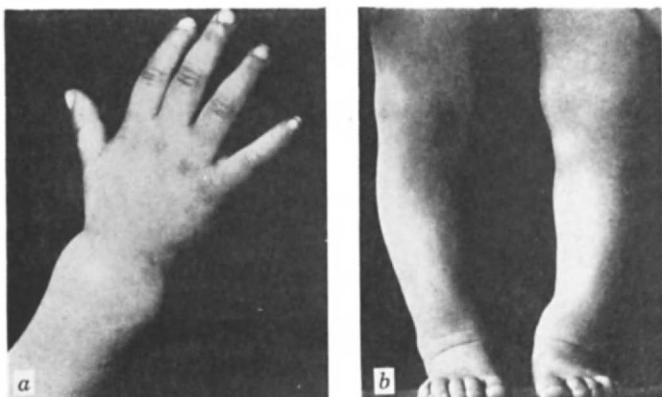


FIG. 13. Enlargement at epiphysis in rickets, causing characteristic deformity at wrist and ankles (from Harris, 1951, after Hunter).

been employed include: irradiated milk (widely used in Germany), irradiated yeast, heliotherapy, and actinotherapy—but these are, in general, less appropriate.

For curative treatment, synthetic vitamin D or halibut-liver oil are preferable to cod-liver oil, as they can be more easily tolerated at a sufficiently high level of dosage. An optimum curative dose is about



FIG. 14. The rachitic rosary (London Hospital Museum).

TABLE V
INCREASED EFFECTIVENESS OF PREVENTIVE TREATMENT OF RICKETS WITH
INCREASING ADEQUACY OF PROTECTIVE DOSE OF VITAMIN D GIVEN
(From data of J. H. Hess *et al.*, 1930)

Treatment	No. of infants examined	No. of cases of rickets after 5-8 months' treatment	
		Severe rickets	Mild rickets
No treatment	34	9	10
Cod-liver oil (1-3 teaspoons to 2 tablespoons)	44	2	4
Vitamin D concentrate (10 drops of "100 D")	6	0	6
Vitamin D concentrate (10-20 drops of "100 D")	63	0	0

1,000 to 3,000 international units daily, the prophylactic dose being 500 to 1,500 I.U. The minimum toxic dose can on occasions be as low as 10,000 I.U. daily; although it must be added that very much more has

often been given with apparent impunity—as in the so-called “bomb therapy” (in which one single massive dose is administered instead of a course of doses). Nevertheless, in the view of the present writer, the more moderate dose levels here recommended should not be exceeded, since the lowest limits of possible toxicity are not far removed from the top levels of effective curative treatment (see Harris, 1932, 1933).

7. INFANTILE TETANY

The direct cause of the infantile tetany, sometimes seen in association with rickets, is the hypocalcemia. For its cure and prevention, the same measures are effective as in uncomplicated rickets. Formerly, theories were in vogue that infantile tetany (see Fig. 15) was attributable to a



FIG. 15. Case of infantile tetany, showing characteristic spasm (from Harris, 1951, after Hunter).

dysfunction of the parathyroid, or to an abnormal guanidine metabolism, but these views can no longer be accepted. Again, treatment with parathyroid hormone was formerly recommended, based on the assumption that parathyroid dysfunction was involved: its use is to be deprecated, except possibly as an emergency measure—since it leads to an aggravation of the responsible metabolic abnormality, causing a still further loss of calcium from the body by withdrawing it from the bones.

8. CELIAC RICKETS

In celiac disease, the failure in fat absorption, including that of the fatty foods containing the vitamin D, can be the cause of “conditioned” rickets. This can be prevented by the exhibition of the vitamin in the form of a concentrate instead of in a fatty menstruum (Parsons, 1927, 1931).

9. JUVENILE RICKETS

In northern America and Western Europe, adolescent or juvenile rickets has been rare, in contrast with infantile rickets. In special circumstances, for example, in parts of Central Europe after World War I,

juvenile rickets was, however, not uncommon. Its occurrence has also been recorded in India (Wilson, 1931; Hutchison and Stapleton, 1924), and among the Finns and Laplanders (Kloster, 1931), and elsewhere. (See Fig. 16.)

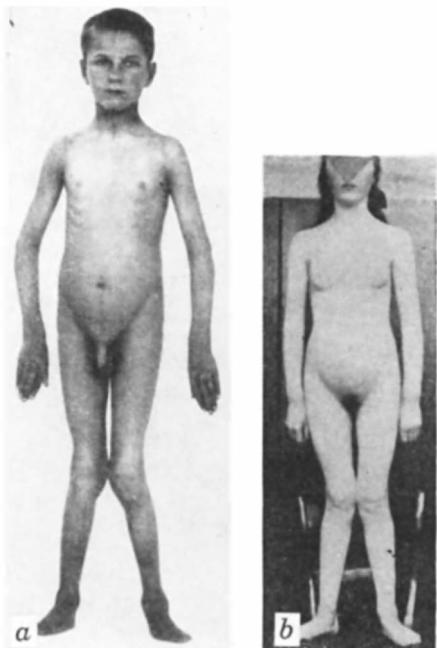


FIG. 16. Adolescent rickets, showing enlarged epiphyses at ankles and wrists, and knock knees (from Harris, 1951, after Feer, and Findlay).

10. OSTEOMALACIA

Adult rickets, or osteomalacia, has frequently been reported in the East, for example in northern China (Maxwell and Miles, 1925), India (Wilson and Surie, 1930; Vaughan, 1929) and other regions. Apart from lack of vitamin D, a shortage of calcium and phosphate in the diet is frequently a contributory cause, and another may be the absence of exposure to sunlight—as when the rite of *purdah* is observed.

Osteomalacia, in these Eastern countries, is often associated with lactation and pregnancy, being accentuated by the physiologically increased loss of mineral elements during these periods.

In Vienna, during the time of food shortage after World War I, osteomalacia occurred among elderly people of both sexes, in the late winter and spring months.

The cause of osteomalacia is essentially the same as that of rickets, the chief pathological difference perhaps being in the *decalcification* of bone, rather than the inadequate calcification seen in the growing end



FIG. 17. Osteomalacia: one of the numerous cases seen in Central Europe after World War I, illustrating the "marmalade legs" (from Harris, 1955, after Frank).



FIG. 18. Contracted pelvis, in a case of osteomalacia. Normal childbirth would be impossible (from Harris, 1951, after Maxwell).

of the bone in the infant. The treatment is as for rickets. Clinical features are illustrated in Figs. 17 and 18.

11. RENAL RICKETS

Renal rickets is not, strictly speaking, to be regarded as a nutritional disorder at all, since it is believed to have its origin in a renal abnormality causing a defective urinary excretion of phosphate. It is characterized by hyperphosphatemia and hypocalcemia—ordinary rickets being usually distinguished by hypophosphatemia with or without accompanying hypocalcemia.

12. RICKETS IN RELATION TO DENTAL CARIES

It has long been known that rickets is often associated with dental caries (see Table VI).

TABLE VI
ASSOCIATION BETWEEN RICKETS AND FAULTY TEETH
(From data of Hall, 1903) *

Type of school	Type of district	Incidence, percent of	
		Rickets	Faulty teeth
Jewish †	Good	5	11
	Poor	7	25
Non-Jewish	Good	8	38
	Poor	50	60

† The lower incidence of rickets in the Jewish families was attributed to their better dietary habits.

* Quoted by L. J. Harris (1955). "Vitamins in Theory and Practice," p. 162. Cambridge University Press, New York. From W. Hall (1903). *Yorkshire Weekly Post*.

The explanation, no doubt, is that vitamin D, by promoting adequate calcification of the teeth (as of other hard structures), insures adequate "dental architecture" and thereby aids them to resist decay.

In clinical trials M. Mellanby (1934) showed that in school children administration of vitamin D had some effect in diminishing the spread of caries, as well as in reducing the development of new caries. However, vitamin D was unable to arrest the disease completely, and therefore its uses are limited (Table VII). Other factors are clearly involved in the control of dental caries, one of the most important being adequacy of the "trace element," fluorine (see e.g. Dean *et al.*, 1942).

TABLE VII
VITAMIN D IN TREATMENT OF DENTAL CARIES
(From data of M. Mellanby, 1934)

Av. No. of carious teeth, per child	Vitamin D in diet	
	With vitamin D supplement	Without vitamin D supplement
New caries	1.7	2.6
Old caries spread further	0.2	0.4

13. VITAMIN D REQUIREMENTS IN PREGNANCY AND LACTATION

Extra vitamin D is desirable for a pregnant or lactating female, not so much for any small effect it may have on the fetus or on the composition of the milk, but rather in order to protect the mother from running

into negative balance of calcium and phosphate as a result of the losses to the fetus or the milk.

VII. Mode of Action of Vitamin D

1. EFFECT OF VITAMIN D ON P AND CA METABOLISM

a. Fecal Excretion of P and Ca

Deficiency of vitamin D, as in active rickets in infants, is characterized, in the first place, by an increased loss of phosphate or calcium, or both, in the feces (Table VIII). Administration of vitamin D suffices to

TABLE VIII
CA AND P EXCRETION BY TYPICAL NORMAL AND
RACHITIC SUBJECTS

Loss in feces	Rachitic subjects	Normal subjects
Ca (as % of intake)	90-100	20-40
P (as % of intake)	60-70	15

correct the abnormality. It follows, therefore, that in the presence of vitamin D there is either an increased absorption of Ca and P from the intestine or else a diminished re-excretion from the body into the alimentary tract. This action of vitamin D can be expressed (Harris, 1932) by saying that it causes an increased "net absorption." (See further below, p. 607.)

b. Blood P and Ca

This increase in net absorption is to be correlated next with a raised concentration of phosphate (the inorganic phosphate fraction) and calcium, or both, in the blood stream (see especially work of Iversen and Lenstrup, 1919; and Howland and Kramer, 1921; and compare Table IX below).

TABLE IX
CA AND P BLOOD LEVELS IN VITAMIN-D DEFICIENCY AND EXCESS
(From Harris, 1955)

Blood level	Vitamin D status		
	Rickets	Normal	Excess
Ca (mg per 100 ml)	7	10	15
Phosphate (inorganic, mg per 100 ml)	3	4	8

c. Explanation of Effect on Bone Calcification

It can be assumed that it is this lowered blood phosphate and calcium (or, to be more precise, a lowered $\text{Ca} \times \text{P}$ product) in rickets that is the direct cause of the defective calcification in the bone. Or, putting it

another way, it is the rise in blood $\text{Ca} \times \text{P}$, brought about by vitamin D, that promotes the increased calcification of bone when rickets is cured or prevented. From the point of view of this theory (Harris, 1928, 1932; Harris and Innes, 1931; Harris and Moore, 1929; Harris and Stewart, 1929) of the mode of action of vitamin D, rickets has to be regarded as being primarily a disorder of the blood and not of the bone. One of the strongest arguments in favor of this explanation is the fact long known, that bone preparations will calcify normally *in vitro*, provided that the saline or the blood serum in which they are bathed contains an adequate amount of phosphate and calcium (Shipley, 1924; Robison and Soames, 1930).

Another piece of evidence in support of this view was derived from observations on the effect of excessive doses of vitamin D. It was found (Harris and Innes, 1928, 1931; Harris and Stewart, 1929) that just as deficiency of vitamin D causes a low retention of phosphate and calcium, low levels in the blood, and deficient calcification, so an excess of vitamin D brought about an increased retention, and excessive levels of blood phosphate and calcium (Table IX). It was to the latter that the excessive calcification seen in hypervitaminosis had presumably to be attributed.

On the basis of this theory also, it was possible to account for such facts as the following: the variation between species in susceptibility to rickets (e.g. dog or man as compared with rat), the etiology of experimental nutritional tetany in contrast with low phosphate rickets, the influence of Ca intake and P-Ca ratio in hypo- and in hypervitaminosis D; and so on. For fuller details of these and other considerations, a review already mentioned may be consulted (Harris, 1932).

2. MECHANISM BY WHICH VITAMIN D CONTROLS P AND CA LEVELS

Although, as we have said, a deficiency of vitamin D causes an increased loss of P or Ca, or both, in the feces, there is little or no evidence that it acts in a direct way upon the function of the alimentary canal as such. The effect on net absorption (p. 606) may be assumed to reside in the primary absorption of P and/or Ca rather than in any re-excretion process: there exists little indication of any active re-excretion of Ca into the alimentary tract other than in the digestive juices. A possible clue as to the mechanism might be sought in the very interesting observation of Zucker and Matzner (1923) that in some circumstances administration of vitamin D can lower the pH of the intestinal contents. This would have the effect of making the calcium and phosphate more soluble and hence would account for the increased net absorption. Nevertheless, any such action on the acid-base relationship does not seem generally applicable; and, moreover, any adequate explanation of this "pH effect" is still lacking.

Evidence based on metabolic experiments with labelled isotopes (e.g. Cohn and Greenberg, 1939) offers the promise of more definite information and has led to the suggestion that a primary effect of vitamin D may be to augment the turnover of organic to inorganic phosphate in the soft tissues. Although a number of such metabolic studies have been made, there is nevertheless no general agreement about the interpretation or even about the facts. In brief, then, for vitamin D, we have no clear knowledge of any role in a coenzyme system such as does exist for various other vitamins, for example for thiamine, nicotinamide, riboflavin, or pyridoxine.

3. VITAMIN D AND PARATHYROID ACTION

A theory widely advocated at one time (e.g. Paton and Findlay, 1916; Bloch and Faber, 1925; Mirvish, 1930; Greenwald and Gross, 1929; Taylor, Braniion, and Kay, 1930) was that vitamin D acted by stimulating the parathyroid secretion. The arguments advanced were that deficiency of parathyroid hormone, like that of vitamin D, caused a fall in the concentration of the blood calcium, and that administration of either substance resulted in a rise in the blood level. Again, with excess of either, calcified deposits were formed in the tissues. Superficially, there is indeed a resemblance; but as against this explanation, it has been shown that the two mechanisms are in fact fundamentally divergent. Whereas parathyroid hormone raises the blood calcium by withdrawing it from the bones and causing a loss to the body as a whole, the essential fact about vitamin D, as indicated above, is that it decreases the fecal loss of calcium and thereby increases its retention in the organism (Harris, 1932).

VIII. Hypervitaminosis D

1. BIOCHEMICAL FEATURES

Some mention has already been made (p. 607) of the ill effects produced by overdoses of vitamin D.

Pfannenstiel (1927), in Germany, was the first to draw attention to the fact that an excessive intake of irradiated ergosterol could be toxic to experimental animals. Kreitmair and Moll (1928), also in Germany, recorded the production of calcified deposits in the soft tissues. However, these observers were unable to throw any light on the meaning of the toxicity, and suggested only that the loss in weight of the experimental animals might seem to indicate an increased basal metabolic rate, and that the lesions could be compared with cholesterol arteriosclerosis. In experimental work that followed at Cambridge, it was shown that vitamin D excess brought about a greatly increased net absorption of calcium and/or phosphate, a liability to hypercalcemia and/or hyper-

phosphatemia (Harris and Stewart, 1929; Harris and Innes, 1931; Harris, 1932) and formation of excessively calcified newly forming bone (Harris and Innes, 1928, 1931) (see Figs. 19 to 24).

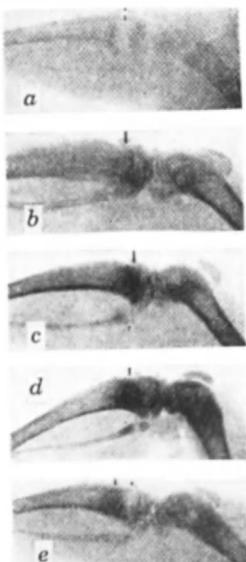


FIG. 19. Radiogram of knee joints of rats, (i) with deficiency, (ii) with normal intake, and (iii) with excess of vitamin D (after Harris, 1932).

19a. Deficiency of vitamin D, showing inadequate calcification at growing end of bone (at the position marked).

19b. Normal intake of vitamin D, showing normal calcification.

19c. Early stage of hypervitaminosis, showing commencement of excessive calcification at growing end.

19d. Same as 19c, but more advanced.

19e. Final stages, complicated by severe illness of rat, cessation of osteogenesis, and new appearance of a less densely calcified band nearest epiphysis (marked by dotted line).

The explanation offered for these results was as follows: "An increased vitamin D intake promotes increased absorption of Ca and/or phosphate from the gut (or diminished excretion into the gut), so tending to raise the level in the blood. With inadequacy of vitamin D, the blood fails to secure sufficient Ca and/or phosphate; with moderate amounts the blood succeeds in maintaining its approximate constancy of composition, while with large overdoses, hypercalcemia and/or hyperphosphatemia cannot be averted" (Harris and Innes, 1931).

Another consequence of hypervitaminosis D, noted by the Cambridge workers (Harris and Moore, 1929; Harris and Stewart, 1929; Ashford, 1930; Watchorn, 1930) was that, as the disorder developed, abnormally large amounts of phosphate and calcium were excreted by the kidney. This was accounted for as the natural response of the kidney, tending to dispose of the excess present in the blood, in the same way as in the hyperphosphatemia or hypercalcemia induced by other means (Harris, 1932).

2. LESIONS OF HYPERVITAMINOSIS D

a. Bones

The overcalcification of bone is clearly seen in the radiogram, or the histological sections (Figs. 19 and 20). It will be noted that the

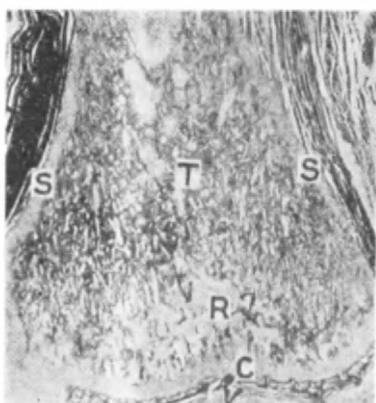


FIG. 20. Advanced hypervitaminosis in a growing rat (after Harris, 1932). Distal end of femur ($\times 14$). Note excessive bone formation, the spongiosa (T) extending far into the marrow cavity.

Secondary resorptive changes are apparent also in the cortex of the shaft (S), and to a less degree in the spongiosa (B) nearest the growth cartilage (C).

excessively densely calcified spongiosa extend far into the marrow cavity. With the excess of vitamin D, osteogenesis is greatly increased, but I have suggested that this does not necessarily indicate any direct action of the vitamin upon the bone cells, for one may imagine that the formation of new bone is stimulated by and dependent on the previous calcification of the osteoid.

b. Teeth

Analogous changes were observed by the writer in the teeth: the cement becomes overcalcified and a remarkable overgrowth of cementoid is formed, which may become ankylosed to the alveolar bone; abnormal new deposits of dentin are laid down (Fig. 21).

c. Calcified Deposits in Soft Tissues

Extensive metastatic calcium deposits are also found in the soft tissues, as originally noted by Kreitmair and Moll (1928), notably in the kidney and in the artery walls (particularly the aorta). There seems little doubt that these are due to the precipitation of the calcium salts from the highly saturated blood, and must be ascribed directly to the hypercalcemia and/or hyperphosphatemia *per se*, for the same result follows also whenever the blood calcium or phosphate is raised to abnormal levels by other means—e.g., by administration of excessive calcium and phosphate, or by means of parathyroid hormone.

It is notable that the kidney was one of the sites most liable to receive these massive calcified deposits. This presumably is a consequence of the immensely increased concentration of calcium salts passed through the organ, but it is not without significance, also, that the kidney is richly supplied with phosphatases, enzymes of the type which in bone are concerned in the process of depositing calcium. The kidney and aorta indeed, according to Robison are among the soft tissues exceptionally

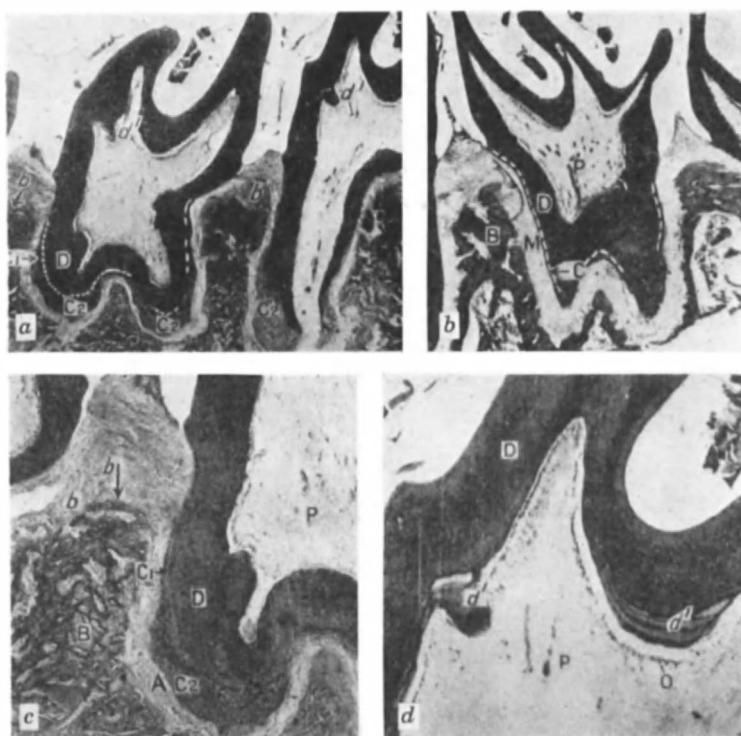


FIG. 21. Effects of excessive vitamin D on dental and periodontal structure, showing overcalcification (after Harris, 1932).

21a. Sagittal section ($\times 10$) of molars of rat suffering from hypervitaminosis; with normal for comparison (21b) (*P*, pulp; *D*, dentin; *C*, cement; *M*, periodontal membrane; *B*, alveolar bone).

Note the overcalcification in hypervitaminosis—e.g., at alveolar crest (*b*) and cement (*C₁*) leading to the formation of extensive cementoid overgrowth (*C₂*) ankylosed to the alveolar bone (some resorption in latter). Formation of abnormally large new deposits of dentin (*d'*).

21c and d. Portions of hypervitaminous tooth, further enlarged, to show: (21c) overcalcification, and new cementoid growth; and (21d) new dentin (lettering as above). (Microphotographs by E. W. Fish)

rich in phosphatase. These are the sites which, after the bone, become most extensively calcified in hypervitaminosis (Fig. 22). (It has been said that the presence of phosphatase in such tissues as kidney need not occasion surprise, for it must be realized that the function of the enzyme is to convert organic P into soluble inorganic phosphate, and that under more normal conditions the secondary effect, the precipitation of calcium salts, would not be involved in sites outside the bone.) To complete the comparison between vitamin excess and deficiency, it may be mentioned

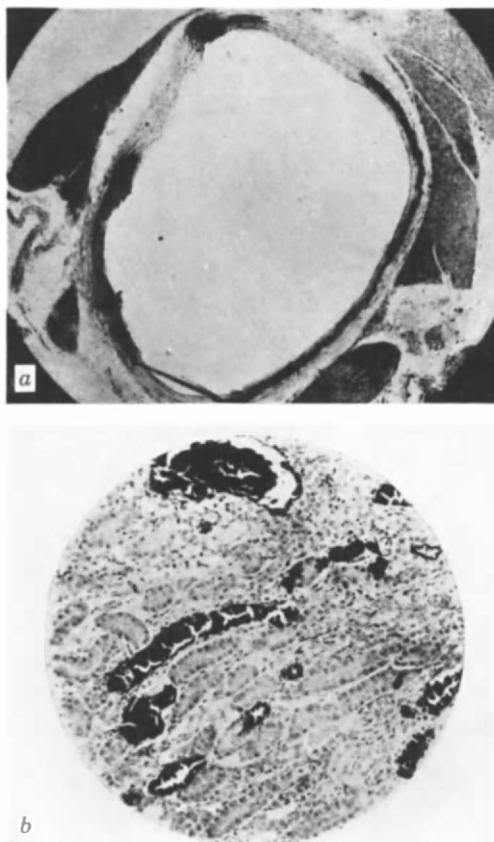


FIG. 22. Metastatic calcification in soft tissues in hypervitaminosis in rat (after Harris, 1955). The areas staining black have become calcified.

- 22a. Aorta.
22b. Kidney.

(Microphotographs by J. R. M. Innes)

that the muscles also have been shown to have an abnormally high Ca or P content in hypervitaminosis, contrasting with the deficiency common in rickets.

3. INFLUENCE OF OTHER FACTORS

As had been foretold, the effects of vitamin D in excess were found to depend largely upon the Ca:P intake, as they do with minimal allowances of the vitamin and in hypo- or avitaminosis. Each increase in the amount of calcium in the diet resulted in an increased severity of hypervitaminosis, for any given overdose of vitamin D (Harris and Innes, 1931; Harris, 1930). This was the result expected; the greater the amount

of Ca available for absorption the greater the ill effects of excessive absorption and the attendant hypercalcemia.

4. SECONDARY EFFECTS IN HYPERVITAMINOSIS D

a. Metabolic

The consequences of hypervitaminosis described above refer essentially to the effects of more *moderate* degrees of overdosing. With *maximal* toxic overdoses, continued for some time, secondary effects may be seen. In these circumstances, the animal begins to lose weight rapidly, and refuses its food, and it may then become impossible to show the characteristic increase of net absorption with each increase of vitamin intake (cf. Figs. 23 and 24). This is due partly to the loss of appetite, so

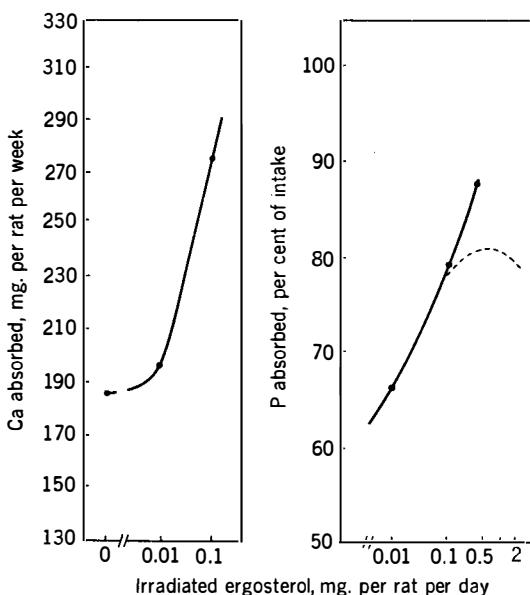


FIG. 23. Increasing net absorption of Ca (left) and P (right) with increasing intake of vitamin D (Redrawn from Harris, 1932, after data of Brown and Shohl).

Amounts of vitamin D were given up to 5,000 times the minimum effective dose. At the maximal level of excess (dotted line), the effect observed on P absorption is complicated by severe illness, with loss of appetite and failure of gut function.

that little or no calcium is being ingested, but partly also to the breakdown of gut function which is seen in any animal when gravely ill. Partially starved controls on a diet normal in composition and restricted only in quantity show a similar failure in percentage net absorption of calcium or phosphate (Watchorn, 1930).

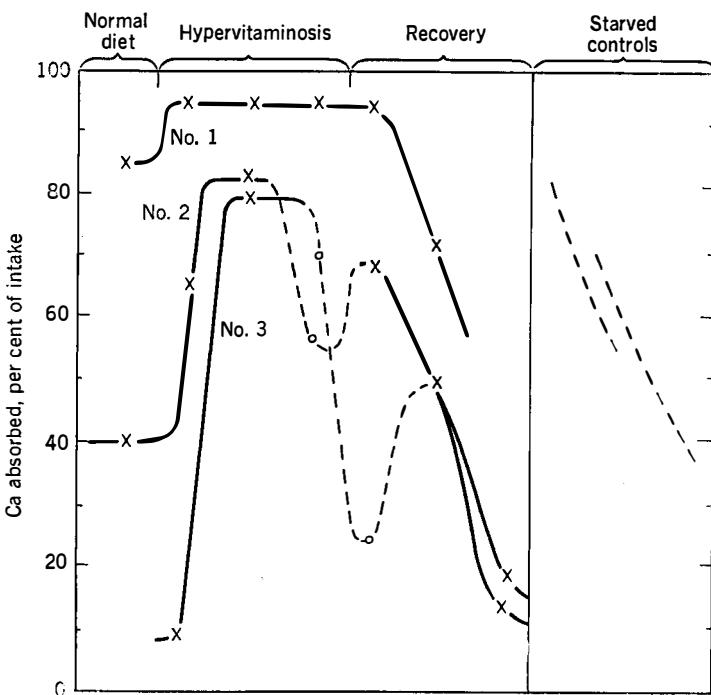


FIG. 24. Increased net absorption of Ca during the course of experimentally induced hypervitaminosis in rats, followed by a diminished net absorption on discontinuation of the treatment (after Harris, 1932).

A secondary effect is seen in the case of rats Nos. 2 and 3 which had become gravely ill in the later stages of the hypervitaminosis, with loss of appetite and failure in gut absorption (dotted lines). A similar failure in absorption is seen in controls partly starved on a normal diet (on right).

b. Bone Changes

A particularly interesting observation is that when maximum degrees of hypervitaminosis are reached and no increased provision of calcium is allowed, there is, especially, clear evidence of resorptive changes in the bone. Figure 20 (from Harris, 1932) illustrates the resorption of compact bone under such circumstances, and indicates how spongiosa which had already become abnormally dense under the action of the vitamin D excess begin later to show a band less densely calcified at the growing end. The explanation seems to be that the bone is called upon as an additional source of calcium for the blood when the alternative available supply in the gut becomes exhausted. In conformity with this view, it has been shown that as the calcium in the diet is reduced a relatively greater proportion of the extra calcium drawn into the blood does come from the bone (Harris, 1932).

It is necessary to add that these "secondary" effects in the severely ill animal should not be allowed to divert attention from the consideration that the primary action of vitamin D, which is of normal physiological importance, and which is equally apparent whether the vitamin is given in suboptimal amounts, in optimal amounts, or in moderate excess, is that described above, namely, increased net absorption of Ca and/or P, raised Ca \times P product, and resulting increased calcification.

5. HYPERVITAMINOSIS: TOXIC BY-PRODUCT THEORY

At one time it was widely held that the toxic effects of excessive amounts of vitamin D were due entirely to the presence of toxic by-products. These were produced, it was claimed (Dixon and Hoyle, 1928; Underhill, 1928) when irradiation of ergosterol was carried out commercially by the German method, in alcohol, but not at all by the English method, in oil. This view was not substantiated, for in experiments on rats highly repurified ergocalciferol isolated from the irradiation mixture was found to have a degree of toxicity sufficient to account quantitatively for the toxicity of the crude irradiated ergosterol from which it was obtained (Harris, 1932). Similarly, pure ergocalciferol gave rise to the characteristic effects of hypervitaminosis in dogs also (Dale, Marble and Marks, 1932). Nevertheless, it has to be added that, as shown by Holtz and coworkers (e.g. Holtz and Schreiber, 1930) and confirmed by others (e.g. Harris, 1932), products (isomers) producing toxic symptoms (hypercalcemia, overcalcification, etc.) at relatively a much lower level of anti-rachitic units can be obtained from irradiated ergosterol. The smaller size of the toxic overdose in proportion to the minimum anti-rachitic dose may perhaps be due to the more complete absorption or retention of these isomers when given in excess, but a full explanation is lacking.

6. HYPERVITAMINOSIS IN OTHER SPECIES; CLINICAL HYPERVITAMINOSIS

Although in the foregoing paragraphs we have been referring particularly to observations on rats, hypervitaminosis has also been produced experimentally in various other species, and is by no means unknown clinically. Allusion to a score or more of typical cases seen by medical practitioners between 1928 and 1931 is made in the article previously cited (Harris, 1932). The signs may include hyperphosphatemia and/or hypercalcemia, hypercalcuria, excessively calcified bone, calcification in kidneys and elsewhere, with pyrexia and malaise. A fatal termination is possible, but recovery can be complete, provided the overtreatment is discontinued in time. As the minimum possible toxic overdose of ergo-

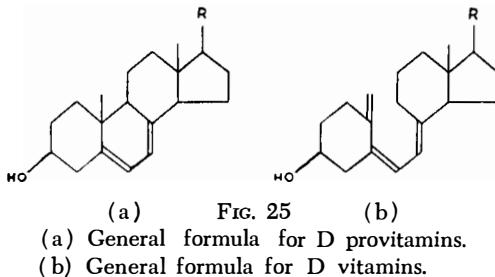
calciferol (about 10,000 I.U. daily) is not far removed from the optimum, or maximum curative dose (say, 3,000 I.U. daily), it is important that the correct dosage (viz. 500 to 1,500 I.U. for prophylaxis and 1,000 to 3,000 I.U. for curative treatment) should be adhered to when concentrated preparations of vitamin D are used clinically. The danger scarcely arises with natural products, 14 teaspoonfuls of cod-liver oil (or 1½ teaspoonsfuls of halibut-liver oil) would be needed to give the same therapeutic effect as 1 mg of crude irradiated ergosterol (the maximum dose necessary for the cure of severe rickets in infants).

IX. Synopsis of Vitamin D Chemistry and Biochemistry

In the chronological introduction at the beginning of this chapter, some preliminary reference was made to the isolation of vitamin D₂, and the subsequent recognition of other forms of vitamin D.

1. STRUCTURE OF THE D VITAMINS

The general type formulae, representing all forms of D vitamins, and the D provitamins from which they are derived, are given in Fig. 25.



In each instance, the activation by ultraviolet irradiation of a provitamin, to produce the corresponding vitamin itself, involves the rupture of the second ring structure at the position indicated. The D vitamins, and pro-vitamins, differ among each other solely in the nature of the aliphatic side chain that they contain, represented by "R" in these formulae.

In vitamin D₂ (ergocalciferol) and in the corresponding provitamin (ergosterol), R is represented by



In vitamin D₃ (cholecalciferol), and in provitamin D₃ (7-dehydro-cholesterol, or cholestadien-3-ol), R is



Other forms of vitamin D, or of substances possessing a biological

activity similar to that of vitamin D, are known but seem of little practical importance.

Vitamin D₁, as a term, is now obsolete. It was formerly applied to a substance first thought to be the pure vitamin, but later found to be a molecular compound of vitamin D₂ and an inactive isomer.

2. PROPERTIES OF VITAMIN D₂

Vitamin D₂ occurs naturally in irradiated vegetable tissues. It is soluble in ether, chloroform, and various other fat solvents. As compared, for example, with vitamin C or vitamin B₁ it is relatively stable to heat. Among its other physical properties is a characteristic ultraviolet absorption spectrum (maximum absorption at 265 m μ) which is used for the assay of pharmaceutical preparations (p. 619).

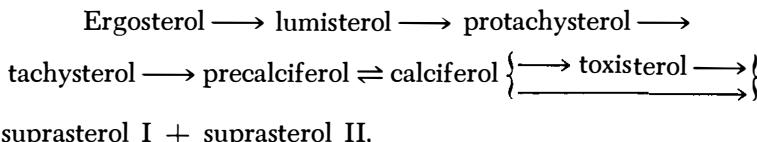
A notable feature of vitamin D₂, as of D₃, is the extraordinarily small concentration in which it occurs, even in the most potent known sources—for example, the amount of vitamin D₃ in cod-liver oil is less than 1 part in 400,000 (p. 586). The daily dose required to cure a rat is about 0.1 µg (= 0.0001 mg). The international unit (I.U.) was first defined as 0.025 µg of a purified standard specimen of vitamin D₂, but has now been replaced by the same quantity of vitamin D₃.

3. PROPERTIES OF VITAMIN D₃

Vitamin D₃, the principal form occurring in fish-liver oils, is prepared artificially by irradiation of 7-dehydrocholesterol. A main point of difference between D₃ and D₂ is that the latter is relatively inactive for poultry and other birds, as compared with vitamin D₃ (Fig. 10). The reason is not yet fully understood. For human beings and rats, and apparently for most other animals, both forms are of approximately equal activity.

4. PHOTOCHEMISTRY OF ACTIVATION

The transformation of ergosterol to ergocalciferol, under the influence of ultraviolet irradiation (of an appropriate wave length) takes the following course, an analogous set of changes occurring likewise with the other provitamins.



5. METABOLISM OF VITAMIN D

That vitamin D can be stored to a significant degree (namely, in the mammalian liver) is apparent from the fact that a single large dose given

to a young animal, or even to its mother, can delay the onset of rickets for a considerable period. Likewise, in "bomb therapy," one single large dose is effective as a preventive or curative measure. When a massive quantity of vitamin D (e.g. 40,000 times the daily protective dose) was administered to a rat, the amount in the liver at first was found to rise sharply, but then to fall quickly during successive days: 17% of the amount given could be accounted for in the feces, and 6% in the body (mostly in the liver), the fate of the remaining 77% being undetermined (Cruickshank and Kodicek, 1953; Cruickshank, Kodicek, and Armitage, 1954).

Very little is known as yet about the metabolic transpositions undergone by vitamin D in the animal organism.

6. DETERMINATION OF D VITAMINS

The most satisfactory methods for determining vitamin D involve:

- (1) Radiological tests on rats (Fig. 26).
- (2) The "line" test, using rats (Fig. 27).

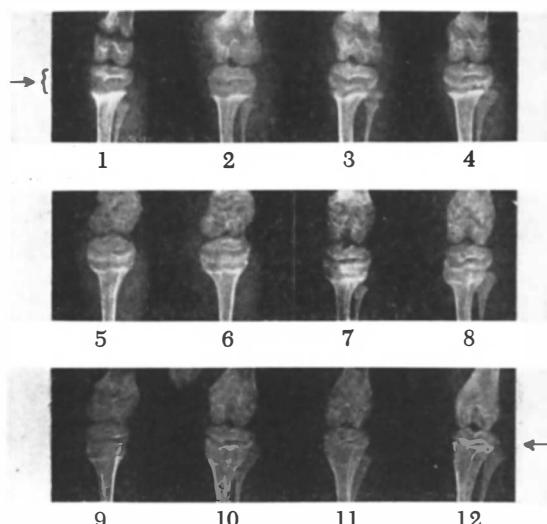


FIG. 26. Graded degrees of recalcification of rachitic bone after administration of graded allowances of vitamin D (from Harris, 1951, after Bourdillon *et al.*, 1931).

In this radiological method for the assay of vitamin D, the criterion used is the degree of healing produced in the bones of rachitic rats. In the X-ray pictures above, reading from left to right, are depicted twelve degrees of healing, corresponding with the increasing graded doses of vitamin D administered.

The appearance of the bone at the position marked by the arrow should be compared: with increased healing the width of the uncalcified gap is diminished.

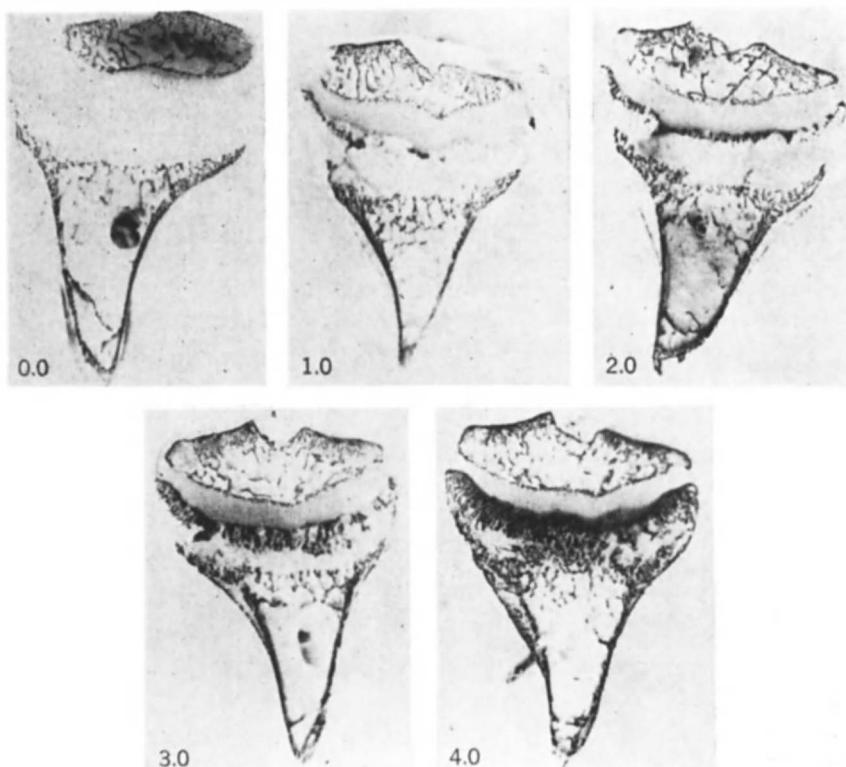


FIG. 27. The "line" test for vitamin D (from Bliss and György, 1951). Varying degrees of recalcification, at epiphyseal end of tibiae of rats, in proportion to amount of vitamin D allowed. Five graded "healing scores" are illustrated.

(3) Analysis of bones, in tests on rats, for their ash content.

Alternatively, chicks may be used in place of rats.

No chemical tests applicable to foodstuffs are yet known. However, concentrated pharmaceutical products such as synthetic preparations of vitamin D can be assayed by various physical or chemical procedures (e.g. by ultraviolet absorption, or by a color test with $SbCl_3$).

A method employing filter-paper chromatography is at present being worked out (Davis, McMahon, and Kalnitsky, 1952; Kodicek and Ashby, 1954), but is not yet applicable to foodstuffs or ordinary animal or vegetable tissues.

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C H A P T E R X X

STEROID HORMONES AND BONE^{1, 2}

MARTIN SILBERBERG and RUTH SILBERBERG

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I. Introduction

Skeletal abnormalities related to the absence or dysfunction of the sex glands have long been known. Aristotle (384-322 B.C.) not only correctly described the skeletal effects of castration but already emphasized the significance of age in determining these effects. He summarized his knowledge as follows: "If castrated at an early age, animals become taller and more delicate than non-castrates; if they are, however, developed (at the time of castration), they do not add to their size." Plinius' (23-79 A.D.) report of a "3 year old boy who was 4½ ft. tall and had a manly voice" suggests that the association of precocious sexual maturity and acceleration of skeletal growth was already known at that time. Phlegon (137 A.D.) tells of a person who was "an infant, an adolescent, a mature man, an old man, and married and begot children all within a space of 7 years." John Hunter (1779) in his treatise on the free-martin noted the increase in standing height and in the length of horns of cattle with congenital hypogonadism. Knowledge of the relationship between dysfunction of the adrenals and skeletal growth dates back to the publication by Cooke (1756) of a case of an adrenal tumor associated with accelerated sexual and skeletal maturation. However, it was not until the second half of the past century that systematic investigations of problems in this endocrine field were initiated: Studies on African eunuchs (Ecker, 1864) and on members of the sect of the Scoptzi (Pelikan, 1876) were followed by experiments in castrate animals. New methods of approach were provided by the demonstration of biologically active substances in the sex glands, placenta, adrenals, and in blood and urine, by the isolation of these substances from their sources, by the recognition of their steroid nature, and by their synthesis in the laboratory. Thus studies of the direct effects of steroids on metabolic functions and structure of tissues were made possible.

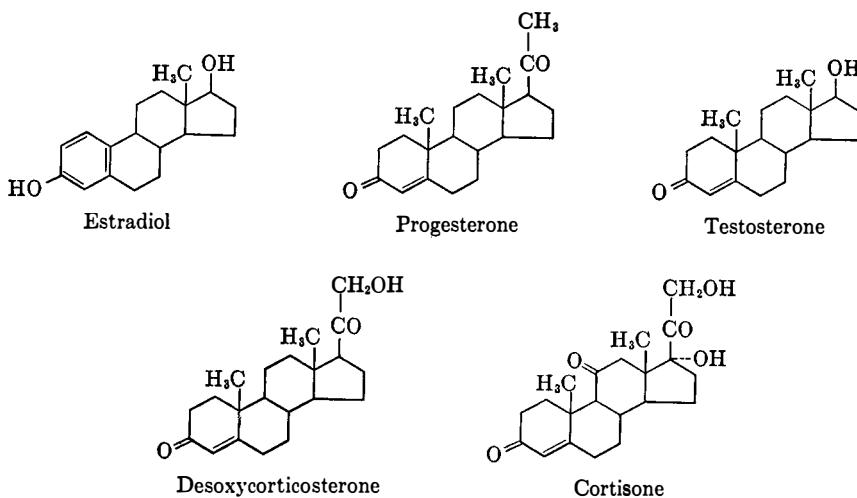
II. Scope of the Problem

Numerous experiments carried out during the past two decades have answered some questions as to the skeletal action of steroids, but have at the same time raised many new problems. These hormones influence growth and development of cartilage and bone, but they also affect other mesenchymal tissues. On the other hand, both skeletal growth and development occur also in the absence of steroids, and the latter may thus merely act as accelerators or inhibitors of the spontaneous processes. Once the problems of spontaneous osteogenesis and osteolysis have been solved, factors that modify these processes will be easier to analyze than they are at the present time. The further question arises as to the relation of the skeletal effects of steroids to steroid action in general. Are

these effects specific or are they merely local manifestations of a single principle, and are they in any way related to or are they independent of the processes of propagation?

None of the classifications of the steroids is entirely satisfactory, and overlapping will occur in all of them. Therefore, a classification of steroids according to their origin from ovaries, testicles, or adrenals, as will be used in this review, seems to be no less objectionable than any other one. The production under certain conditions of androgen by the ovaries, of estrogen by the testes and placenta, and of progesterone by the adrenals may be presently discounted.

The steroids to be discussed are: The *ovarian* steroids, estrogen and progesterone, produced primarily by the theca interna of the follicle and by the lutein cells of the corpus luteum respectively; the *testicular* steroids and the *adrenal cortical* steroids of which the androgenic 17-ketosteroids, the mineralocorticoids (desoxycorticosterone group) and the glucocorticoids (cortisone group) will be considered separately. These hormones have in common the steroid nucleus, but they vary in number and position of hydroxyl- and ketogroups and of other radicals. The basic structure of some representative steroids is given below:



Steroids have been studied as to their action on size and shape of the skeleton as a whole and of individual bones; on the proportion of individual bones to each other and to the skeleton as a whole; on weight, breaking strength, chemical composition and X-ray diffraction patterns of bone; on microscopic structure; and on the metabolism of essential

TABLE I
PUBLICATIONS DEALING WITH THE SKELETAL EFFECTS OF OVARIECTOMY, AND MAIN FINDINGS REPORTED

Species	Author	Skeletal findings
MAN		
	(1) H. Sellheim, <i>Arch. Frauenheilk. Konst. forsch.</i> 10 , 215 (1923).	Decreased stature and disproportionate growth of bones
	(2) R. Roessle and J. Wallart, <i>Beitr. allgem. Pathol. pathol. Anat.</i> 84 , 401 (1930).*	Decreased stature and disproportionate growth of bones
	(3) G. Pich, <i>Beitr. allgem. Pathol. pathol. Anat.</i> 98 , 218 (1937).*	Decreased stature and disproportionate growth of bones
	(4) R. F. Varney, A. T. Kenyon, and F. S. Koch, <i>J. Clin. Endocrinol.</i> 2 , 137 (1942).	Decreased stature and disproportionate growth of bones
	(5) F. Albright, P. H. Smith, and R. Fraser, <i>Am. J. Med. Sci.</i> 204 , 625 (1942).	Decreased stature and disproportionate growth of bones
	(6) K. Kaijser, <i>Acta Endocrinol.</i> 3 , 351 (1949).	Decreased stature and disproportionate growth of bones
	(7) G. Hellinga and J. Groen, <i>Acta Physiol. Pharmacol.</i> 2 , 150 (1951).	Eunuchoid with increased stature
CATTLE		
	(8) S. G. Shattock and C. S. Seligmann, <i>Proc. Roy. Soc. Med. Pathol.</i> 3 , 102 (1909).	Pelvis asexual
	(9) J. Tandler and K. Keller, <i>Arch. Entwicklungs-mech. Organ.</i> 31 , 289 (1910).	Horns lengthened
PIG		
	^a S. G. Shattock and C. S. Seligmann, <i>Proc. Roy. Soc. Med. Pathol.</i> 3 , 102 (1909).	Inconclusive
SHEEP		
	(10) Franz, cited by R. Disselhorst, <i>Kiuhn-Arch.</i> 5 , 29 (1914).	Pelvis asexual; epiphyseal closure retarded
DOG		
	(11) H. Sellheim, <i>Beitr. Geburtshilf. u. Gynäkol.</i> 2 , 236 (1899).*	Tubular bones elongated, closure of epiphyses and sutures retarded

	(12) G. Schickele, <i>Z. Morphol.</i> 18 , 525 (1914).	Tubular bones elongated
RABBIT	a S. G. Shattock and C. S. Seligmann, <i>Proc. Roy. Soc. Med. Pathol.</i> 3 , 102 (1909); a G. Schickele, <i>Z. Morphol.</i> 18 , 525 (1914).	Pelvis narrowed Tubular bones elongated
GUINEA PIG		
	(13) P. Bouin and P. Ancel, <i>Compt. rend.</i> 142 , 232 (1906).	Tubular bones elongated
	(14) C. R. Moore, <i>Biol. Bull.</i> 43 , 285 (1922).	Body size decreased; tubular bones elongated
	(15) M. Silberberg and R. Silberberg, <i>Proc. Soc. Exptl. Biol. Med.</i> 37 , 446 (1937)*; <i>Am. J. Pathol.</i> 15 , 55 (1939).*	Development of epiphyseal and articular cartilage retarded
RAT		
	(16) J. M. Stotsenburg, <i>Anat. Record</i> 7 , 183 (1913).	Bone growth retarded
	(17) S. Hatai, <i>J. Exptl. Zool.</i> 18 , 1 (1915).	Growth of body and of tubular bones increased
	(18) R. M. McKeown, G. C. Harvey, and R. W. Leumsden, <i>Arch. Surg.</i> 26 , 433 (1933).*	Callus formation and breaking strength of bones decreased
	(19) C. B. Freudenberger and E. I. Hashimoto, <i>Am. J. Anat.</i> 62 , 93 (1937); <i>Proc. Soc. Exptl. Biol. Med.</i> 41 , 530 (1939).	Bone growth temporarily increased; also age factor
MOUSE		
	(20) M. Silberberg and R. Silberberg, <i>Anat. Record</i> 95 , 97 (1946).*	Development of epiphyses and joints retarded
	(20a) R. Silberberg and M. Silberberg, <i>Lab. Invest.</i> 3 , 228 (1954).*	Degenerative joint disease decreased and retarded

* Indicates microscopic studies.

▲ References without numbers have been cited previously in the tables.

components of skeletal tissues, in particular Ca and P. The subject has been reviewed from time to time (Riddle, 1941; Silberberg and Silberberg, 1943, 1949; Gardner and Pfeiffer, 1943; McLean, 1943; Murray, 1947; Parker, 1949; Baker, 1950; Dallemagne, 1950, 1951).

III. Sexual Dimorphism of the Skeleton

The sexual dimorphism of the skeleton is in most of its aspects genetically determined (Stevenson, 1924; Todd, 1933). However, certain of its features become accentuated with the appearance of sex hormones. Therefore, the latter play a limited role in determining the ultimate state of the skeleton. Many observations illustrate the interaction of genetic and hormonal factors in skeletal development: Sex differences in the structure of the human pelvis are present from the fifth month of gestation (Fehling, 1876) and gradually increase thereafter. During puberty these differences become still more accentuated, while in eunuchs this phase of pelvic development fails to occur. Centers of ossification are better developed in girls than in boys (Hill, 1939). Still, in children with ovarian or testicular deficiency, ossification of those centers that ordinarily appear late in childhood is retarded. Conversely in sexually precocious children with androgen- or estrogen-producing tumors of the adrenals or sex glands, ossification of these centers is accelerated. Epiphyseal closure likewise follows essentially a genetic pattern, but again in adolescent boys, epiphyseal closure and skeletal age in general are more closely related to sexual development (Francis, 1940; Greulich, 1940) and to the amounts of urinary 17-ketosteroids than to chronological age (Talbot *et al.*, 1943; Nathanson, 1947).

IV. Ovarian Steroids

1. SKELETAL EFFECTS OF OVARIAN DEFICIENCY

Skeletal changes have been studied in cases of primary ovarian deficiency as occurs spontaneously in the freemartin and in humans with agenesis or hypoplasia of the ovaries, and following therapeutic or experimental ovariectomy. On the whole, there is agreement regarding the skeletal effects of ovarian deficiency as seen in Table I.³ Discrepancies that do exist are of a minor nature and are obviously due to differences in the age of the test animal at the time of ovariectomy

³ The aim of the tables is to give an objective account of the often contradictory findings made by different investigators. No claim is made as to completeness of the list. In the case of investigators having published a series of articles on the subject, the earliest and the most recent papers are usually quoted. (Concluded March 31, 1954).

or to variations in the interval between operation and the time of observation.

In the *growing* skeleton, ovarian deficiency affects endochondral and intramembranous ossification: The bones are delicate and thinner than usual, their breaking strength is decreased, and callus formation is retarded. The epiphyseal growth zones and cranial sutures close later than ordinarily, and centers of ossification are delayed in appearance. The pelvic diameters are shortened, and horns continue to grow beyond their normal length. Linear growth of the body as a whole, of certain tubular bones, and of tails is temporarily increased. However, this stimulation is followed by a slowdown of the rate of growth. The ultimate size of the skeleton, therefore, does not exceed that seen ordinarily; it may remain even below average: Girls suffering from ovarian agenesis show a characteristic "Kleinwuchs" (decrease of stature) with disproportionately long extremities.

Microscopic studies deal chiefly with the influence of spaying on endochondral ossification. During the early stages following ovariectomy, the epiphyseal growth zones contain increased numbers of cartilage cells, while less spongiosa is formed than usual. The hyperplasia and hypertrophy of the epiphyseal cartilage may be due partly to an intensification of growth and partly to a temporary delay of the regressive changes that ordinarily contribute to epiphyseal closure. The retardation of skeletal development and ageing also affects the articular cartilage and is reflected in a lower incidence and decreased severity of degenerative joint disease in spayed as compared to non-spayed animals (20a).⁴

In the *adult* organism, ovarian deficiency fails to exert noticeable skeletal effects. This confirms with expectations, since aged cartilage reacts less readily to changes in the endocrine equilibrium than young cartilage. Ovarian deficiency may, however, lead to post-castrational osteoporosis. Proof that ovarian deficiency is also the cause of post-menopausal osteoporosis has still to be forthcoming (Anderson, 1950).

In summary—Ovarian deficiency delays skeletal development and decreases ossification. These effects are most conspicuous in those bones that are least developed at the time the hormonal disturbance sets in. An individual bone retarded in development may continue to grow for an abnormal length of time, and the result may be a certain disproportionality of the skeleton as a whole.

2. SKELETAL EFFECTS OF OVARIAN STEROIDS

(a) *Estrogen*

The metabolism of bone is intimately related to that of minerals.

*This and all subsequent figures refer to references given in the tables.

TABLE II
PUBLICATIONS DEALING WITH THE VALUES OF SERUM CA AND P IN ESTROGEN-TREATED ANIMALS AND MAIN RESULTS REPORTED^a

Species	Author	Observation	Ca mg%	P mg%
GUINEA PIG				
	(21) G. Poumeau-Delille and J. Fabiani, <i>Compt. rend. soc. biol.</i> 138 , 238 (1944).	Control Estrogen (Dipropionate only)	9.5–11.0 up to 29.0	— —
RAT				
	(22) H. G. Day and R. H. Follis, Jr., <i>Endocrinology</i> 28 , 83 (1941).	Control ^b Estrogen ^b	10.59–11.22 11.58–11.93	— —
	(23) J. J. Pindborg, <i>Acta Pathol. Microbiol. Scand.</i> 22 , 290 (1945).	Control ^c Estrogen ^c (1 animal)	9.60–11.70 8.50–11.90 14.7	— — —
DUCK				
(Mallard)	(24) W. Landauer, C. A. Pfeiffer, W. U. Gardner, and J. C. Shaw, <i>Endocrinology</i> 28 , 458 (1941).	Control male Estrogen male	9.76–10.40 22.40–62.40	2.8–4.0 5.4–14.0
(Pekin)		Control male Estrogen male	8.64–10.40 23.52–68.0	2.0–5.8 5.6–10.6
CHICKEN				
(Adult)	(25) B. Zondek and L. Marx, <i>Arch. intern. pharmacodynamie</i> 61 , 77 (1938).	Control male Estrogen male	to 15.0 16.6–117.6	approx. 8.0 to 11.4
	(26) W. Landauer, C. A. Pfeiffer, W. U. Gardner, and E. B. Man, <i>Proc. Soc. Exptl. Biol. Med.</i> 41 , 80 (1939).	Control male Estrogen male	8.96–9.61 11.4–78.1	— —
(Cockerel)	(27) K. Salomon, B. W. Gabrio, E. Reinhard, and R. Silberberg, <i>Arch. Pathol.</i> 43 , 76 (1947).	Control male Estrogen male	8.91–13.77 8.10–13.45	— —
PIGEON				
	(28) C. A. Pfeiffer and W. U. Gardner, <i>Endocrinology</i> 23 , 485 (1938).	Control female Estrogen female	8.0–10.72 13.12–21.65	— —

(29) O. Riddle, V. M. Rauch, and G. C. Smith, <i>Endocrinology</i> 36 , 41 (1945).	Control	8.9-11.4	-
	Estrogen	15.1-26.0	-
	Estrogen hypophysectomy	19.1-24.7	-
	Estrogen parathyroidectomy	14.5-20.6	-
(30) M. J. Dallemagne, <i>Endocrinology</i> 48 , 443 (1951).	Control male	5.89-7.73	-
	Control egg-laying	7.17-7.75	-
	Estrogen male	15.72-25.13	-
SPARROW			
(31) C. A. Pfeiffer, A. Kirschbaum, and W. U. Gardner, <i>Yale J. Biol. and Med.</i> 13 , 279 (1940).	Control male	9.70	-
	Control egg-laying	to 20.0	-
	Estrogen male	to 77.1	-

a Only those articles are quoted in which the investigators have correlated the serum mineral levels to findings in the bones.

b Pooled material.

c Material from individual animals.

Therefore, investigations of skeletal effects of steroids have necessarily been concerned with the action of these substances on mineral metabolism. Only those results will be considered in which observations of the mineral metabolism have been correlated to changes in the bones.

i. *Biochemical findings in the blood serum.* Calcium: The levels of serum Ca in untreated animals vary considerably according to species, age, sex, and degree of maturity, egg-laying activity, and generally depend on the amount of estrogen circulating in the blood. Results attributed by some investigators to experimental conditions fall within the normal limits established by others (Table II). Some of the discrepancies in the results reported can be explained by differences in methods of sampling blood and of Ca determination; in particular pooling of blood may fail to disclose significant individual differences.

In birds, hypercalcemia occurs during the egg-laying cycle or after administration of estrogen, and there is a definite positive correlation between the dose of hormone given and the rise in the level of the serum Ca. Most investigators do not distinguish between the non-ultrafiltrable and the ultrafiltrable fractions of serum Ca. However, in pigeons estrogens increase primarily the non-ultrafiltrable fraction (McDonald and Riddle, 1945). In mammals, the serum Ca responds to estrogen less consistently and by far less conspicuously than in birds. Slight and temporary rises as well as declines in the levels of serum Ca have been reported. Young animals react more vigorously to the steroid than old individuals (22).

Phosphorus: In birds, estrogens—endogenous as well as exogenous—increase the serum levels of P. The latter closely parallel those of Ca and seem to concern all (Mandel, Clavert, and Mandel, 1946), but especially the non-ultrafiltrable fractions (Riddle and McDonald, 1945). The few reports on the effect of estrogen on serum P in species other than birds are inconclusive, and so are the available data regarding their action on the serum phosphatase (22).

The mechanism by which estrogens call forth the changes in the serum Ca and P are unknown. In pigeons, fasting, parathyroidectomy, or hypophysectomy did not significantly modify the effects of estrogen (29). However, in drakes parathyroidectomy inhibited and hypophysectomy decreased the hypercalcemia produced by estrogen (Benoit, Clavert, and Fabiani, 1942). Thus, hypophysis and parathyroids may augment the action of estrogens on serum Ca and P, but they do not seem indispensable in this respect. While considerable effort has been made to determine the influence of estrogens on the mineral levels in the blood serum, little information is available concerning their effect on the uptake, turnover, and elimination of these minerals. Abnormal serum

levels of any substance indicate a disequilibrium of its release into and its removal from the serum. However, disturbances of mineral metabolism are not necessarily accompanied by abnormal serum levels of the mineral involved. The mere establishment of serum levels, therefore, may fail to disclose major metabolic disturbances and besides gives no information on the uptake of Ca from the diet, the release of Ca from the bones, or its elimination by the kidneys, the intestine or the oviduct. However, without adequate data concerning these phases of the action of estrogen, the understanding of the subject remains fragmentary. In pigeons, estrogens markedly accelerated the uptake and considerably retarded the elimination of Ca^{45} . The exchange of Ca ions between the serum and the skeleton was slow (30). Data on the effects of estrogen on the Ca and P balance are few and contradictory: The negative Ca and P balance prevailing in old individuals reverted to positive after administration of estrogen (Albright, Smith, and Richardson, 1941), while in prepuberal girls estrogen caused the normally positive balance to become negative (Johnston, 1941).

In summary—The marked response of serum Ca to estrogen in birds and the lack of such a conspicuous response in mammals in which there is no relationship between serum Ca and reproduction suggest that in birds the hypercalcemia is incidental to egg-laying. The cyclic accretion of bone taking place in female birds may be at least partly due to this hypercalcemia and thus differ from apparently similar changes induced by estrogen in mammalian bone.

ii. *Biochemical and biophysical changes in bone.* The conflicting views on normal osteogenesis are reflected in the different interpretations of the effects of steroids on formation and resorption of bone. Various answers have been given to questions like the following: Is the excessive bone seen after administration of estrogen identical with ordinary bone and is it formed in a similar fashion? Specifically, do estrogens cause deposition in the uncalcified matrix of CaCO_3 or of bicalcium phosphate, and do they promote the conversion of the latter into tricalcium phosphate? Is the increase in the amount of bone caused by estrogens relative or absolute, and is this a primary effect or is it secondary in nature? The available data are often difficult to integrate not only because of the divergence of the material tested but also because different investigators use different methods of standardization.

Estrogens increase the percentage of total bone ash in animals of various species. However, the overmineralization is not uniform throughout the skeleton, and some bones such as the os ilii and the symphysis pubis may actually undergo demineralization (77); (Dallemande, 1950, 1951; (30)). In pigeons receiving estrogen, the percentage of Ca in

the tubular bones was doubled, while the Ca content of the remaining skeleton increased only 25% (Clavert and Benoit, 1942; Clavert, Cabannes, and Grangaud, 1945). If Ca^{45} was injected together with an estrogen, the specific activity of femurs, tibiae, radii, and cubiti was increased, while that of the remaining skeleton was not. As confirmed by injections of radioactive isotopes, the turnover of P in the skeleton was intensified after administration of estrogens, and thus closely followed that of Ca (Dallemande *et al.*, 1950, 1951 (30)).

The chemical nature of the mineral laid down under the influence of estrogen has not been unequivocally established. The low Ca/P ratio in the bone ash suggested an early deposition of bicalcium phosphate which was supposed to be speedily converted into tricalcium phosphate (Ettori *et al.*, 1942). However, X-ray diffraction studies failed to demonstrate the pattern of bicalcium phosphate (Dallemande, 1951), and therefore the role of this mineral in estrogen-induced osteogenesis remains in doubt.

In baby rats, estrogen did not change the percentage of total ash (Ely and Philips, 1940), while in the femurs of weanling rats (22) and mice (Wentworth, Smith, and Gardner, 1940) bone ash was increased. In rats, the steroid raised the Ca/P ratio of the skeleton about 10%, while the percentage of total Ca remained essentially unchanged. The alteration in the Ca/P ratio was therefore attributed to a loss of phosphate from the bone ash associated with an increase in carbonates (Macola, 1937). This view was seemingly supported by X-ray diffraction studies of bones of estrogen-treated C3H mice in which the pattern of CaCO_3 was identified (Reed, Reed, and Gardner, 1946). However, subsequent experiments carried out in mice of strain C57BL and A failed to reduplicate the earlier results (Reed, Reed, and Silberberg, unpublished data).

The significance of the changes induced by estrogen in bone ash may have been generally overrated, while major changes in the non-asheous components of bone were disregarded. Thus in young rats receiving estrogen, the water content and the non-asheous solids of bone decreased progressively. If however, the values were corrected for body weight, the proportion of asheous to non-asheous material was the same as in non-injected animals (69). The effect of the steroid thus manifests itself in decreased formation of organic matrix, and the apparent increase in the ash content constitutes a concentration of ash within a smaller volume of organic matrix rather than an absolute increase. This interpretation appears to be the only one that links the biochemical changes in the long bones with the inhibition of linear growth caused by estrogens.

The close relationship of overmineralization of bone and inhibition of linear growth is also indicated by the decreased effectiveness of the

steroid in animals which no longer grow. In old mice or in young hypophysectomized mice given estrogen, bone ash increased less than in growing mice treated correspondingly (83, 83a). The overmineralization is thus, to a large extent, due to the concentration of ash in a decreased volume of organic matrix. As will be discussed later, some of the excessive bone ash can be accounted for by decreased resorption of cortical bone and by secondary accretion of bone.

In summary—In birds, the overmineralization of certain bones occurring under the influence of estrogen is closely related to the simultaneous hypercalcemia. The deposition of minerals in the skeleton is usually considered a process preparatory to the formation of the egg shell, but it may also be a mechanism protecting the animal from the injurious effects of the hypercalcemia. In mammals, the overmineralization of the bones is in part relative, due to concentration of ash in decreased amounts of organic matrix; partly it is attributable to decreased resorption of cortical bone, and only partly to accretion of osseous substance. Overmineralization of some bones is accompanied or preceded by demineralization of others.

iii. *Gross observations.* Estrogens may exert three major effects on the structure of the skeleton— inhibition of linear growth, acceleration of skeletal development and ageing, and condensation of bone (Figs. 5, 7).* In addition, these steroids may cause pubic relaxation.

In growing individuals of all species investigated, estrogens inhibit growth of long and flat bones (Table III).³ Acceleration of development is indicated by hastening of epiphyseal closure and by premature appearance of centers of ossification. In the long bones of female birds, ossification of the marrow cavity occurs prior to egg laying. Bony spicules are laid down along the endosteum, and encroach upon and obliterate the marrow cavity. With the laying of the egg, the excess bone is resorbed. Similar changes can be produced by the administration of estrogens to male birds and to certain mammals. In mice and, to a lesser degree, in individuals of other species, there appears in the metaphysis of the long bones a closely knit network of bony trabeculae which gradually involves both the diaphyseal cavity and the shaft. This network always decreases in density towards the center of the shaft. According to expectation, the breaking strength of bones thus altered is increased.

Pubic relaxation is related to parturition and therewith to reproduction. It is, therefore, not surprising that the pelvic structures respond to estrogens. In virgin female and castrate male rodents, resorp-

* Figs. 1–4 in Plate I; Figs. 5–9 in Plate II (see pp. 665–667).

TABLE III
INVESTIGATIONS DEALING WITH MORPHOLOGICAL EFFECTS OF ESTROGENS IN BONES AND MAIN FINDINGS REPORTED

Species	Author	Skeletal findings
MAN		
	(32) C. Sternberg, <i>Jahrbuch der Wiener Krankenanstalten</i> 5 , 47 (1896).	Generalized osteosclerosis with malignant ovarian tumor
	(32a) R. Neurath, <i>Ergeb. inn. Med. u. Kinderheilk.</i> 4 , 46 (1909); <i>Z. Kinderheilk.</i> 19 , 209 (1919).	Premature skeletal development with precocious sexual maturity; comprehensive references
	(33) J. Lenz, <i>Arch. Gynäkol.</i> 99 , 67 (1913).	Premature skeletal development with precocious sexual maturity; comprehensive references
	(34) M. S. Sherman, <i>J. Bone and Joint Surg.</i> 30A , 915 (1948).	Increased bone formation (case complicated by Pagets disease)
	(35) L. M. Hurxthal and N. Musulin, "Clinical Endocrinology." Lippincott, Philadelphia, 1953.	Premature skeletal development with precocious sexual maturity (complete endocrine references)
MONKEY		
	(36) C. G. Hartman, C. F. Geschickter, and H. Speert, <i>Anat. Record</i> 79 , 31 (1941).	Tubular bones shortened
DOG		
	(37) M. Tausk and P. deFremery, <i>Acta Brevia Neerl. Physiol. Pharmacol. Microbiol.</i> 5 , 19 (1935).*	Increased ossification; also effect of castration
	(38) R. G. Hills and J. A. Weinberg, <i>Bull. Johns Hopkins Hosp.</i> 68 , 238 (1941).*	Callus formation accelerated
	(39) C. J. Sutro and L. Pomerantz, <i>Arch. Pathol.</i> 33 , 305 (1942).*	Premature cessation of growth
	(40) W. Bauer, <i>J. Am. Coll. Dentists</i> 12 , 192 (1945).*	Inhibition of growth, increased bone formation
	(41) M. R. Urist, A. M. Budy, and F. C. McLean, <i>Trans. 7th Josiah Macy Jr. Conf. on Metabolic Aspects of Convalescence</i> , p. 79 (1948).	Inhibition of growth, increased ossification

CAT

- ^a R. G. Hills and J. A. Weinberg, *Bull. Johns Hopkins Hosp.* **68**, 238 (1941).*
- ^a R. Urist, A. M. Budy, and F. C. McLean, *Trans. 7th Josiah Macy Jr. Conf. on Metabolic Aspects of Convalescence*, p. 79 (1948).

RABBIT

- (42) G. Coryn, *Arch. intern. méd. exptl.* **11**, 135 (1936).*
- ^a R. Urist, A. M. Budy, and F. C. McLean, *Trans. 7th Josiah Macy Jr. Conf. on Metabolic Aspects of Convalescence*, p. 79 (1948).

GUINEA

PIG

- (43) F. L. Hisaw, *Proc. Soc. Exptl. Biol. Med.* **23**, 661 (1926).
- (44) L. Brouha, *Compt. rend. soc. biol.* **113**, 406 (1933).
- (45) C. I. Parhon and C. Parhon-Stefanescu, *Compt. rend. soc. biol.* **114**, 327 (1933).*
- (46) G. Bankoff, *Arch. klin. Chir.* **179**, 256 (1934).*
- (47) F. Dessau, *Act. Brevia Neerl. Physiol. Pharmacol. Microbiol.* **5**, 138 (1935).
- (48) M. Silberberg and R. Silberberg, *Arch. Pathol.* **28**, 340 (1939);* *Anat. Record* **102**, 141 (1948).*
- (49) C. J. Sutro and L. Pomerantz, *Arch. Surg.* **39**, 992 (1939).*
- (50) F. E. Emery and A. Lawton, *Am. J. Physiol.* **151**, 134 (1947).
- (51) R. V. N. Talmage, *Anat. Record* **99**, 571 (1947); *Endocrinology* **47**, 75 (1950).*

Doubtful effect on callus formation

Inhibition of growth, increased bone formation

Hypertrophy of epiphyseal cartilage increased, also effect of castration

Inhibition of growth, increased bone formation

Pubic relaxation

Pubic relaxation

Inhibition of growth

Callus formation increased

Pubic relaxation

Inhibition of growth and of bone resorption; also effects of sex, age and vitamin C deficiency

Slight acceleration of epiphyseal closure

Pubic relaxation

Pubic relaxation; also Ca, P, H₂O and alkaline phosphatase

TABLE III (Continued)

Species	Author	Skeletal findings
	^a R. Urist, A. M. Budy, and F. C. McLean, <i>Trans. 7th Josiah Macy Jr. Conf. on Metabolic Aspects of Convalescence</i> , p. 79 (1948).	Inhibition of growth, ossification increased
	(52) M. X. Zarrow, <i>Endocrinology</i> 42 , 129 (1948).	Pubic relaxation
	(53) W. C. Young and F. E. Emery, <i>Federation Proc.</i> 8 , 174 (1949).	Pubic relaxation
POCKET GOPHER	(54) F. L. Hisaw, <i>J. Exptl. Zool.</i> 42 , 411 (1925).	Pubic relaxation
HAMSTER	(55) A. A. Koneff, M. E. Simpson, and H. M. Evans, <i>Anat. Record</i> 94 , 169 (1946).*	Decreased bone formation
	^a R. Urist, A. M. Budy, and F. C. McLean, <i>Trans. 7th Josiah Macy Jr. Conf. on Metabolic Aspects of Convalescence</i> , p. 79 (1948).	Inhibition of growth, ossification increased
RAT	(56) J. Spencer, F. E. d'Amour, and R. G. Gustavson, <i>Proc. Soc. Exptl. Biol. Med.</i> 28 , 500 (1931); <i>Am. J. Anat.</i> 50 , 129 (1932).	Inhibition of growth
	(57) B. Zondek, <i>Lancet</i> 231 , 2, 842 (1936).*	Inhibition of growth, increased bone formation, also effect of age
	(58) R. L. Noble, <i>Lancet</i> 233 , 2, 192 (1938).	Inhibition of growth, also effect of age
	(59) J. H. Gaarenstroom and L. H. Levie, <i>J. Endocrinol.</i> 1 , 420 (1939).*	Inhibition of growth, ossification increased; also effect of anterior hypophysis
	(60) N. B. Talbot, <i>Endocrinology</i> 25 , 325 (1939).	Accelerated appearance of centers of ossification
	(61) J. L. Bremer, <i>Arch. Pathol.</i> 32 , 200 (1941);* <i>J. Bone and Joint Surg.</i> 24 , 32 (1942).*	Increased bone resorption, also role of parathyroid
	(62) M. Silberberg and R. Silberberg, <i>Am. J. Anat.</i>	Inhibition of growth and of resorption; increased bone

- MOUSE
- | | |
|---|---|
| (69) 295 (1941); * <i>Endocrinology</i> 29 , 475 (1941). | formation |
| (63) H. G. Day and R. H. Follis, Jr., <i>Endocrinology</i> 28 , 83 (1941).* | Inhibition of growth and of resorption; increased bone formation |
| (64) H. L. Williams and E. M. Watson, <i>Endocrinology</i> 29 , 258 (1941). | Bone formation, bone resorption and bone phosphatase |
| (65) G. A. Pollock and R. K. Ghormley, <i>J. Bone and Joint Surg.</i> 23 , 273 (1941).* | Doubtful effect on callus formation |
| (66) E. A. Kibrick, M. E. Simpson, H. Becks, and H. M. Evans, <i>Endocrinology</i> 31 , 93 (1942).* | No effect of estrogen after hypophysectomy |
| (67) M. Griffiths and F. G. Young, <i>J. Endocrinol.</i> 3 , 96 (1942). | Growth inhibition; also effect of hypophyseal hormone |
| (68) G. H. Bell and D. P. Cuthbertson, <i>J. Endocrinol.</i> 3 , 302 (1942). | Bone strength increased |
| (69) H. N. Lippman and J. B. de C. Saunders, <i>J. Endocrinol.</i> 3 , 370 (1942).* | Inhibition of growth, increased bone formation; also bone ash |
| (70) C. H. Whicher and E. M. Watson, <i>Endocrinology</i> 33 , 83 (1943).* | Increased bone formation and bone resorption; bone phosphatase |
| (71) H. V. Brush, <i>Am. J. Anat.</i> 76 , 339 (1945);
a J. J. Pindborg, <i>Acta Pathol. Microbiol. Scand.</i> 22 , 290 (1945).* | Callus formation slightly delayed
Inhibition of growth and of resorption of bone; also effect of age |
| (72) H. D. Armstrong, M. Knowlton, and M. Gouze, <i>Endocrinology</i> 36 , 313 (1945). | Estrogen effective in denervated limbs |
| (73) B. L. Baker and J. H. Leek, <i>Am. J. Physiol.</i> 147 , 522 (1946).* | Inhibition of growth and of resorption of bone; also effect of parathyroidectomy |
| (74) J. Duckworth and G. M. Ellinger, <i>J. Endocrinol.</i> 7 , 7 (1950).* | Effect of estrogen after calcium depletion |
| (75) A. M. Budy, M. R. Urist, and F. C. McLean, <i>Am. J. Pathol.</i> 28 , 1143 (1952).* | Inhibition of growth, increased bone formation; also interaction with testosterone |
| (76) H. Burrows, <i>J. Physiol. (London)</i> 85 , 159 (1935). | Pubic relaxation |

TABLE III (Continued)

Species	Author	Skeletal findings
DUCK	(77) W. U. Gardner, <i>Am. J. Anat.</i> 59 , 459 (1936);* <i>Anat. Record</i> 76 , 22 (1940).*	Pubic relaxation; also interaction of progesterone
	(78) W. U. Gardner and C. A. Pfeiffer, <i>Proc. Soc. Exptl. Biol. Med.</i> 37 , 678 (1937);* (a) 38 , 599 (1938);* (b) <i>Endocrinology</i> 32 , 149 (1943).*	Bone formation increased; also breaking strength; effect of age, strain, and sex differences; also testosterone
	(79) C. J. Sutro, <i>Proc. Soc. Exptl. Biol. Med.</i> 44 , 151 (1940).*	Inhibition of growth; increased formation and resorption of bone
	(80) M. Silberberg and R. Silberberg, <i>Am. J. Anat.</i> 69 , 295 (1941);* (a) <i>Am. J. Pathol.</i> 22 , 1033 (1946).*	Inhibition of growth and of resorption, increased formation of bone; also strain and sex differences, effects of age, thyroid, hypophysis and testosterone
	(81) E. W. Miller, J. W. Orr, and F. C. Pybus, <i>J. Pathol. Bacteriol.</i> 55 , 137 (1943).*	Increased resorption of bone; strain differences
	(82) A. Segaloff and W. M. Cahill, <i>Proc. Soc. Exptl. Biol. Med.</i> 54 , 162 (1943).*	Strain differences in bone formation (strain CHI); also vitamin D deficiency
	(83) W. U. Gardner and H. C. Clouet, <i>Anat. Record</i> 88 , 433 (1944);* (a) 91 , 275 (1945).*	Bone formation increased after hypophysectomy and bone formation in old mice; also bone ash
	(84) W. U. Gardner and M. L. Mahan, <i>Anat. Record</i> 94 , 462 (1946).*	Strain differences in bone formation and resorption (strain BC); also effects of age, sex, thyroid and protein Pubic relaxation
	(85) K. Hall and W. H. Newton, <i>J. Physiol. (London)</i> 106 , 18 (1947);* <i>J. Endocrinol.</i> 7 , 54 (1950).*	Inhibition of growth and of resorption of bone; increased bone formation, strain differences, effect of age
	(86) M. R. Urist, A. M. Budy, and F. C. McLean, <i>J. Bone and Joint Surg.</i> 32A , 143 (1950).*	Increased bone of alveolar processes
	(87) S. S. Stahl, J. P. Weinmann, I. Schour, and A. M. Budy, <i>Anat. Record</i> 107 , 21 (1950).*	Bone formation increased; also strain differences and effect of testosterone
	^a W. Landauer, C. A. Pfeiffer, W. U. Gardner, and J. C. Shaw, <i>Endocrinology</i> 28 , 458 (1941).*	

- (88) J. Benoit, J. Messerschmitt, and R. Grangaud, *Compt. rend. soc. biol.* **135**, 1593 (1941).*
- (89) J. Benoit and J. Clavert, *Bull. histol. appl. physiol. et pathol. et tech. microscop.* **20**, 23 (1943);* *Compt. rend. assoc. anat.* **50**, 27 (1947).*
- (90) W. Landauer and B. Zondek, *Am. J. Pathol.* **20**, 179 (1944).*
- CHICKEN**
- a B. Zondek and L. Marx, *Arch. intern. pharmacodynamie* **61**, 77 (1938).*
- a W. Landauer, C. A. Pfeiffer, W. U. Gardner, and E. B. Man, *Proc. Soc. Exptl. Biol. Med.* **41**, 80 (1939).
- (91) J. L. Bremer, *Anat. Record* **76**, 9 (1940).*
- (92) L. V. Domm and W. Bloom, *Anat. Record* **81**, 91 (1941).*
- a W. Landauer and B. Zondek, *Am. J. Pathol.* **20**, 179 (1944).*
- a K. Salomon, B. W. Gabrio, E. Reinhard, and R. Silberberg, *Arch. Pathol.* **43**, 76 (1947).*
- (93) J. Hublé, *Acta Endocrinol.* **13**, 317 (1953).*
- PIGEON**
- (94) P. Kyes and T. S. Potter, *Anat. Record* **60**, 377 (1934).
- (95) C. A. Pfeiffer and W. U. Gardner, *Endocrinology* **23**, 485 (1938).*
- (96) M. A. Bloom, W. Bloom, L. V. Domm, and F. C. McLean, *Anat. Record* **78**, 143 (1940);* **83**, 99 (1942).*
- (97) O. Riddle, V. M. Rauch, and G. C. Smith, *Anat. Record* **90**, 295 (1944);* *Endocrinology* **36**, 41 (1945).*
- Bone formation increased; also parathyroidectomy
- Increased bone formation; after parathyroidectomy decreased bone formation; effect of low Ca diet
- Formation and resorption of bone increased; also strain differences and effect of age
- Inhibition of growth; increased formation of bone
- Bone formation increased
- Increased bone resorption
- Ossification during egg laying cycle
- Increased formation and resorption of bone
- Inhibition of growth; bone formation increased, bone resorption decreased
- Inhibition of growth
- Ossification during egg laying cycle
- Bone formation increased
- Bone formation increased; also testosterone
- Ossification during egg laying cycle; bone formation increased after hypophysectomy and parathyroidectomy

TABLE III (Continued)

Species	Author	Skeletal findings
	(98) J. Benoit and J. Clavert, <i>Compt. rend. soc. biol.</i> 139 , 728 (1945);* <i>Arch. Anat. microscop. et morphol. exptl.</i> 37 , 41 (1948).*	Bone formation increased, role of decreased bone resorption
QUAIL		
	(99) A. R. Ringoen, <i>J. Morphol.</i> 77 , 265 (1945).*	Ossification during egg laying cycle
SPARROW		
	(100) A. Kirschbaum, C. A. Pfeiffer, J. van Heuverswyn, and W. U. Gardner, <i>Anat. Record</i> 75 , 249 (1939).*	No effect of estrogen on bone formation
	(101) C. A. Pfeiffer, A. Kirschbaum, and W. U. Gardner, <i>Yale J. Biol. and Med.</i> 13 , 279 (1940).*	Increased bone formation, also testosterone interaction
	^a A. R. Ringoen, <i>J. Morphol.</i> 77 , 265 (1945).*	Ossification during egg laying cycle

* Indicates microscopic studies.

^a References without numbers have been cited previously in the tables.

tion of the pubic symphysis can be induced by injections of ovarian extracts or of estrogens. In small doses, the steroid exerts a priming effect sensitizing the tissue to the action of relaxin, a non-steroid hormone which is primarily responsible for pubic relaxation. However, if administered over prolonged periods of time, estrogen may effect pubic separation in the absence of relaxin (Table III).

iv. *Microscopic studies.* Growth of cartilage is inhibited, while hyalinization and calcification of the ground substance are intensified; erosion by capillaries of the provisional cartilage is delayed, and the latter persists longer than ordinarily (Figs. 1, 4). Provided the cartilage is not too badly damaged, growth may be resumed after cessation of treatment, or even under continued treatment, and the excessive spongiosa may also be resorbed. In the articular cartilage, estrogens render the ground substance resistant to breakdown and liquefaction, and thus may counteract the development of spontaneous osteoarthritis and of joint lesions induced by anterior hypophyseal hormone (80a).

There are some differences in the response to estrogen of avian and mammalian bones. In birds, the increased medullary deposition of bone originates in the diaphysis, while in mammals the process sets in in the metaphysis of the long bones. The tissue changes are complex and variegated (Table III). It is, therefore, understandable that various investigators have stressed various details of observations and have minimized others. The evaluation of the static microscopic picture is rendered particularly difficult by the rapid succession and interaction of osteogenic and osteolytic processes. Emphasis has variously been placed on osteoblastic proliferation, on damage to the bone marrow with secondary deposition of bone, and on inhibition of resorption of provisional cartilage and bone followed by accretion of osseous substance.

While estrogens may increase the number of osteoblasts, it is doubtful whether this is due to a direct stimulation by the hormone or whether it is secondary to changes called forth by the steroid in the bone marrow. Such early changes consist of hyperemia, stimulation of hemopoesis, and subsequent deposition of collagenous fibrils and ossein. The latter undergoes calcification to form trabeculae. The newly formed spicules contain delicate doubly refractile fibrils which are less serrated and less regular than those of the pre-existent bone. The new bone does not form lamellae and is not arranged according to mechanical stress as is ordinary bone (88, 98).

On the other hand, particularly in growing rodents, early changes produced by estrogen are, inhibition of resorption of provisional carti-

lage and of primary spongiosa. A poorly vascular fibrillar and hyalinized connective tissues replaces the metaphyseal bone marrow usually seen between the primary trabeculae. Osteoclasts are scanty or absent, and the spongiosa does not undergo resorption as usual. Osteoblasts accumulate in close approximation to the heavily calcified spicules and deposit organic matrix. The latter calcifies and forms bone between the trabeculae which thus link up with one another. This dense bony network encroaches upon the diaphysis and extends to the endosteum. The cortex is thickened, the vascular canals are narrow and contain fibrillar connective tissue (22, 80, 80a, 86).

In adult and old animals, there is no consistent effect of estrogen on bone. In old mice of strains with a natural tendency to heavy bone formation, the steroid may still induce osteosclerosis. However, in mice of strains with a natural tendency to bone resorption, the hormone may intensify the latter processes prior to formation of bone. This interplay of osteogenesis and osteolysis results in a peculiar disorganization of the diaphyseal architecture, particularly near the residual epiphyseal plate and the adjacent parts of the cortex. The shaft becomes porotic, and the bone marrow fibrous with interspersed fragments of bony spicules (62, 80) (Figs. 8, 9).

In young animals treated with estrogen, the spongiosa of flat bones, such as calvaria, vertebrae, and mandibles, may undergo osteosclerotic changes (Table III).

Because of its condensing effect on bone, the hormone has been used in the treatment of osteogenesis imperfecta. Decreased fragility was noted during the period of treatment (Ropes, Rossmeisl, and Bauer, 1946). No objective evidence has as yet been put forward to demonstrate a beneficial effect of estrogen on the bone changes of menopausal osteoporosis (Anderson, 1950) and its effect on callus is equivocal (38, 46, 65, 71). Microscopic studies likewise failed to disclose increased bone formation in patients treated with estrogens for metastatic bone cancers (Kennedy *et al.*, 1953).

Estrogen causes gradual replacement of the symphyseal cartilage by connective tissue (Table III). This process in pelvic relaxation is to be distinguished from the effects of relaxin, which induces rapid disintegration of the cartilage of the symphysis.

In summary—Estrogen inhibits linear growth and accelerates skeletal development; in certain locations the hormone retards the resorption of the spongiosa and causes fibrosis and hyalinization of the bone marrow. These processes are followed by accretion of osseous tissue. The changes are reversible. In old animals, estrogen may intensify resorption of bone, an effect associated with or followed by new formation of osseous tissue.

v. *Factors modifying the skeletal effects of estrogen.* The effects of estrogen vary considerably with the conditions under which the hormone is tested. The modifying factors may be exogenous and related to the hormone itself, such as the type of estrogen, the duration of treatment, and the mode of administration; or the modifiers may be endogenous and due to species, strain, sex, age, or to local conditions within individual bones (62). Of the numerous natural and synthetic steroids, the benzoic and propionic esters of estradiol as well as estrone and diethylstilbestrol elicit the most vigorous skeletal response (21, 86). The relative potencies of these steroids are difficult to establish because their effects vary not only in degree but also in time of appearance and in duration. Steroids dissolved in sesame oil are most stable (Barnicot, 1951). A given amount is usually more effective if administered in divided doses than if given at one time. Estrogens have been administered orally, intraperitoneally, subcutaneously, percutaneously, topically, or by implantation of pellets. However, the rate of absorption, esterification and excretion of the steroid are more important in determining the results than its mode of application (Folley, 1942; Cowie and Folley, 1944; Wilder-Smith and Williams, 1946). The amount of bone deposited is usually proportionate to the metabolized amount of the estrogen. There is no consistent quantitative correlation between the effects on bone and the estrogenicity of a compound (Parkes, 1947; (86)).

Effects of age. The susceptibility of the skeleton to estrogen decreases with advancing age of the individual (23, 27, 57, 58, 63, 78, 80, 90). However, the inhibition of growth is less marked in newborn than in prepuberal animals. This may be due to higher growth potential of the cartilage of the newborn animal—harder to suppress than that of older animals which grow at a slow rate. Aged cartilage cannot respond to further inactivation by the hormone.

Effects of species. While administration of estrogen inhibits growth in young animals of all species, species differences exist in regard to the reactivity of bone proper. The osteogenic response of pigeons and ducks exceeds that of chickens and sparrows; of mammalian bones, those of mice react most vigorously to the steroid (24, 42, 86). These species differences may be due to species differences in the metabolic degradation of estrogen or in tissue susceptibility.

Effects of strain. Strain differences have been observed in birds as well as in mammals: Pekin drakes are more responsive than Mallards (24, 90). In mice of strain C57BL treated with estrogen, metaphyseal bone is abundant, while in mice of strains DBA and NBT resorption of bone is conspicuous from the beginning (62, 81); in some mice of strain CHI, there was no endosteal bone formation (82); in mice of strains BC,

DBA, and presumably in others, spontaneous resorption of bone occurred with prolonged administration of estrogen, while in mice of strains C3H and A the excess bone may persist indefinitely (78, 84). Some of these differences are apparently related to the genetically different rates of skeletal growth, development, and ageing of the different strains. The skeleton of rapidly ageing individuals is at any given chronological age physiologically older than that of individuals with a slow rate of skeletal development. In other words, estrogen given to animals of the same chronological age but of various strains will in fast-ageing strains act on bone in which resorption is already prominent, while in slowly developing strains bone formation may still be in progress. Strain differences in nutritional requirements and in endocrine activity may likewise play a role in the different skeletal response of different strains to the steroid. In view of the conspicuous effects that even small amounts of hormones may exert as primers, such a role should certainly not be denied to the endogenous hormones.

Effects of sex. Sex differences in the skeletal response—particularly conspicuous in mice—may be partly explained by the higher physiological bone age of growing females as compared to that of males of the same chronological age. Male mice of most strains react more vigorously to estrogen than females. However in some strains of mice such as C3H, estrogen exerted a more marked reaction in the bones of females than of males. This observation has been considered as due to an antagonism of the injected estrogen and the endogenous androgen present in the male. Numerous experiments (24, 75, 78a, 80, 84, 101) have failed to establish unequivocally the existence of an androgen-estrogen antagonism regarding the skeleton. Actually in pigeons, small priming doses of androgen were necessary for medullary bone formation to occur under the influence of estrogen (96). Moreover, in various strains of mice the two steroids do not antagonize each other when injected simultaneously. The time element in particular is an important factor in the divergence of the results obtained. Synergistic action of androgens and estrogens during early stages of administration may cause the maximum effect to be reached prematurely and the reversal likewise to set in prematurely. Thus an actual synergism may simulate the existence of an antagonism. Pelvic relaxation caused by estrogen may be prevented by androgens (78a). This antagonism, however, is related to the role of sex hormones in propagation and may, therefore, be governed by rules that do not apply to extragenital effects of these steroids.

Response of individual bones. The susceptibility of individual bones to estrogen decreases in the following order: Lower femur, upper tibia, upper femur, humerus, and cubitus (30, 41, 88). The rule that the

younger a bone physiologically at the beginning of the treatment the more vigorous its reaction is, applies therefore also to individual bones. The os ilii is remarkable in that it actually undergoes resorption under the influence of estrogen (77, 86).

vi. *Mechanism of action of estrogen.* One of the major questions regarding the mode of action of estrogen on bone is, whether or not the hormone acts on the skeleton directly or through intermediation of other endocrine glands. Several possibilities have been investigated:

Role of hypophysis. Changes in the structure and function of the anterior hypophysis seen following administration of estrogen suggest that the hormone exerts its skeletal effects by inactivation of the former. This might seem even more plausible since both estrogen and hypophysectomy inhibit linear growth. However, the microscopic changes seen in the epiphyseal cartilage under the two conditions are distinctive: Following hypophysectomy, growth, development and ossification of the provisional cartilage are retarded and decreased, while estrogen not only inhibits growth of cartilage but also intensifies ossification and regressive changes. The latter changes affect the cartilage so severely that they may suffice to inhibit growth on a local level. Moreover, estrogens may act on both serum Ca and bone in the absence of the hypophysis (29, 67, 83, 97). The failure of estrogen to elicit skeletal effects in hypophysectomized animals (66) does not necessarily indicate an intermediary function of the hypophysis: after hypophysectomy growth of cartilage is maximally depressed so that further inhibition by estrogen is impossible. Similarly, bone formation is at a minimum following hypophysectomy so that there is little or no substratum for the estrogen to act on. If estrogen and hypophyseal hormone are given simultaneously to animals with intact hypophyses, both substances counteract each other in regard to growth of cartilage (59, 80a). Both hormones, however, act synergistically with respect to calcification and ossification. In conclusion, while it cannot be disproven that the growth inhibition caused by estrogen may at least be partly mediated through the hypophysis, this possibility is not a likely one. Most of the skeletal effects exerted by estrogen can be accounted for without intervention of the hypophysis.

Role of parathyroids. Since the skeletal effects of estrogens can be observed in the absence of the parathyroids (29, 73, 88, 89, 97), the latter may be disregarded as a factor essential to the action of estrogen on bone. However, in some species, estrogens might stimulate the parathyroids (61, 89, 91, 97), and parathyroid hormone may contribute to the peculiar combination of formation and resorption of bone observed.

Role of nutritional factors. Estrogen did not accelerate the repletion of body Ca following Ca depletion (74). The hormone exerted its effects

also on bone of animals kept on a vitamin D deficient (82) or a low Ca diet (89). If however, the serum Ca falls below a critical level as has been shown in parathyroidectomized ducks given a low Ca diet (89), the effects of estrogen are obscured by the appearance of osteoid tissue indicative of rickets-like conditions. Therefore, the results of these experiments should not be construed to prove that estrogen causes deposition of organic matrix and that this effect is independent of the serum Ca. A low protein diet (84) did not alter the skeletal response to estrogen, and in guinea pigs estrogen was capable of acting in the absence of vitamin C, while at the same time retarding the development of acute scorbutic lesions (48). Estrogen also exerted its effects on long bones whose nerve supply had been severed (72).

Role of local factors. As has been discussed, secondary endocrine and nutritional factors play only a minor role in determining the action of estrogen on bone. Therefore, the skeletal effects of these steroids seem to be to a large extent local in character. Direct observations as well as circumstantial evidence have failed so far to explain their true nature. This may be due to the fact that this action takes place on a hitherto unexplored level and that all studies made to date deal actually with secondary manifestations of one or several primary effects. Estrogens cause degenerative changes, in particular hyalinization of connective tissue (Loeb *et al.*, 1938; Loeb, 1940; Miner and Hechter, 1953). In addition, estrogens as well as other steroids influence the water content of various tissues (Zuckerman, Palmer, and Bourne, 1939). A similar susceptibility of the cartilaginous and pre-osseous ground substance to hormones has been repeatedly demonstrated (48, 62, 80) (Loeb, 1940). These changes may be primary; they may, however, also be due to or coincide with changes in the composition or permeability of the vessel walls. Whatever the underlying mechanism, the combination of such changes in cartilage connective tissue and bone may set off the sequelae characteristic of the action of estrogen. With the increasing interest in structure and function of ground substance in general, progress should be made also regarding the effects of steroids on the matrix of the skeleton.

The inhibiting effect exerted by estrogen on skeletal growth is safely established. However, not all observers have considered the inhibition of growth as of significance in the evolution of the subsequent changes in metaphysis and shaft. Based on the observations described above four major theories have been advanced regarding the mode of action of estrogen on the skeleton: (1) Estrogen stimulates primarily osteoblastic growth. This view (Gardner and Pfeiffer, 1938 (78); 1943) presupposes a primary anabolic effect of the steroid on osteoblasts. How-

ever, there is as yet no supporting evidence for an anabolic effect being exerted by this hormone on any mesenchymal tissues. The remaining three hypotheses agree on a primary injurious effect exerted by estrogen on the mesenchyme, either on cells, or on the ground substance, or on both. (2) Estrogen induces hyalinization of the ground substance. This change interferes with linear growth, vascularity, and resorptive processes, while new formation of osseous tissue goes on. The ensuing condensation of bone is thus primarily due to decreased resorption, and only to a limited degree to deposition of new material (Silberberg and Silberberg, 1939 (48), 1943; Day and Follis, 1940 (63); Lippman and deSaunders, 1942 (69)). (3) Estrogen causes primary osteoclasia and osteolysis associated with or followed by excessive medullary bone formation (Benoit and Clavert, 1943 (89)). This view has been arrived at predominantly from observations on birds. It seems likely that the fluctuations of serum Ca and bone mineral and the necessity of a rapid response to the requirements of the developing egg create in birds metabolic conditions that do not apply to mammals. Yet, basically the response of the avian and mammalian mesenchyme may be similar but may be modified in birds by the simultaneous hypercalcemia. (4) Estrogen injures reticulum cells of the bone marrow, and the subsequent excessive bone formation constitutes a result of cicatrization (Landauer and Zondek, 1944 (90); Urist, Budy, and McLean, 1948 (41)). This theory combines some of the views discussed above. It is more specific than the former in regard to the injurious effects of estrogen, which are supposed to be directed against the cells rather than matrix. The term cicatrization for the changes taking place in the bone marrow seems unfortunate. Cicatrization denotes a permanent pathological change, while the changes appearing during egg-laying are certainly physiological and are, as those induced by exogenous estrogens, reversible to an extraordinary degree. In conclusion, much new factual material is needed in order to establish the basic reactions caused by estrogen in the skeleton. Probably this effect will turn out to be related to that exerted by estrogens on connective tissue in general.

(b) *Progesterone*

The effects of this steroid on Ca metabolism and on bone are not conspicuous and are of interest only because of their relation to the action of other hormones. Progesterone has no influence on ultimate body size (Sosa-Gallardo and Arias, 1941); it is unable to prevent the estrogen-induced changes in the bones of mice (77). In young guinea pigs, the hormone slightly stimulates epiphyseal growth and temporarily retards regressive changes and ossification of the cartilage (Silberberg and Silberberg, 1941). The effect resembles that seen at early stages following

TABLE IV
PUBLICATIONS DEALING WITH THE SKELETAL EFFECTS OF ORCHIECTOMY AND MAIN FINDINGS REPORTED

Species	Author	Skeletal findings
MAN		
	(102) F. Bergmann, <i>Arch. per lo studio delle tradizioni popolare</i> 2, <i>Palermo</i> (1883).	Disproportionate growth of trunk, tubular bones and skull; pelvis asexual
	(103) C. Breus and A. Kolisko, "Pathologische Beckenformen," Vol. 1, Deuticke, Wien, 1904.	Disproportionate growth of trunk, tubular bones and skull; pelvis asexual
	(104) J. Tandler and S. Gross, <i>Arch. Entwicklungsmech. Organ.</i> 27, 290 (1909).	Disproportionate growth of trunk, tubular bones and skull; pelvis asexual
	(105) W. Engelbach and A. MacMahon, <i>Endocrinology</i> 8, 1 (1924).	Disproportionate growth of trunk, tubular bones and skull; pelvis asexual
	(106) F. Wagenseil, <i>Z. Morphol. Anthropol.</i> 26, 264 (1926).	Disproportionate growth of trunk, tubular bones and skull; pelvis asexual
	(107) F. Altmann, <i>Virchow's Arch. pathol. Anat. u. physiol.</i> 276, 455 (1930).*	Disproportionate growth of trunk, tubular bones and skull; pelvis asexual
	(108) E. Pittard, "La castration sur l'homme," Libr. de l'Acad. de méd. Masson, Paris, 1934.	Disproportionate growth of trunk, tubular bones and skull; pelvis asexual
	(109) J. B. Hamilton, <i>Recent Progr. in Hormone Research</i> , 3, 257 (1948).	Disproportionate growth of trunk, tubular bones and skull; pelvis asexual
	(110) P. Ravault, G. Vignon, and H. Fraisse, <i>Rev. rheum.</i> 17, 247 (1950).	Disproportionate growth of trunk, tubular bones and skull; pelvis asexual
	(111) P. Horstmann, <i>Acta Endocrinol. (Suppl.)</i> 5, No. 3 (1953).	Osteoporosis in eunuchs Disproportionate growth of trunk, tubular bones and skull; also endocrine influences (therapeutic)
HORSE		
	(112) A. Poncet, <i>Mém. et compt. rend. soc. sci. méd. Lyons</i> , 17, 178 (1878).	Tubular bones elongated
	^a H. Sellheim, <i>Beitr. Geburtshülf. u. Gynäkol.</i> 2, 236 (1899).*	Epiphyseal closure retarded
	^a Huschke, cited by R. Disselhorst, <i>Kühn-Arch.</i> 5, 29 (1914).	Pelvis asexual

CATTLE		
	^a H. Sellheim, <i>Beitr. Geburtshülf. u. Gynäkol.</i> 2 , 236 (1899).*	Epiphyseal closure retarded
	^a J. Tandler and K. Keller, <i>Arch. Entwicklungs-mech. Organ.</i> 31 , 289 (1910).	Horns of asexual type
DEER		
	^a J. Tandler and K. Keller, <i>Arch. Entwicklungs-mech. Organ.</i> 31 , 289 (1910).	Antlers not shed
	(113) H. Sellheim, <i>Z. Geburtshülf. u. Gnyäkol.</i> 74 , 362 (1913).*	Growth of antlers retarded; whig antlers
	(114) J. C. Aub and G. B. Wislocki, <i>Cancer Research</i> 6 , 501 (1946).	Growth of antlers prevented; or antlers not shed
PIG		
	^a H. Sellheim, <i>Beitr. Geburtshülf. u. Gynäkol.</i> 2 , 236 (1899).*	Epiphyseal closure retarded
SHEEP		
	(115) N. Tscherwinsky, <i>Arch. mikroskop u. Entwick-lungsmech. Anat.</i> 75 , 522 (1910).	Growth increased; also age factor
	^a Franz, cited by R. Disselhorst, <i>Kühn-Arch.</i> 5 , 29 (1914).	Pelvis asexual, epiphyseal closure retarded
	(116) F. H. A. Marshall and J. Hammond, <i>J. Physiol. (London)</i> 48 , 171 (1914).	No growth of horns
	(117) J. Hammond, "The Growth and Development of Mutton Qualities in the Sheep." Oliver and Boyd, London, 1932.	Growth increased; skeleton delicate
CAT		
	(118) A. Poncet, <i>Assoc. franç. l'avance sci. compt. rend. 6e sess., LeHavre</i> , p. 893 (1877).*	Tubular bones elongated
RABBIT		
	^a A. Poncet, <i>Assoc. franç. l'avance Sci. compt. rend. 6e sess., LeHavre</i> , p. 893 (1877).	Tubular bones elongated
	(119) W. R. Poppe, <i>Kühn-Arch.</i> 34 , 61 (1934).	Disproportionate growth of flat bones

TABLE IV (Continued)

Species	Author	Skeletal findings
GUINEA PIG		
	(120) A. Poncet, <i>Compt. rend. soc. biol.</i> 55 , 65 (1903); a P. Bouin and P. Ancel, <i>Compt. rend.</i> 142 , 232 (1906). a C. R. Moore, <i>Biol. Bull.</i> 43 , 285 (1922).	Tubular bones elongated Tubular bones elongated
	(121) M. D. Sumulong, <i>Philippine J. Sci.</i> 27 , 325 (1925). a M. Silberberg and R. Silberberg, <i>Am. J. Pathol.</i> 15 , 55 (1939).*	Body size decreased; tubular bones absolutely shortened, relatively lengthened Body size increased
RAT	a S. Hatai, <i>J. Exptl. Zool.</i> 18 , 1 (1915). (122) G. van Wagenen, <i>Am. J. Physiol.</i> 84 , 461 (1928). (123) J. J. Lawless, <i>Anat. Record</i> 66 , 455 (1936). (124) H. S. Rubinstein, A. R. Abarbanel, and A. A. Kurland, <i>Endocrinology</i> 25 , 397 (1939). (125) G. R. Pomerat and R. C. Coe, <i>Endocrinology</i> 29 , 1015 (1941).	Development of epiphyseal and articular cartilage retarded Body growth retarded, long bones and tail elongated Growth of body and bones decreased Body growth decreased Tail and body growth decreased Tubular bones shortened
MOUSE	a M. Silberberg and R. Silberberg, <i>Anat. Record</i> 95 , 97 (1946).*	Development of epiphyses and joints retarded
CHICKEN	a H. Sellheim, <i>Beitr. Geburtshilf. u. Gynäkol.</i> 2 , 236 (1899).* a Pirsche, cited by A. Poncet, <i>Compt. rend. soc. biol.</i> 55 , 65 (1903). (126) W. Landauer, <i>Anat. Record</i> 69 , 247 (1937).	Disproportionate growth of bones and body Body and bone growth increased Bone growth not affected

* Indicates microscopic studies.

a References without numbers have been cited previously in the tables.

ovariectomy and may thus be due to inhibition of endogenous steroids that hasten skeletal development.

The role of progesterone in pubic relaxation has been repeatedly studied. Since the hormone does not produce relaxation in the absence of the uterus, its effect is now considered an indirect one due to stimulation of relaxin production in the uterus (Hisaw *et al.*, 1942; Hisaw, 1944; Fugo, 1943; Zarrow, 1948). In mice, progesterone inhibits pubic relaxation caused by estrogen (Hall, 1949).

V. Testicular Steroids

1. SKELETAL EFFECTS OF TESTICULAR DEFICIENCY

In the *growing* skeleton, effects of orchietomy manifest themselves in the tubular and flat bones and in such secondary sex characteristics as horns and antlers. Skeletal development is retarded and the more so the earlier in life castration is performed (Table IV). Growth zones that usually close before puberty are not at all or least effected; those of tibia, femur, radius, and ulna that unite during puberty show retardation of epiphyseal closure of varying degree and those that ordinarily close after puberty, such as those of the iliac crest, may never unite. The delay of epiphyseal closure is followed by prolongation of the growth period; however, growth is not accelerated but may proceed at a subnormal rate. Therefore, gigantism does not occur, and there may indeed be a moderate degree of stunting. Irrespective of their ultimate body size, eunuchs show a disproportionate increase in the length of those bones whose growth zones are open at the time of castration; they thus have an increased arm spread, and abnormally long hands, legs, and feet as well as elongated facial skeletal features resembling those seen in acromegalic individuals. The sitting height of eunuchs is comparatively short. A decrease in sitting height, however, does not necessarily imply a decrease in height of the individual vertebrae, since the length of the vertebral column depends partly on the state of ligaments and intervertebral discs (109). The castrate skeleton as a whole is delicate and sometimes porotic (110, 117). This condition may be a direct effect of the lack of sex hormone, but it may also be related to the decreased bulk and strength of the muscles of the castrate. The castrate pelvis retains an infantile pattern.

The findings in orchietomized animals are similar to those seen in eunuchism (Table IV). Large castrate animals, such as steers, geldings, wethers, and barrows have an increased standing height, disproportionate

growth of skull, asexual pelvis, and generally delicate bones. Small castrate rodents, such as guinea pigs and rats, usually show decreased body size associated with an absolute decrease but relative increase in the length of the tubular bones.

Horns have a bony core, and antlers are entirely osseous, and as secondary sex characters these structures respond readily to changes in the serum levels of sex hormones. The horns of castrate cattle are of an intermediate or asexual type, and they grow longer than ordinary (9), while in castrate rams horns do not develop (116). In deer orchietomized before puberty, antlers are not formed, and in young adult deer castrated while the antlers are in the velvet, development is arrested until the following summer, when new antlers grow from the stumps. These antlers ossify slowly and are not shed as usual (114). Thus, due to repeated annual increment "whig" antlers form (113).

Microscopically, the growth zones of the long bones of castrates contain an increased number of columnar cartilage cells, while hypertrophy of these cells is delayed. Regressive changes and replacement of the provisional cartilage by bone are likewise retarded (20).

In *adult* individuals, deficiency of male sex hormone is supposed to give rise to osteoporosis, and in old age this disease is believed to become accentuated due to the decline in the production of androgens (Heller and Shipley, 1951). There is, however, in aged patients an overlapping of effects of androgen deficiency with those of adrenal cortical steroids so that definite conclusions should be withheld until adequate experimental data are available.

In summary—The skeletal effects of testicular deficiency resemble those caused by ovarian deficiency. All phases of skeletal development are retarded. However, at the sites of retarded epiphyseal closure, growth is not accelerated and may indeed be slowed down.

2. SKELETAL EFFECTS OF TESTICULAR STEROIDS

(a) *Biochemical and Biophysical Findings*

The testicular 17-ketosteroids are not known to affect the level of Ca or P in the serum or bones of normal individuals (Fox and Kochakian, 1944; Kennedy *et al.*, 1953). Androgens may, however, interfere with certain skeletal effects exerted by estrogen: in mice of some strains, the increase in breaking strength and in bone ash noted after treatment with estrogen was decreased by simultaneous administration of testosterone (78b). Moreover, in aged humans suffering from osteoporosis, testosterone increases the retention of N, Ca, and P, (Reifenstein and Albright, 1947). Similarly in patients with osteogenesis imperfecta, the fragility of bones was slightly decreased during treatment with androgen (Ropes *et al.*, 1946).

(b) *Gross Observations*

Administration of testicular extracts or transplantation of testicular tissue retards growth of the long bones (Table V). These effects are due to the presence in the grafted tissue of 17-ketosteroids. Androgens whose effects have been most thoroughly investigated are testosterone propionate and methyltestosterone. 17-Ketosteroids with androgenic potencies may act as growth stimuli. The prepubertal growth spurt occurring in normal boys and the temporary acceleration of growth seen in sexually precocious children are attributed to the growth-promoting action of testosterone. While small doses of the hormone stimulate, large doses delay or suppress linear growth. No matter, whether given in small or large doses, testosterone always hastens skeletal development: the steroid causes premature appearance of those centers of ossification that appear late in childhood, and it produces premature epiphyseal closure. The latter accounts for the ultimate deficiency in body size of animals receiving large amounts of testosterone and of children with precocious sexual maturity.

In deer, administration of testosterone interferes with the cyclic growth of antlers: males thus treated may shed the velvet, and ossification of the antlers is accelerated. Conversely, in castrate males or in females who never had antlers, such structures will develop following treatment with the hormone. The role of testicular secretions in antler growth seems to consist of several phases (114, 136): a priming effect preceding actual growth; a maturation-promoting effect regulating calcification and ossification of the fibrocartilaginous material of which the antlers are composed; and a maintaining effect that prevents the shedding of the antlers, which occurs with declining testicular activity.

(c) *Microscopic Studies*

Testosterone in large doses suppresses proliferation of cartilage, but intensifies hypertrophy, hyalinization, calcification, and ossification (Fig. 1, 3). Resorption of both the provisional cartilage and primary spongiosa is slightly inhibited, but at no time is there as much bone in the metaphysis as after treatment with estrogen (156). The hormone has no effect on bone repair, if tested under strictly controlled conditions (137).

(d) *Factors Modifying the Skeletal Effects of Androgen*

As was shown for estrogens, the skeletal effects of testosterone are subject to a number of modifying influences. Among these factors, the dose is important, since it determines whether or not growth will be stimulated. Furthermore, the susceptibility to the hormone changes with the physiological age of the skeleton: a maximum response is elicited shortly before puberty, while newborn and adult individuals show a

TABLE V
INVESTIGATIONS DEALING WITH THE MORPHOLOGICAL EFFECTS OF ADMINISTRATION OF TESTICULAR 17-KETOSTEROIDS ON
BONE AND MAIN FINDINGS REPORTED

Species	Author	Skeletal findings
MAN		
	(127) H. Gilford, "The Disorders of Postnatal Growth and Development." Adlar & Son, London, 1911.	Comprehensive clinical references
	(128) L. de Gennes and R. Rogé, <i>Gaz. méd. France</i> 44 , 821 (1937).	Growth stimulated in hypogenital boys
	(129) M. Villaret, L. Justin-Besençau, and A. Rubens-Duval, <i>Compt. rend. soc. biol.</i> 127 , 599 (1938).	Growth stimulated in eunuchism
	(130) J. E. Howard and S. A. Vest, <i>Am. J. Med. Sci.</i> 198 , 823 (1939).	Development accelerated
	(131) B. Webster and W. Hoskins, <i>Proc. Soc. Expt. Biol. Med.</i> 45 , 72 (1940).	Growth stimulated in hypogenital boys
	(132) E. P. McCullagh and F. J. McGurl, <i>Endocrinology</i> 26 , 377 (1940).	Development accelerated in enuchism
	(133) L. Wilkins, "The Diagnosis and Treatment of Endocrine Disorders in Childhood and Adolescence." C. C Thomas, Springfield, 1950.	Comprehensive references
	(134) N. B. Talbot, E. H. Sobel, J. W. MacArthur, and J. D. Crawford, "Functional Endocrinology." Harvard Univ. Press, Cambridge 1952.	Comprehensive references
MONKEY		
	(135) G. van Wagenen, <i>Federation Proc.</i> 6 , 219 (1947); <i>Endocrinology</i> 45 , 544 (1949).	Temporary acceleration of growth; acceleration of development
DEER		
	^a J. C. Aub and G. B. Wislocki, <i>Cancer Research</i> 6 , 501 (1946).	Accelerated development of antlers
	(136) G. B. Wislocki, J. C. Aub, and C. M. Waldo, <i>Endocrinology</i> , 40 , 202 (1947).*	Accelerated development of antlers

DOG

- (137) W. D. Armstrong, *Trans. 3rd Josiah Macy, Jr. Conf. on Bone and Wound Healing*, p. 74 (1942).

No effect on healing of fractures

RABBIT

- (138) L. Dor, J. Maisonnare, and R. Monziols, *Compt. rend. soc. biol.* **59**, 673 (1903).^{*}
a G. Coryn, *Arch. intern. méd. exptl.* **11**, 135 (1936).^{*}
- (139) C. G. Bergstrand, *Acta Endocrinol.* **4**, 91 (1950).^{*}
- (140) S. Amante and E. Bidone, *Minerva chir.* **5**, 779 (1950).^{*}

Growth inhibited

Development accelerated

Growth inhibited

Callus formation accelerated

GUINEA

PIG

- (141) H. Seemann, *Endokrinologie*, **18**, 225 (1937).^{*}

Epiphyseal growth inhibited

RAT

- (142) C. S. McEuen, H. Selye, and J. B. Collip, *Proc. Soc. Exptl. Biol. Med.* **36**, 390 (1937).
- (143) L. H. Levie, *Acta Brevia Neerl. Physiol. Pharmacol. Microbiol.* **8**, 53 (1938).
- (144) P. H. Sainton, H. Simmonet, and M. Petrescu, *Compt. rend. soc. biol.* **128**, 698 (1938).
- (145) F. W. Clausen, and C. B. Freudenberg, *Endocrinology* **25**, 585 (1939).
- (146) H. S. Rubinstein, A. A. Kurland, and M. Goodwin, *Endocrinology* **25**, 724 (1939); H. S. Rubinstein and M. L. Solomon, *Endocrinology* **28**, 112 (1941).
- (147) R. R. Green, M. W. Burrill, and C. A. Ivy, *Am. J. Anat.* **67**, 305 (1940).^{*}
- (148) H. H. Turner, E. Lachmann and A. A. Hellbaum, *Endocrinology* **29**, 425 (1941).

Growth not inhibited

Growth of tail inhibited

Growth inhibited

Growth accelerated

Growth inhibited by large doses, accelerated by small doses; also effect of castration

Development of os priapi accelerated

Development of tuberositas ischii and of os priapi accelerated; also castration

TABLE V (Continued)

Species	Author	Skeletal findings
	(149) R. F. Tyslowitz, <i>Trans. 4th Josiah Macy, Jr. Conf. on Metabolic Aspects Convalescence</i> , p. 123 (1943).	Growth stimulated after hypophysectomy and treatment with testosterone
	(150) M. E. Simpson, W. Marx, H. Becks, and H. M. Evans, <i>Endocrinology</i> 35 , 309 (1944).*	Slight stimulation of growth after hypophysectomy and treatment with testosterone
	(151) M. Reiss, J. E. Fernandez, and Y. M. L. Golla, <i>Endocrinology</i> 38 , 65 (1946).	Development of bones accelerated, growth of tails not accelerated
	(152) C. R. Noback, J. C. Barnett and H. S. Kupperman, <i>Anat. Record</i> 103 , 49 (1949).*	Development accelerated
	(153) W. R. Lyons, E. Abernathy, and M. Gropper, <i>Proc. Soc. Exptl. Biol. Med.</i> 73 , 193 (1950).	No stimulation of growth; development of os penis accelerated
	(154) A. Lichtwitz, R. Parlier, G. Thiery and M. Delaville, <i>Acta phys. therap. rheumat. Belg.</i> 5 , 116 (1950).	Stimulation of growth; also sex differences in response to testosterone
	(155) A. E. Light and J. A. Turnaben, <i>J. Nutrition</i> 51 , 365 (1953).	Growth of tail stimulated
MOUSE		
	(156) M. Silberberg and R. Silberberg, <i>Arch. Pathol.</i> 32 , 85 (1941)*; <i>Anat. Record</i> 95 , 97 (1946).*	Development accelerated; also castration and dose; sex differences in response to testosterone
	(157) R. P. Fox and C. D. Kochakian, <i>Federation Proc.</i> 3 , 10 (1944).	Data on P turnover and ash content of bones
DUCK		
	(158) H. Bulliard and A. Ravina, <i>Compt. rend. soc. biol.</i> 127 , 525 (1938).	Growth temporarily accelerated
CHICKEN		
	(159) G. H. Ettinger, <i>Anat. Record</i> 82 , 408 (1942).	Growth inhibited

* Indicates microscopic studies.

a References without numbers have been cited previously in the tables.

decreased reaction or none at all (156). Female mice respond more vigorously to the age-promoting action of testosterone than males (156). This may be due to the relatively older physiological age of the female as compared with that of the male of corresponding chronological age. In the physiologically older female, the hormone merely accentuates a prevailing trend, while in the physiologically younger male, the steroid has to overcome such growth potencies as may still be present.

(e) *Mechanism of Action of Androgen*

The growth-promoting action of testosterone has been observed only in undersized hypogenital boys (128-134), and in castrate (146, 154-156), or hypophysectomized animals (149, 150). This suggests that the growth-stimulating capacity of testosterone is limited. Androgen-induced growth is apparently related to the increased N retention caused by the hormone (Kochakian and Murlin, 1936; Kenyon *et al.*, 1940). In the presence of a growth potential, N retention may stimulate both proliferation and hypertrophy of the cartilage as well as accretion of bone. In the light of these findings, the effects of castration on food consumption (Holt, Keeton and Vennesland, 1936; Tang, 1941) lose their significance. Since the growth-promoting effect of testosterone is seen also in hypophysectomized animals (149, 150), it is independent of the hypophysis.

The growth-inhibiting effect of large doses of the hormone has been attributed to suppression of the hypophysis by the androgen. This view has its counterpart in the interpretation of the acromegaloid features of the castrate skeleton as manifestations of secondary hyperpituitarism developing in the absence of testicular secretions. However, it is not at all certain how specific for hyperpituitarism these changes are. In both pituitary gigantism and eunuchism, the patency of a number of growth zones is the basis for the increased growth of the extremities. These open epiphyseal discs represent purely local conditions conducive to continued growth with or without intervention of the hypophysis. Furthermore, the effects of testosterone on the growth zones are those of acceleration of the normal sequelae and not those of a slowdown due to inhibition of the hypophyseal function. In addition, the premature appearance of centers of ossification seen in the presence of excessive amounts of testosterone are incompatible with decreased hypophyseal activity. On the other hand, the local changes caused by the steroid in the ground substance may directly interfere with growth and nutrition of cartilage and thus bring about acceleration of skeletal development.

As is true for the estrogens, the nature of the effects of testosterone on

the ground substance is unknown. These effects seem to be concentrated in the extracellular compartment of the mesenchyme, and it is in this respect that estrogens and androgens behave similarly. The two steroids differ, however, in that the androgens are far less potent and in that they fail to affect osteogenesis and osteolysis to the same extent as the estrogens.

In summary—Aside from a limited growth-promoting effect, androgenic 17-ketosteroids cause acceleration of skeletal development by promoting condensation of the ground substance and ossification.

VI. Adrenal Cortical Steroids

1. SKELETAL EFFECTS OF ADRENAL CORTICAL DEFICIENCY

In adrenalectomized rats, growth of epiphyseal cartilage is arrested (Coryn, 1936 (42); Ingalls and Hayes, 1941; Wyman and Tum-Suden, 1945). This effect is apparently non-specific and caused by decreased food consumption. In adrenalectomized animals, maintained with chlorides and desoxycorticosterone, the long bones are somewhat enlarged. This slight growth stimulation supposedly results from the elimination of growth-inhibitors present in the adrenal cortex (Maassen, 1952a).

2. SKELETAL EFFECTS OF ADRENAL CORTICAL HORMONES

(a) *Crude Extracts and Transplants of Adrenals*

Adrenal cortical extracts injected into the yolk sac of Leghorn eggs increase the incidence of micromelia but do not affect the rumplessness induced by insulin. These extracts thus have no effect during the early stages of skeletal development, when rumplessness is initiated (Landauer, 1947). In mice, cortical secretions given off by grafted adrenals counteract the growth processes in the articular cartilage and thus decrease the incidence of degenerative joint disease called forth by anterior hypophyseal homeografts (Silberberg, Silberberg, and Opdyke, 1954).

(b) *17-Ketosteroids*

The adrenal cortex produces androgenic steroids that appear in the urine as 17-ketosteroids. The output of these hormones is increased under pathological conditions associated with adrenal cortical hyperplasia or benign or malignant cortical tumors (Kussmaul, 1862; Lenz, 1913 (33); Gross, 1940; Hurxthal and Musulin, 1953 (35)). The skeletal effects exerted by these 17-ketosteroids are identical with those exerted by 17-ketosteroids of testicular origin (Figs. 5, 6). These effects have been fully described above, and a further discussion of their action seems therefore unnecessary.

(c) *Mineralocorticoids*

Administration of desoxycorticosterone or of similar mineralo-

corticoids slightly inhibits body growth of chicken embryos (Stock, Karnofsky, and Sugiura, 1951), and retards growth of the long bones and bone repair in dogs (Fontaine, Mandel, and Wiest, 1952). Desoxycorticosterone acetate also counteracts the growth spurt produced by hypophyseal growth hormone in the tail of rats (Maassen, 1952b). The mode of action of these cortical steroids is unknown.

(d) *Glucocorticoids*

Many of these substances inhibit growth of chicken embryos (Karnofsky, Ridgway, and Patterson, 1950), but they do so to different degrees. Applied to the chorioallantois, cortisone is more effective than if injected into the yolk sac, and divided doses are more potent than single large doses. Cortisone, and presumably other steroids of this group, are ineffective until the eighth or tenth day of incubation. In young rodents, cortisone inhibits linear growth (Wells and Kendall, 1940) by interfering with the proliferation of the epiphyseal cartilage and with the production of cartilaginous matrix. The primary spicules are poorly ossified, inadequately resorbed, and may persist for an abnormally long period of time (Follis, 1951; Mandel *et al.*, 1953) (Figs. 1, 2). In accordance with the retarding effect of cortisone on growth of connective tissue, callus formation is delayed (Blunt *et al.*, 1950).

Cortisone also increases the incidence of malformations, in particular of cleft palate in newborn mice whose mothers were treated with high doses of the hormone during pregnancy (Fraser and Fainstat, 1951). This effect might be considered as due to inhibition of growth, were it not for the fact that cleft palate is produced also if the steroid is given at a time when the naso-maxillary sinuses are already closed. It has been suggested that cleft palate may result from regressive changes and resorption of bone caused by cortisone.

No experimental data are as yet available regarding the effects of cortisone on adult bone. However, glucocorticoids have been implicated as a cause of senile osteoporosis (Albright, 1947) and of osteoporosis associated with Cushing's syndrome (Cushing, 1932). In old age, the production of glucocorticoids by the adrenals does not decrease as does that of 17-ketosteroids (Heller and Shipley, 1951), and Cushing's syndrome is characterized by an overproduction of glucocorticoids. The anti-anabolic glucocorticoids are supposed to prevent the formation of osseous matrix, and thus shift the balance between formation and resorption of bone in favor of the latter. However, the few microscopic studies carried out on bones of patients with Cushing's syndrome showed neither decreased osteogenesis nor increased osteolysis (Follis, 1950).

Mechanism of action of glucocorticoids: Rats given cortisone and fed ad libitum consume less food than non-injected controls. However, if the weights of untreated rats are controlled so as to equal those of cortisone-treated rats the non-treated animals require only two thirds

of the food intake of the cortisone-treated animals (Winter, Silber, and Stoerk, 1950). Therefore, decreased food consumption can be ruled out as a cause for the skeletal changes. The former may in fact be the consequence rather than the cause of the decrease in anabolic processes. Decreased skeletal growth and ossification as seen after administration of cortisone are consistent with a state of decreased anabolism. However, the decrease of cellular growth and of deposition of organic matrix constitute merely a quantitative change, while the inhibition of bone resorption points to qualitative alterations in the intercellular substance. Such changes may also be responsible for the inhibiting effect of adrenal cortical transplants on the development of degenerative joint disease. Evidence is accumulating that adrenal cortical steroids produce major changes in the ground substance and fibrils of ordinary connective tissue (Baker, 1950; Opsahl, 1950; Miner and Hechter, 1953), and it may be only a question of time before such changes can be demonstrated in cartilage and bone.

In summary—The 17-ketosteroids of adrenal cortical origin exert the same skeletal effects as those originating in the testicle. Mineralocorticoids have not yet been shown to exert any skeletal effects. Adrenal glucocorticoids inhibit skeletal development probably by way of their anti-anabolic action. These steroids may also cause qualitative changes in the specialized skeletal ground substance.

VII. Summary and Conclusions

Many of the steroid hormones influence skeletal growth, development, and ageing; sex steroids in addition regulate the development of osseous structures representing secondary sex characters, such as horns and antlers. In birds, these steroids also control skeletal changes that are incidental to egg-laying, namely medullary bone formation. The effects of steroid hormones on bone are thus manifold, and even though some of the end results may appear similar, they may be brought about by different mechanisms. Direct and circumstantial evidence strongly indicates that steroid hormones may act on the skeleton directly without mediation of other endocrine glands.

Only a limited number of investigations have been carried out, and so far little information has been forthcoming concerning the skeletal effects of progesterone, of desoxycorticosterone and related substances, and even of the glucocorticoids.

Of the steroid hormones tested, only the androgenic 17-ketosteroids in small doses have been shown to increase linear growth; this effect seen only in individuals, whose body growth is retarded due to certain endocrine deficiencies, is probably related to the increased N retention caused by the 17-ketosteroids. Androgenic 17-ketosteroids in large doses

as well as estrogens retard skeletal growth but, at the same time, they advance development and ageing of cartilage and bone. Adrenal glucocorticoids inhibit skeletal growth as well as development apparently because of their antianabolic action.

Notwithstanding the large amount of work done, little is known about the fundamental processes whereby these hormones influence the transformation of the juvenile into the adult skeleton. These processes are primarily regressive in character and are conducive to bone formation; they affect the cells and ground substance of cartilage and of various other mesenchymal tissues. The underlying mechanisms, although presently unknown, will probably turn out to be similar to those released by the respective hormones in non-specialized connective tissue. The close relationship of such changes in cartilage and connective tissue respectively is illustrated by the sequence of events following administration of estrogen. In young animals thus treated, alterations of both cartilage and connective tissue result in the persistence of bone that ordinarily would have been resorbed. This inhibition of resorative processes contributes primarily to the excess bone found in animals receiving estrogen, while actual accretion of bone plays a secondary role. In avian species, the marked hypercalcemia developing in response to estrogen and related to egg-laying creates special conditions which distinguish the reaction of birds from that of mammals. The excessive amounts of available Ca together with the changes in the bone marrow account for the rapid and abundant accretion of bone in birds.

The early myelofibrosis and osteolysis seen in estrogen-treated adult and old animals provide additional evidence for the occurrence of a primary disturbance in the connective tissue. Accordingly, accretion of bone seems to be secondary and to take place only if additional metabolic prerequisites of general or of local character are met. A concept of this kind facilitates an understanding of the diverse skeletal effects of estrogens which range from overmineralization and hyperostosis to demineralization and fibrous replacement even within the same individual.

In contrast to their significance for skeletal growth and development the role of steroid hormones in the homeostasis of the adult skeleton is not clear. It has been claimed but not proven that these hormones are essential for the maintenance of the normal adult skeleton.

Controlled studies are needed in order further to define conditions under which a given steroid influences skeletal tissues in a certain manner. Such investigations should avoid the pitfalls of generalizations and dogmaticisms in regard to substances whose effects like those of steroid hormones are determined by a large variety of extrinsic and intrinsic factors. This applies in particular to a target tissue like bone in which the homeostatic mechanisms of osteogenesis and osteolysis have a pronounced tendency to resist extraskeletal influences.

PLATE I

Sections through the epiphyseal growth zones of the upper tibia of 3 month old male mice of strain C57BL. $\times 100$.

1. Untreated control: the cartilage and the metaphyseal trabeculae show regular arrangement.
2. Treatment with cortisone: 0.5 mg 5 times weekly for 2 months starting at the age of 1 month. The growth zone is wider and contains less intercellular substance than that of the untreated control. The metaphyseal trabeculae are scantier, thinner and shorter than usual, and some are oriented in a transverse direction indicating arrest of growth at some time during the experiment.
3. Treatment with testosterone propionate: 0.25 mg 3 times weekly for 2 months starting at the age of 1 month. The growth zone shows areas of degeneration of cartilage; the metaphyseal trabeculae are more numerous, longer, and thicker than in the control.
4. Treatment with α -estradiol benzoate: 0.03 mg once weekly for 2 months starting at the age of 1 month. The epiphyseal growth zone is narrow and heavily calcified. The metaphyses and upper diaphyses contain a dense network of coarse interlacing bony trabeculae.

PLATE I

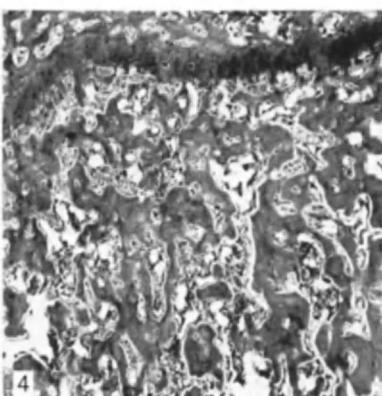
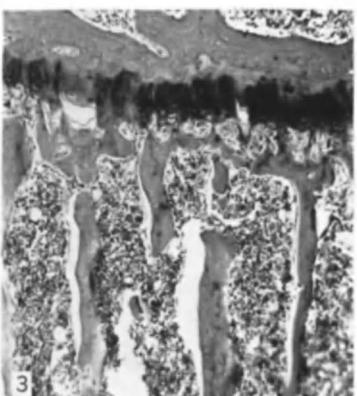
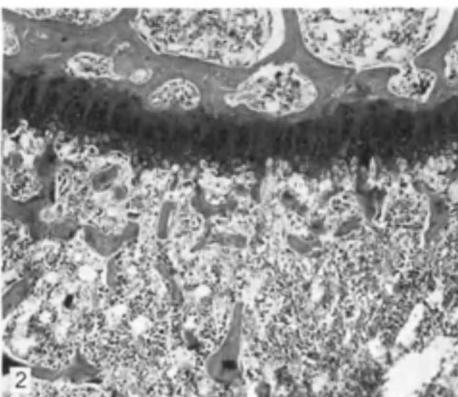
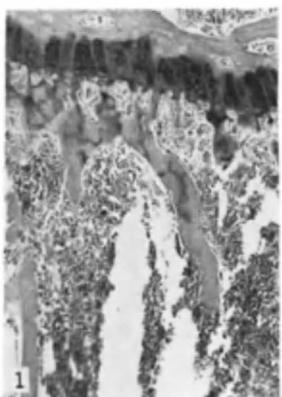


PLATE II

5. X-ray photograph of the wrist of a normal 3-year-old girl: two wrist bones are developed. Courtesy: Dr. Don C. Weir, Department of Radiology, St. Louis City Hospital.

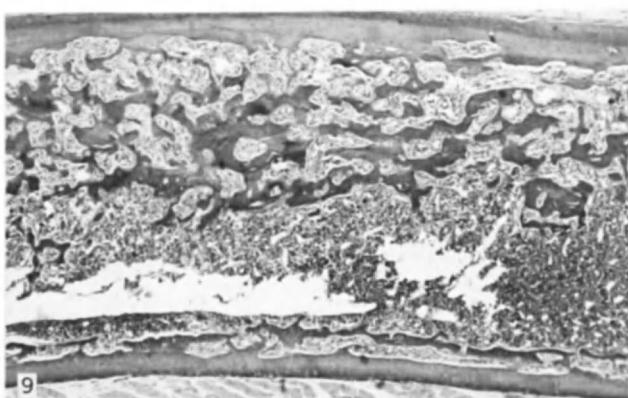
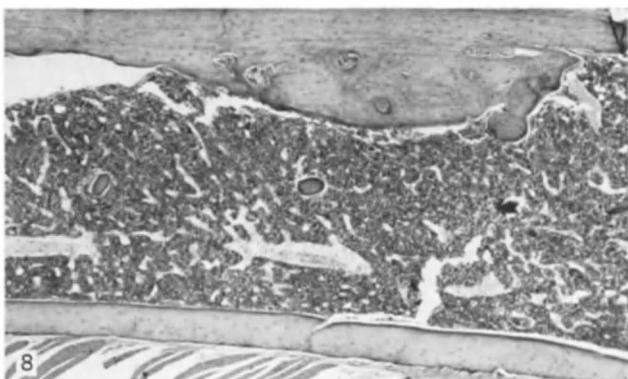
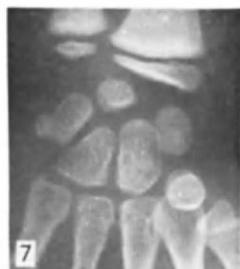
6. X-ray photograph of the wrist of a 3-year-old girl with an adrenal tumor producing 17-ketosteroids. All seven wrist bones are present; physiological bone age approximately 9 years. Courtesy: Dr. Willard M. Allen, Department of Obstetrics and Gynecology, Washington University, St. Louis.

7. X-ray photograph of the wrist of a 4-year-old girl with hyperestrinization: All wrist bones are present; physiological bone age about 9 years. Courtesy: Dr. Don C. Weir, Department of Radiology, St. Louis City Hospital.

8. Section through the shaft of the tibia of a 14-month-old untreated male mouse of strain C57BL: the bony protuberance projecting into the marrow cavity is regularly found in the tibiae of adult mice. $\times 50$.

9. Section through the shaft of the tibia of a male mouse of strain C57BL treated with 0.03 mg of α -estradiol benzoate twice weekly for 2 months starting at the age of 12 months. The section is taken from an area corresponding to that shown in Fig. 8. There is marked resorption of bone on both sides of the shaft with new formation of connective tissue and small osseous spicules. If one is unaware of the presence of the focal thickening of the normal cortex, one might be led to attribute the changes in the upper part of the picture to primary new formation of bone. $\times 50$.

PLATE II



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CHAPTER XXI

ANTERIOR PITUITARY REGULATION OF SKELETAL DEVELOPMENT

C. W. ASLING and H. M. EVANS

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I. Introduction

The purpose of this chapter is to describe the present state of knowledge of anterior pituitary hormonal regulation of skeletal development. As in other endocrine studies, this requires detailed analysis of (1) the consequences of deficiency (in this case, chiefly those induced by hypophysectomy), (2) the repair of these consequences (by pituitary or related hormones), and (3) the effects of administration of such hormones in excess.

Attention will be chiefly devoted to data obtained from rats, where both the general body of information and the specific experience of this laboratory are greatest. This is due to the devising, by P. E. Smith (1930), of a simple and dependable technique for hypophysectomy in the rat, by means of which substantial numbers of these animals may

be made available for study. However, before proceeding to describe the results of such studies, mention must be made of other pioneer papers in the field. For example, Dandy and Reichert (1938), in studies on hypophysectomized puppies, demonstrated by radiographs that both bony growth and maturation (i.e., epiphyseal union) were markedly retarded by pituitary removal. Dwarfism and failure of epiphyseal union were also the main findings in the histologic study of the human skeleton by Erdheim (1916) in a case in whom a destructive lesion of the pituitary gland was demonstrable at autopsy. On the other hand, the repeated clinical observations of pituitary hyperfunction in human gigantism received experimental substantiation when Evans and Long (1922) induced excessive growth in intact rats by administering crude anterior lobe extracts of the pituitary gland. Other classic reports have been cited in the bibliographies of such reviews as those by Silberberg and Silberberg (1943), H. Houssay (1947), Simpson, Asling, and Evans (1950), Asling, Simpson, and Evans (1951), and McLean and Urist (1955).

II. Experimental Conditions

Brief mention should be made of some of the experimental conditions necessary to successful study of hypophysectomized rats. Diet is of primary importance; it is routine to feed a diet from natural sources, high in protein and containing supplements of essential vitamins and minerals. Measured feeding and, in some experiments, paired feeding may be necessary. These dietary and other pre- and post-operative measures which have markedly reduced the morbidity and mortality of this delicate and sensitive test animal have been summarized recently by Fortgang and Simpson (1953). When hormonal replacement therapy is to be studied, the hormones should be of the maximal purity obtainable when tested both physicochemically and biologically. In the case of pituitary hormones this requires proteins of a single molecular species as demonstrated by such physicochemical tests as electrophoresis, and free from contamination by other pituitary principles in doses of many multiples of the minimal effective dose when tested biologically by injection into hypophysectomized rats. For example, an attainable objective in studies using growth hormone is a preparation of a minimal effective dose of 10 μg (tibia line test, Evans *et al.*, 1943; Greenspan *et al.*, 1949) which contains no detectable contamination by other hormones when 5 mg are administered to hypophysectomized rats over a 4-day period (Li, Evans, and Simpson, 1945).

Of techniques specifically directed toward the skeleton, mention may be made of three. *Gross measurements*, directly from bones (or from radiographs when suitable precautions against parallax errors can be

taken) are made by vernier calipers reading to 0.1 m. The observer's error is usually less than 2 parts per 100. *Radiographic studies*, particularly for defining the state of patency of an epiphyseal cartilage plate, require an emulsion of finer grain than that used clinically. The X-ray film commonly used in our laboratory is Eastman Industrial Type A. The patency of even such tiny epiphyseal plates as those of the phalanges may be established with greater than 90% accuracy when such films are examined at $7.5 \times$ magnification. The film's slower speed, coupled with the relatively long tube-to-emulsion distance (30 to 36 inches) customarily used to reduce errors of parallax, gives no problem when autopsy material is being examined, but necessitates anesthesia when the living animal is to be studied. The infrequent administration of anesthesia, with administration of antibiotics to prevent post-anesthetic pneumonia, gives us the great advantage of making serial studies on the same animal in long-term experiments. Finally, *histologically*, information of great value may be obtained from such a simple technique as nitric acid decalcification, nitrocellulose embedding, 8-10 micron sectioning, and hematoxylin-and-eosin or modified Mallory staining. The chief use thus far made has been the observation and measurement of growth and maturation processes at epiphyseal cartilage plates. However, this histologic field offers excellent promise for profitable use of histochemical and radioautographic procedures, especially with the development of alternative methods of decalcification of the specimens.

In this account, in addition to hypophysectomy, some mention will also be made of the effects of thyroidectomy. By this operation not only is a primary thyroid deficiency created; a substantial degree of pituitary deficiency also ensues, as a result of the diminished level of pituitary activity in the absence of thyroxin. Additional interest attaches to thyroidectomized rats since it is in this operation that there has been the greatest success in attempting to provoke a maximal defect of skeletal growth by interfering at the earliest possible age; Salmon (1936, 1938, 1941), Scow and Simpson (1945), and Ray *et al.* (1950a) have succeeded in maintaining rats thyroidectomized on the first day of life. The earliest age at which hypophysectomy has been successfully performed on substantial numbers of rats is 6 days (Walker *et al.*, 1950, 1952).

III. Effects of Hormonal Deficiency on Skeletal Growth

1. HYPOPHYSECTOMY

Hypophysectomy, when performed on growing rats, results in a marked retardation of growth. Figure 1 is a graph of body lengths of a group of female rats hypophysectomized when 26-28 days of age (the age most commonly employed in our laboratory). Individual bones (tibia,

Fig. 2, and third metacarpal, Fig. 3) show the same general type of curve (Becks and Evans, 1953).

The growth retardation following hypophysectomy is, to some extent,

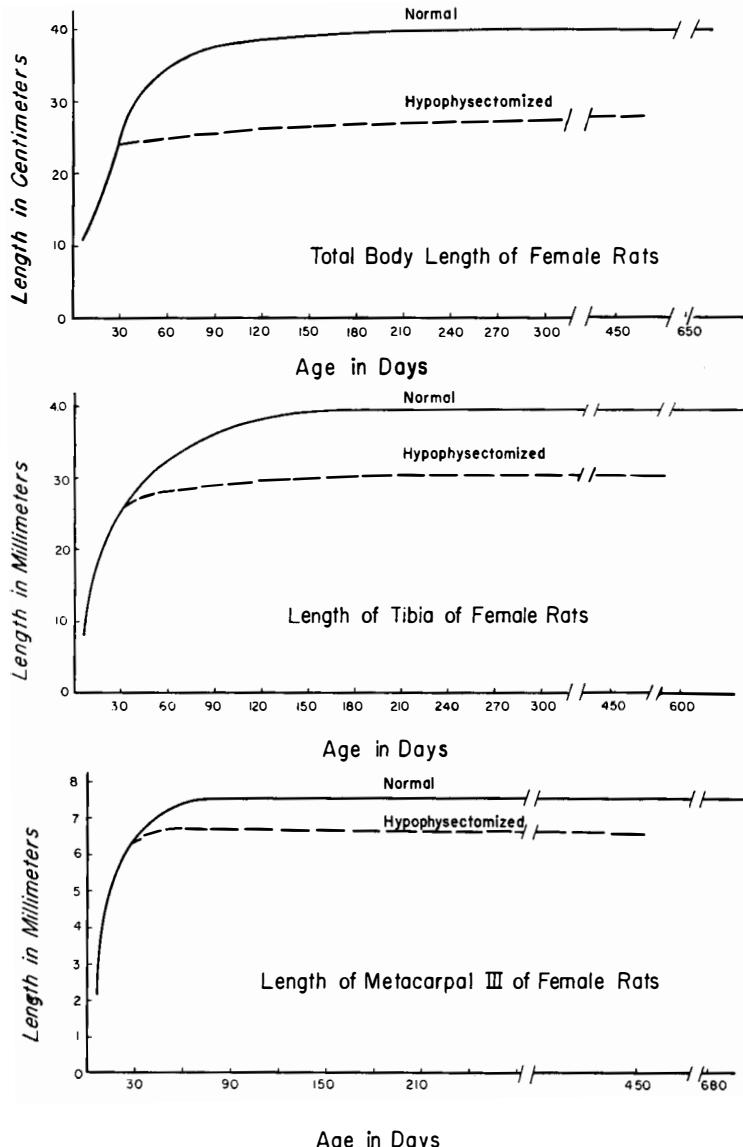
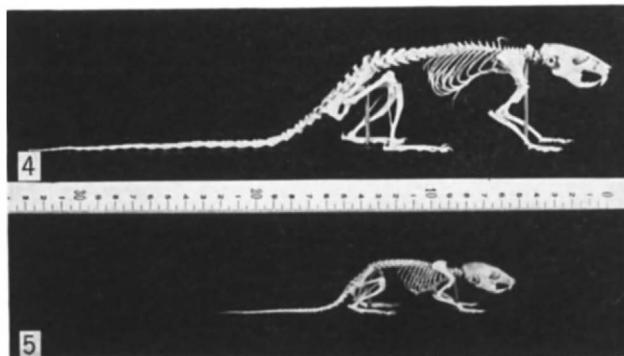


FIG. 1. Growth in body length of normal female rats, and the retardation following hypophysectomy at 28 days of age (Becks and Evans, 1953, after Asling).

FIG. 2. Growth of tibia of normal female rats, and the retardation following hypophysectomy at 28 days of age (Becks and Evans, 1953, after Asling).

FIG. 3. Growth of third metacarpal of normal female rats, and the retardation following hypophysectomy at 28 days of age (Becks and Evans, 1953, after Asling).

dependent upon the age at the time of the operation. When rats are hypophysectomized when 6-days-old, growth continues, although at a clearly reduced rate, for some weeks (Walker *et al.*, 1950). The data presented in Table I show the percent increase in length over that at the time of hypophysectomy for animals of different ages at the time of operation. Although in every case growth was retarded, the extent of the retardation was least in the animals hypophysectomized at the earliest age. In ultimate size, however, these animals show extreme dwarfism, as is shown by the skeletons in Figs. 4 and 5. Thus, it appears that very



Figs. 4 AND 5. Mounted skeletons of 60-day-old female rats, with scale in centimeters. Above, normal; below, hypophysectomized when 6 days of age (after Walker *et al.*, 1952).

young animals have some capacity for growth independent of the pituitary hormonal stimulus; this capacity is progressively reduced as they grow older, and dependence upon the pituitary increases. It has been shown by graphic analysis of these data that this dependence on pituitary hormones probably becomes maximal in month-old rats (Simpson *et al.*, 1950).¹

The histologic changes which follow hypophysectomy and which give us, for example, a thinner cortical bone, are perhaps most marked at the epiphyseal cartilage plates; those at the proximal epiphyseal plate of the tibia have been most extensively studied, and appear to be representative of the majority of regions of endochondral osteogenesis (Becks and Evans, 1953). As may be seen by comparing Figs. 6 and 8, proliferation of chondrocytes, and their transformation from flattened to vacuolated forms ceases, and as a result the plate is narrowed. The vascular tufts from the marrow which normally impinge upon the vacuolated cells

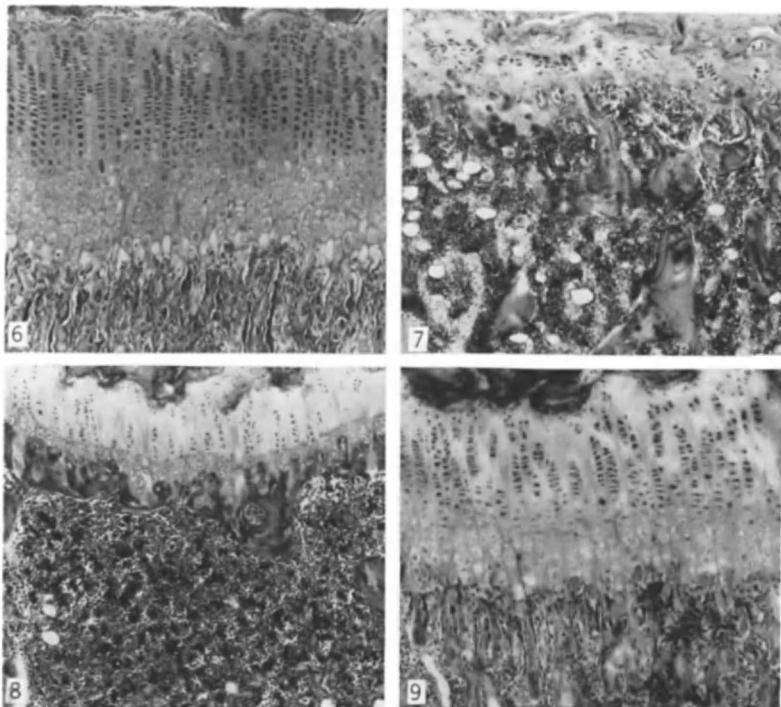
¹ It is possible, however, that even at that time, a very slight capacity for growth persists; rats hypophysectomized at 28 days of age were found to increase in length during the following 8 months, although the rate of gain was so slight that the increase did not become statistically significant until after 7 weeks (Frank, 1953).

TABLE I
DIFFERENCES IN GROWTH IN LENGTH AMONG FEMALE RATS
HYPOPHYSECTOMIZED AT VARYING AGES ^a

Age at operation	Age at autopsy	Length at autopsy	Increase in length ^b	Increase normal for same period ^b
days	days	cm	%	%
6	60	20.2	85	200
13	60	22.4	51	123
21	60	24.6	33	78
28	60	26.1	14	45
75	111	35.7	0	6

^a Data from Asling *et al.* (1949), Walker *et al.* (1950) and Ray *et al.* (1950b)

^b Percentage increase in length = $\frac{\text{Gain in length during experiment}}{\text{Length at onset}} \times 100$



Figs. 6 TO 9. Median saggital sections of proximal tibial epiphyseal cartilage plate of female rats (H and E, $\times 55$). (After Becks *et al.*, 1945a, b; 1946.)

6. Normal, 30 days of age.
7. Normal, 307 days of age.
8. Hypophysectomized at 28 days of age, 326 days after operation.
9. Hypophysectomized at 28 days of age, 324 days after operation, with growth hormone injections for last 39 days.

disappear, and the fine trabecular bone of the spongiosa is resorbed. Eventually calcification of the juxtamedullary portion of the cartilage ensues, and finally it is sealed from the marrow by a transverse lamina of bone (Becks, Simpson, and Evans, 1945b). These transformations closely resemble those observed in growth stasis from other causes. Among the other circumstances in which similar appearances can be found are normal ageing (in the proximal tibial epiphysis, Fig. 7, and certain other epiphyses, Becks, Simpson, and Evans, 1945a), thyroidectomy (Becks, Kibrick and Evans, 1942 and Becks *et al.*, 1942), vitamin A deficiency (Wolbach, 1946, 1947), riboflavin deficiency (Nelson *et al.*, 1947), pantothenic acid deficiency (Nelson *et al.*, 1950), protein deficiency (Frandsen *et al.*, 1954), caloric restriction (Handler, Baylin, and Follis, 1947), and radiation injury (Heller, 1948). It is likely that the radiographic "lines of arrested growth" (Harris, 1933) observed in children suffering acute infectious disease may represent the same structures.

2. REPLACEMENT THERAPY FOLLOWING HYPOPHYSECTOMY

Administration of the growth hormone of the anterior lobe of the pituitary is effective in repairing the growth defect of hypophysectomized rats. The animals are responsive to its effect whether the injections are commenced shortly after the operation (Simpson, Evans, and Li, 1949) or whether the delay is a year or longer (Becks *et al.*, 1946). Although

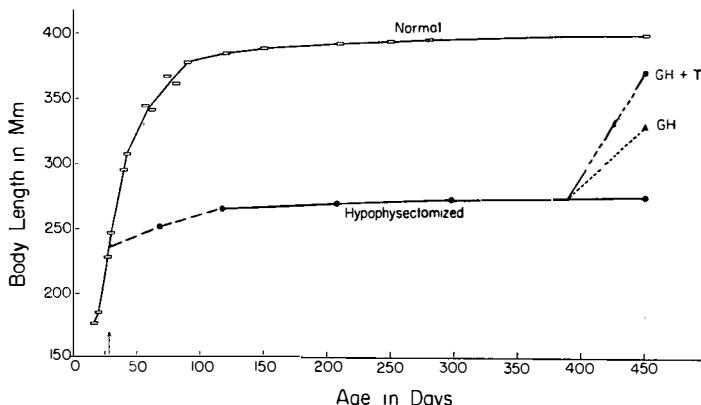


FIG. 10. Re-establishment of growth in body length by growth hormone (GH) injections started one year after hypophysectomy at 28 days of age, and augmentation of the growth hormone by thyroxin (Tx). (Adapted from Asling *et al.*, 1954.)

the majority of experiments have been made on rats hypophysectomized when 28 days old, the age at the time of hypophysectomy is not a factor, since animals hypophysectomized when as young as 6 days of age

(Walker *et al.*, 1952) and as old as 6-7 months of age (Moon *et al.*, 1951), have been shown to be responsive to administration of the growth hormone. A typical instance of the effect of growth hormone on body length is illustrated by the graph in Fig. 10. The question of differences amongst bones in their response to the hormone will be dealt with in a subsequent section describing the effect of hypophysectomy and replacement therapy on body proportions.

Growth hormone administrations rapidly restore the histologic appearance of epiphyseal cartilage plates to that characterizing young, actively growing bones (Fig. 9, from Becks *et al.*, 1946). Promptly (in 5 days or less) chondrogenesis is reawakened, vascular tufts from the marrow again impinge on the vacuolated cells (the sealing-bone being broken up, if it has had time to form) and the delicate bony trabeculae of a re-established spongiosa appear, bearing an abundance of epithelioid osteoblasts on their surfaces. The cortical bone thickens, and one may eventually see (by differential staining) lines which distinguish the recently formed bone from the older, thinned cortex. The widening of the epiphyseal cartilage plate is, within a broad range of dosages, proportionate to the amount of hormone administered (Becks *et al.*, 1941). This has given rise to a method of assay of the potency of hormone preparations (Greenspan *et al.*, 1949), which is sensitive to as little as a total of 5 μg . of hormone given over a 4-day period.

Although growth hormone is effective as described, and although growth paralleling (or even exceeding) normal may be maintained in hypophysectomized rats by careful grading of the dose, it is possible to augment its effect markedly through a synergistic action of the thyroid gland. Thyrotropic hormone has been demonstrated to show this synergism with growth hormone (Smith, 1933; Marx, Simpson, and Evans, 1942), but it has not been available in sufficiently large amounts to allow study of its skeletal effects. Reliance has therefore been placed on the injection of solutions of crystalline thyroxin in the study of the pituitary-thyroid relationship. A typical instance of the synergism is shown in Fig. 10. Under some circumstances the augmentation of growth hormone's effect even extends to the widening of the epiphyseal cartilage plates.

The question of whether thyroid hormone alone will stimulate growth in hypophysectomized rats has been raised by several workers (Laqueur, Dingemanse, and Freud, 1941). In some short-term experiments (i.e., 30 days) low dosages of thyroid hormone (2.5 $\mu\text{g}./\text{day}$ or less) have enabled hypophysectomized animals to exceed their controls slightly but definitely in skeletal dimensions (Ray *et al.*, 1950b). Recently a prolonged (14 month) experiment showed that although appreciable (but subnormal) growth could be maintained when thyroxin was administered

immediately after hypophysectomy (at 28 days of age) this growth was not continued (Asling *et al.*, 1954). As shown by Fig. 11, after 40 days of injections the full growth retardation characteristic of hypophysec-

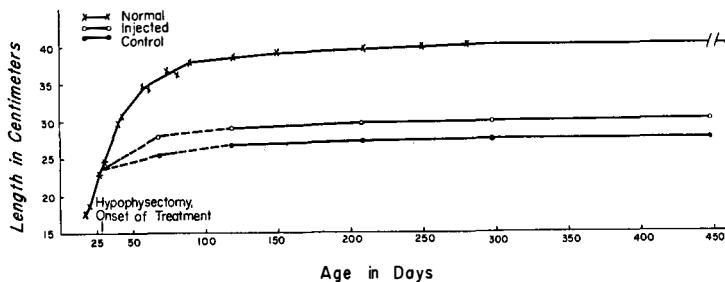


FIG. 11. Effect of prolonged thyroxin treatment on body length of hypophysectomized female rats. Solid circles: hypophysectomized controls; open circles: hypophysectomized, thyroxin-injected rats; \times - \times : normal controls. (Adapted from Asling *et al.*, 1954.)

tomized rats developed and the rats persisted in this condition for the remainder of the long injection period.² Histologically, after 14 months their proximal tibial epiphyseal cartilage plates showed the same inactivity and atrophy as did the hypophysectomized controls. Thyroxin is, therefore, not a growth-promoting hormone *per se*, since one of the established criteria for such a hormone is that administration over a prolonged period should result in sustained growth of the hypophysectomized rat.³

3. THYROIDECTOMY

As was mentioned earlier, almost as much interest attaches to the effects of thyroidectomy on skeletal growth as to those of hypophysectomy. The skeleton is thus deprived of any direct action of thyroid hormone (including augmentation of growth hormone's action). Removal of thyroid hormone also results in a deficient pituitary function, one of the first signs of which is degranulation of the acidophils, the presumptive source of growth hormone. The skeleton of such rats has been studied by several groups of workers. Evans, Simpson, and Pencharz (1939) showed a growth retardation following thyroidectomy at 35–45

² Both initially and at the end of the experiment the food consumption of the thyroxin-treated hypophysectomized rats was nearly 50% higher than that of their hypophysectomized controls (*i.e.*, 7.0 vs. 4.8 g/day/rat).

³ The other two criteria to which we have adhered are: (a) growth exceeding that which can be induced by non-specific stimulants, and (b) growth proportional to dosage over a wide range of dosages.

days of age much like that which follows hypophysectomy. That it may not have been quite as severe is suggested by the observation that thyroidectomized-hypophysectomized rats had body lengths still shorter than those only thyroidectomized. Becks *et al.* (1942a) presented histologic data from the tibias of these same rats and, in another study (Becks *et al.*, 1942b), from adult male rats which had been thyroidectomized when 26–28 days of age. The epiphyseal cartilage plate's regression after thyroidectomy corresponded in essential features to those described after hypophysectomy.

Ziskin, Salmon, and Applebaum and coworkers (1940), Scow and Simpson (1945), Scow *et al.* (1949), and Ray *et al.* (1950a) have studied the skeletons of rats thyroidectomized when 1 day of age. The most nearly comparable data from hypophysectomized rats are those obtained from animals 6 days old at the time of operation (Walker *et al.*, 1950, 1952). It will be remembered that these latter showed definite growth (Table I). Likewise, the thyroidectomized rats of Ray *et al.*, gained 140% over their original body length in 2 months (while their intact controls were gaining 300%). The data of all these workers suggests that the greatest part of these gains was achieved before the rats were a month old. Some excess of growth of thyroidectomized rats over that of rats hypophysectomized and observed at comparable ages might well be expected. Thyroid hormone is known to be relatively long-lasting, and the full effect of its deprivation (including pituitary hypofunction) might not be developed immediately after thyroidectomy, whereas growth hormone has a very short survival time (Van Dyke *et al.*, 1950) and would be expected to disappear promptly after hypophysectomy. With this reservation, it may be said that the effect of the two deficiencies on skeletal growth are quite similar. The similarity extends to histological structure of the proximal tibial epiphyseal cartilage plate when rats operated upon at an early age are compared. In both deficiencies the plates remain wider, and the expansion of the epiphyseal ossification center is less complete than in rats operated upon at older ages.

4. REPLACEMENT THERAPY FOLLOWING THYROIDECTOMY

The problem of reinstating growth after thyroidectomy differs in some respects from that after hypophysectomy. Thyroxin injections will stimulate active growth in thyroidectomized rats (Scow *et al.*, 1949; Ray *et al.*, 1950a). Concurrently pituitary function is restored (as attested by evidence from gonads and adrenals that gonadotropic and adrenocorticotropic hormones are again in good endogenous supply). The repair of pituitary structure, and particularly the regranulation of acidophilic (Koneff *et al.*, 1949) makes it likely that the growth results from an endogenous supply of growth hormone. It must be remembered that the conditions necessary for growth hormone-thyroxin synergism are

satisfied by this therapy, and that optimal growth may thus be approximated. It is not surprising, therefore, to find observers differing on whether additional (exogenous) growth hormone will stimulate still more active growth; Scow *et al.* (1949) believed the further growth insignificant, while Ray *et al.* (1950a) found substantial enhancement.

In contrast to Salmon (1936, 1938, 1941), who found thyroidectomized rats to be insensitive to pituitary growth hormone, all of the other investigators mentioned have been able to reawaken growth by this hormone in the absence of the thyroid. It is true that the animals are much less responsive than hypophysectomized rats, and large doses of the hormone may be required.⁴ The explanation of the difference in sensitivity may lie in the aforementioned augmenting action of thyroid hormone. Whereas thyroidectomized rats are virtually devoid of such hormone, there is evidence that slight thyroid function persists after hypophysectomy (Morton *et al.*, 1942). The level of function, even though low, may yet be enough to enhance the effect of growth hormone administration.

It is appropriate to mention here that the response of rats to hypophysectomy and thyroidectomy combined, and to replacement therapy, has been studied by Evans *et al.* (1939) in rats approximately 40 days of age when operated upon, and by Ray *et al.*, (1954) who followed thyroidectomy on the first day of life by hypophysectomy on the 20th day of age. The need for such studies is obvious, for (as shown) the presence of each gland complicates the analysis of results obtained when administering products of the other. Growth stasis in these rats was, of course, marked. Thyroxin administration had negligible effects on growth. Analysis of the results of growth hormone administration was complicated by variability in response and condition of these delicate test animals, but it was clear that some of the rats were responsive to its stimulus. Concurrent administration of growth hormone and thyroxin stimulated vigorous growth, even surpassing the normal rate. The differing responses of the distal end of the humerus to these various treatments is illustrated in Figs. 24 to 27, in connection with the discussion of bone maturation.

5. EFFECTS OF GROWTH HORMONE IN EXCESS

The overgrowth of the skeleton which should follow administration of growth hormone in excess of normal requirements has been demonstrated on several occasions. In a short-term (30 day) experiment, in which the hormone was administered to normal growing female rats starting at 81 days of age, growth in body and tibia length was pushed

⁴ Ray *et al.* (1950a) have tabulated the difference in sensitivity to growth hormone in the two deficiencies.

beyond normal by 1 mg of hormone per day (Asling *et al.*, 1948, 1950b). Widening of the tibia's proximal epiphyseal cartilage plate was also found. Long-term experiments, with gigantism as an objective, had been performed much earlier; an instructive one was that of Evans and Simpson (1931) who treated adult rats, both male and female, both intact and gonadectomized, for 8 months with a potent pituitary extract containing growth hormone. Gigantism was induced in all the treated rats. The intact males receiving the hormone exceeded their controls in body length by over 9%; the corresponding females exceeded their controls by 15%. Gonadectomized rats were similarly responsive; in fact, the response seemed even greater in ovariectomized rats. Later experiments, performed when procedures for extracting growth hormone of a high degree of purity had been developed, confirmed the responsiveness of intact rats to growth hormone. Twice (Evans, Simpson, and Li, 1948; Moon *et al.*, 1950a) groups of intact adult female rats have been injected with such hormone for periods exceeding a year. Gigantism was attained, most of the skeletal dimensions exceeding normal by 12–16%. Intact adult males have also been stimulated to gigantism by highly purified growth hormone (unpublished data). Two experiments have been performed on hypophysectomized female rats. In one the pituitary was removed when the rats were 28 days of age, and the hormone was injected for 14½ months starting 12 days after hypophysectomy (Simpson *et al.*, 1949). The dosage was only one fifth to one tenth that given to intact rats. These hypophysectomized rats, while exceeding normality in weight, just maintained normality in length and in dimensions of the individual bones. In another experiment marked gigantism was induced in hypophysectomized rats, under different experimental conditions (Moon *et al.*, 1951). The rats were hypophysectomized when adult, and the dosage of hormone (started immediately and continued for 13 months) closely approximated that given the intact rats which had become gigantic. Such rats exceeded in some skeletal dimensions even the intact giants (Asling *et al.*, 1955).

Reinhardt and Li (1953) have reported the development of a chronic arthritis in knees and ankles of gonadectomized-adrenalectomized rats under prolonged treatment with growth hormone. Joint deformities were also developed in both intact and hypophysectomized rats of the series just mentioned.⁵ They were found most constantly in the vertebral

⁵ Other disorders induced by growth hormone overdosage in rats have included tumors of lungs, suprarenal medulla, and other viscera (Moon *et al.*, 1950a, b, c; Koneff *et al.*, 1951). Derangements of carbohydrate metabolism (e.g., diabetes) which are easily provoked in carnivores by growth hormone, are difficult to produce in rats. The diabetes of the alloxan-treated rat is enhanced by growth hormone (Bennett and Li, 1947, Bennett *et al.*, 1948).

column, knee, and ankle joints. The incidence and the degree of deformity was much greater in hypophysectomized rats than in intact rats similarly treated. The structures are still under analysis, but the changes accord closely with the findings (by Kellgren, Ball, and Tutton, 1952) in human acromegalic joint disease. The radiographic appearance of knee and ankle, and the exuberant and irregular osteogenesis shown histologically at the distal end of the tibia are illustrated in Figs. 12 and 13. The kyphosis and the characteristic overgrowth of bone on the

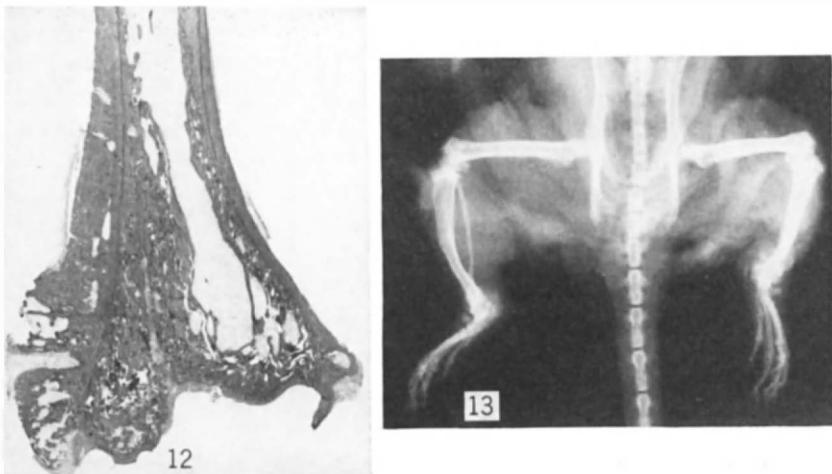


FIG. 12. Excessive bone formation at distal end of tibia and fibula of 16-month-old rat hypophysectomized when 6 months of age and treated with growth hormone for the ensuing ten months. (H and E, $\times 6$.) (After Asling *et al.*, *Hypophyseal Growth Hormone*, McGraw-Hill, 1955.)

FIG. 13. Radiograph of lower extremities of rat after treatment like that described in Fig. 12. Note resultant osteo-arthropathy of knee and ankle (one-half natural size. (After Asling *et al.*, *Hypophyseal Growth Hormone*, McGraw-Hill, 1955.)

anterior aspect of the vertebral bodies, as described by Erdheim (1931) for human acromegalic joint disease, was clearly identifiable in these rats. The kyphosis even prevented accurate determinations of body length in the animals.

The relation of gigantism to excessive growth hormone would appear to have been established on experimental grounds by the experiments just mentioned. The diagnostic features of acromegaly in the human skeleton are well established, but it is not clear what changes in the rat skeleton would be required to diagnose experimentally produced acromegaly. Certain comparisons can be made. The paws become thickened both laterally and dorsoventrally, in rats treated chronically with growth hormone, although the bones of the paws do not elongate beyond normal. The thickening seems chiefly in skin and subjacent connective tissues.

Epiphyseal fusion occurs during the treatment if it has not already taken place before the hormonal injections began. The incisor malocclusion of mandibular prognathism is not demonstrable in the rat, perhaps due to the special features of incisal occlusion in rodents, whose incisors form and erode continuously throughout life. However, overgrowth and deformity of the mandible's zone of endochondral ossification—that cartilage of the condyle subjacent to the "temporomandibular" (actually squamosomandibular) articular cartilage—has been shown (Becks *et al.*, 1948a, b and c; Collins *et al.*, 1949). Malocclusion of the molar teeth, by antero-displacement of the mandibular molars as a result of the excessive condylar growth, also is demonstrable. The bony attachments of the temporal and suboccipital muscles become very rugged and exaggerated. Overgrowth of some of the prominences of the appendicular skeleton has also been shown; for example, that of the deltoid tuberosity of the humerus is extremely marked. Perhaps the best evidence that acromegaly has been induced experimentally by purified growth hormone may be the development of the acromegalic joint disease which has been described (Asling *et al.*, 1955).

IV. Effects of Hormonal Deficiency on Skeletal Maturation

1. CRITERIA OF MATURATION

Much attention has been given to a peculiarity of development in the skeleton of rodents, in which many epiphyseal ossification centers remain separated from the diaphysis until the senescent period of the animal's life (Dawson, 1925). It is this persistence of some of the epiphyseal cartilage plates which makes it possible to stimulate the skeleton of adult rats to renewed growth (and ultimately, gigantism) by growth hormone. However, this same persistence of the epiphyseal plates or "lapsed union" has impressed some investigators with the unsuitability of rats as experimental animals for comprehensive studies on skeletal development (Washburn, 1946). We shall attempt to refute this contention. It is true that a number of growth centers in rats maintain this patency until 2½–3 years of age. Among those in the axial skeleton are the vertebral epiphyses, and the occipitophenoid and basisphenoid-presphenoid synchondroses. In the appendicular skeleton these centers include the proximal end of the humerus, both ends of the ulna, the distal end of the radius, both ends of the femur, and the proximal ends of the tibia and fibula. Conversely, there are cartilage plates which undergo resorption early in the rat's life—before 4 months of age—with synostosis of the adjacent bony masses. In the axial skeleton the exo-occipital synchondrosis of the skull shows this resorption; in the appendicular skeleton the processes of complete maturation of epiphyseal plates

TABLE II
DETERMINATION OF SKELETAL AGES

Ossification center	Chronological age in days												Latent period	940	1006-	1091	1135+
	12	15	18	21	25	30	40	50	60	80	100	120					
Humerus, proximal	P															A
distal	P															A
medial epicondyle					P											A
Radius, proximal	P															A
distal	P															A
Ulna, proximal	P															A
distal	P															A
Metacarpal, distal	P															A
Phalanges	PI	PII														A
row I, II, III		PIII														A
Femur, proximal					P											A
trochanter																A
distal	P															A
Patella					P											A
Tibia, proximal	P															A
distal	P															A
Metatarsal, distal	P3	P2	P4	P1												A
				P5												A
Pelvis, acetabulum lip						P-A											
Triradiate						A											

"P" represents present; "A" represents absent.

From Simpson, Asling, and Evans (1950); Adapted from Scow *et al.* (1949), Ray *et al.* (1950a) and Dawson (1925).

may be studied at the distal end of the humerus (Becks *et al.*, 1948b), the proximal end of the radius, the metacarpal and phalangeal epiphyses (Becks *et al.*, 1948d), the conjoined distal cartilage of the tibio-fibula (Asling *et al.*, 1950b), the calcaneus, the metatarsals, and phalanges. A fusion also occurs at the triradiate cartilage in the acetabulum and at the supra-acetabular tuberosity (Ray *et al.*, 1950a). These cartilage resorptions are not the only maturation phenomena which may be studied. In young rats, these may be seen also in the establishment and expansion of the ossification centers for the epiphyses which occur during the first two months of life.

It becomes possible, therefore, to determine the usual times of these various events in the skeleton, combining radiographic and histologic data when necessary, and to arrange them in a table of *maturity indicators* for any given age. A condensed version of the table used for the "skeletal age" diagnoses in endocrine deficiency experiments is shown in Table II.

An additional type of observation, solely histological, offers information of interest when applicable. Advantage may be taken of the presence of several centers of development in some bony regions, to detect in the same histologic section any interference with their maturation over a period of several weeks. The distal end of the humerus is such a region; it has at least two epiphyseal ossification centers whose cartilage plates are disposed at such an angle as to be clearly separable. As seen in Table III, critical events in the maturation of this region follow closely one on another for 5 weeks.

The majority of the observations to be reported here are supported by evidence gained both from radiographic and histologic criteria.

2. HYPOPHYSECTOMY

It is obvious, from what has been said earlier, that it would be impractical to attempt to detect any delaying effect of hypophysectomy on maturation in the rat by study of the proximal end of the tibia. After hypophysectomy in young rats, these "lapsed union" epiphyses take on prematurely the narrow, atrophic, "sealed" appearance which characterizes the same area in normal senescent rats (Figs. 7 and 8). It would be necessary to wait until after 3 years, when delayed union would normally finally have occurred, to detect any further effect of hypophysectomy.

Attention must therefore be directed to the group of epiphyses whose union to the diaphysis occurs at earlier, more easily observable ages. In these, it is found that following hypophysectomy at 28 days of age, epiphyseodiaphyseal union fails to occur where it should normally during the third and fourth month of age, and that patency is maintained even

TABLE III

PROGRESSIVE STAGES IN THE DEVELOPMENT OF THE DISTAL END OF THE HUMERUS
IN NORMAL FEMALE RATS BETWEEN 10 AND 45 DAYS OF AGE

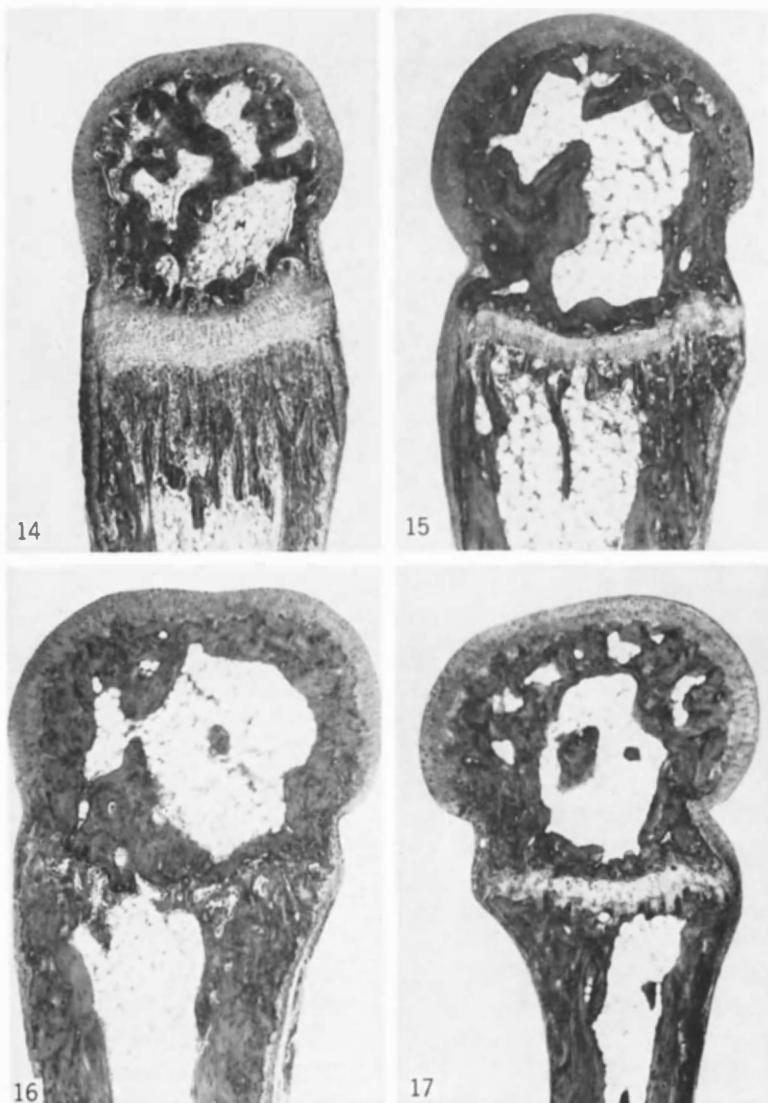
Age in days	Ossification center	
	Main (capitulum-trochlea)	Medial epicondyle
10	Cartilaginous	Cartilaginous
15	Cartilaginous, central masses of calcified matrix and enlarged, vacuolated cells foreshadow ossification center	Cartilaginous
20	Ossification center active, expanded; cartilage plate established, often already perforated at one point	Cartilaginous, central masses of calcified matrix and enlarged, vacuolated cells foreshadow ossification center
25	Cartilage plate further interrupted, reduced to several bars of cartilage	Ossification center active, expanded; cartilage plate established
30	Small fragments of cartilage, except for substantial bar in <i>lateral</i> epicondyle	No further change
35	Narrow cartilage bar in <i>lateral</i> epicondyle only	No further change
40	No cartilage remaining; epiphyseal fusion complete	No further change
45	No further change	No further change

Adapted from the report of Becks *et al.* (1948b).

if the observation period be prolonged to 14 or more months (Becks and Evans, 1953). The events in the third metacarpal bone typify the defect (Collins *et al.*, 1948), which has also been studied in phalanges, at the distal end of the tibiofibula (Asling *et al.*, 1954), at the medical epicondyle of the humerus (Becks *et al.*, 1948b), at the exoccipital synchondrosis, and at the proximal end of the radius. Normally, as growth slows, chondrogenesis becomes less active and the cartilage plate narrows (Figs. 14 and 15). Erosion and resorption of cartilage continues from the diaphyseal aspect of the plate, however, and the cartilage finally disappears completely with at first bony and subsequently marrow continuity between medullary cavities of diaphysis and epiphysis (Fig. 16). After hypophysectomy, both chondrogenesis and cartilage resorption are retarded to an equal degree. The latter probably fails through the disappearance of vascular tufts from the marrow subjacent to the cartilage plate. Eventually this narrow plate of cartilage becomes "sealed" from the diaphyseal marrow by a bony lamina (Fig. 17), in every respect like that described earlier for the proximal tibial epiphyseal plate. It is the

persistence of this "sealed" cartilage plate which allows the diagnosis of lapsed union to be made by X-ray.

When discussing growth, the time required for the retardation fol-



Figs. 14 to 17. Distal end of third metacarpal bones of female rats (H and E, $\times 40$). (After Becks *et al.*, 1948d, e.)

14. Normal, 30 days of age.

15. Normal, 90 days of age.

16. Normal, 110 days of age.

17. Hypophysectomized at 28 days of age, 346 days after operation.

lowing hypophysectomy to become evident was mentioned. The question also arises in the case of retarded maturation. It was observed that, although almost all of the epiphyses of old rats showed lapsed union after hypophysectomy at 28 days of age, the distal end of the humerus was an exception. Synostosis of the capitulum-trochlea with the diaphysis (normally completed at 40 days of age, Table III), also proceeded to completion in the hypophysectomized animals. The answer appears when one studies the skeletal ages attained by rats after hypophysectomy at varying chronological ages (Table IV). (These are the same animals whose body growth retardation was evaluated in Table II.)

It will be seen that, irrespective of the age at which the animals were hypophysectomized, the limited advance in maturation which they achieved before ultimate arrest was virtually the same in each case, and represented the amount undertaken by normal rats during a 2-3 week period. Therefore, the extent of retardation of maturation following hypophysectomy is a constant which is independent of the age at which the operation is performed.

TABLE IV
COMPARISON OF THE EFFECTS OF THYROIDECTOMY AND HYPOPHYSECTOMY
ON SKELETAL MATURATION IN THE RAT *

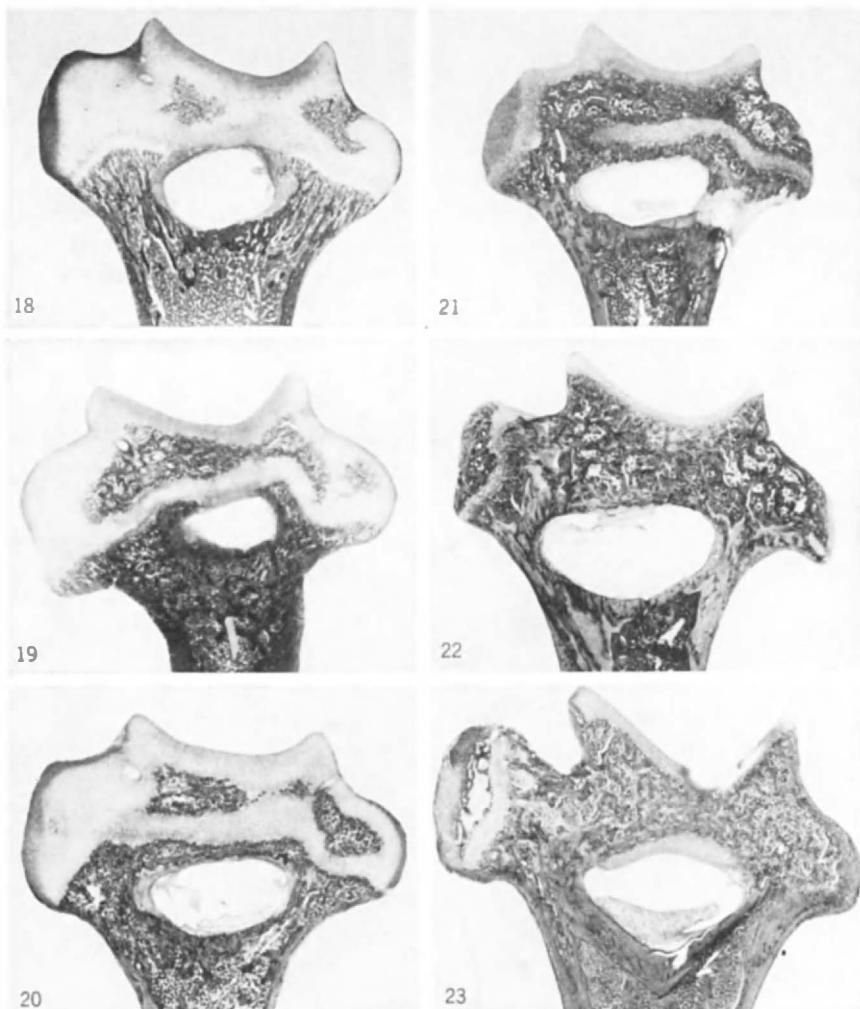
Operation	Age at operation	Skeletal age at 60 days		Observer	Advance in skeletal age after operation
		days	days		
Thyroidectomy	1	15	Ziskin <i>et al.</i> (1940)	(1940)	14
	1	18	Scow <i>et al.</i> (1949)	(1949)	17
	1	23	Ray <i>et al.</i> (1950a)	(1950a)	22
	7	23	Ziskin <i>et al.</i> (1940)	(1940)	16
Hypophysectomy	6	24	Walker <i>et al.</i> (1950)	(1950)	18
	13	33	Walker <i>et al.</i> (1950)	(1950)	20
	21	42	Ray <i>et al.</i> (1950b)	(1950b)	21
	28	47	Walker <i>et al.</i> (1950)	(1950)	19
	(75)	(95)	Asling <i>et al.</i> (1949)	(1949)	20
Thyroidectomy and Hypophysectomy	1	18	Ray <i>et al.</i> (1954)	(1954)	17
	20				

* From Simpson, Asling, and Evans (1950)

3. EFFECT OF THYROIDECTOMY, AND OF THYROIDECTOMY-HYPOPHYSECTOMY

As seen in Table IV, essentially the same degree of delay of skeletal maturation is demonstrable after thyroidectomy as after hypophysectomy. In rats thyroidectomized when 1 day old the full effects are not demon-

strable histologically even after 100 days, in that the cartilage plates remain wider than after hypophysectomy and the "sealing" lamina of bone does not appear within this time. The excessive width results partly



Figs. 18 to 23. Distal end of humerus of female rats, 60 days of age except as noted (H and E, $\times 10$). (After Ray *et al.*, 1950a, 1954; Asling *et al.*, 1950a; Simpson, Asling, and Evans, 1950.)

18. Normal, 18 days of age.
19. Thyroidectomized on first day of life.
20. Thyroidectomized on first day of life, and hypophysectomized at 20 days of age.
21. Hypophysectomized at 6 days of age.
22. Hypophysectomized at 28 days of age.
23. Normal, 60 days of age.

from the failure of the epiphyseal ossification center to expand completely (itself an instance of retarded maturation—Ray *et al.*, 1950a; Becks *et al.*, 1950).

The retarded maturation of the distal humeral epiphysis which follows these various operations is shown histologically in Figs. 18 to 23.

4. REPAIR OF RETARDED SKELETAL MATURATION

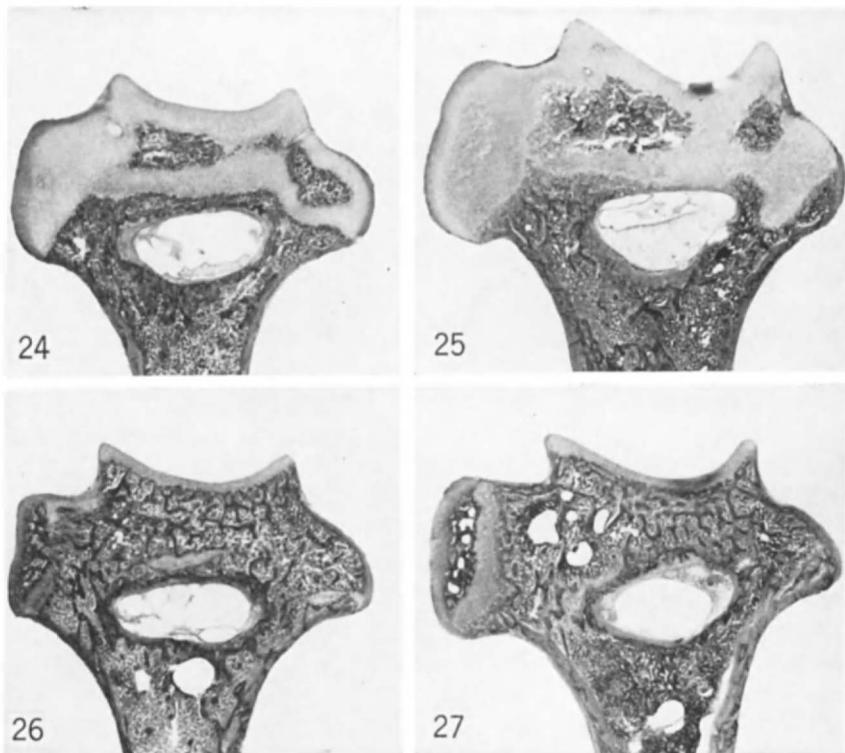
In seeking the hormonal factor which might specifically repair the failure of skeletal maturation, the observation that skeletal maturation may continue for a limited period after hypophysectomy at any age is instructive. It directs attention to a hormone characterized by some capacity for persistence—thyroxin. This implication is supported by numerous experiments, in all of which skeletal maturation has been enhanced by this hormone. In hypophysectomized rats, thyroxin will maintain skeletal maturation at a rate parallel to that in normal rats when its administration is begun immediately after hypophysectomy (Asling *et al.*, 1949, 1954; Ray *et al.*, 1950b); on the other hand, it will re-establish maturation even after a delay of a year (Becks *et al.*, 1948b; Collins *et al.*, 1948). It has proved effective when tested in rats hypophysectomized at 21, 28, or 75 days of age (Asling *et al.*, 1949).

Skeletal maturation was also advanced by thyroxin treatment of thyroidectomized rats (Becks *et al.*, 1948c; Ray *et al.*, 1950a) and thyroidectomized-hypophysectomized rats (Ray *et al.*, 1954). As mentioned earlier, only in the thyroidectomized rats was growth also stimulated to any substantial degree.

A brief remark on the effects of excessive thyroid hormone is required. No critical experiment has been performed with overdosage of this hormone in hypophysectomized rats; it appears that old hypophysectomized rats tolerate high dose levels poorly (Becks *et al.*, 1946). Intact rats have been treated with an excess of thyroid hormone with greater success; the skeletal development of nursing rats was accelerated (Noback, Barrett, and Kupperman, 1949), precocious metacarpal epiphyseal union was brought about in 82-day-old rats (Christensen and McLean, unpublished), and even the extreme of resorption of the epiphyseal plate of the proximal end of the tibia was demonstrated by Smith and McLean (1938), although with dosages to which most of the rats succumbed. (Premature epiphyseal closure in adult mice has also been effected, by Silberberg and Silberberg, 1940.) However, only when the pituitary gland is lacking can it be demonstrated that the skeletal-maturing effect of thyroxin is a direct one, and not mediated through some other pituitary hormone.

Growth hormone is without effect on skeletal maturation. In normal rats it neither accelerated nor delayed epiphyseal union (Asling *et al.*, 1948, 1950b). When given to deficient rats (hypophysectomized, thv-

roidectomized, or thyroidectomized-hypophysectomized) the skeletal age of the injected rats remained the same as the untreated, retarded controls.⁶ The sections of humerus shown in Figs. 24 to 27 illustrate the



Figs. 24 to 27. Distal end of humerus of female rats, 60 days of age, following thyroidectomy on first day of life, hypophysectomy at 20 days of age, and replacement therapy as indicated from the 30th to the 60th day of age. (See Figs. 18 and 23 for intact controls.) (H and E, $\times 10$). (After Ray *et al.*, 1954.)

- 24. Untreated control.
- 25. Growth hormone.
- 26. Thyroxin.
- 27. Growth hormone and thyroxin.

⁶ One unexplained observation requires the holding of some reservations on the relation of growth hormone to skeletal maturation. When rats hypophysectomized at 28 days of age were treated for fourteen months with growth hormone, it was found that metacarpal epiphyseal closure took place during the treatment; it could be demonstrated that this occurred at about the 8th month of the experiment, when the metacarpal bone had attained the length which normally characterizes it (Becks *et al.*, 1949). Two explanations are possible. The attainment of a bone's definitive length may assist in promoting its maturation. On the other hand, it may be that although the standard 10-day tests for purity of a hormone show the presence of no hormonal contaminants, the prolonged injection of large amounts might disclose side effects due to minimal amounts of other hormones. It is presumed that the thyrotropic hormone of the pituitary might promote skeletal maturation (acting through the thyroid). The crucial experiment, yet to be performed, is the chronic administration of growth hormone to thyroidectomized rats.

growth without parallel maturation induced by growth hormone, the maturation without growth induced by thyroxin, and the balance in development restored by combined therapy.

V. Effects of Hormonal Deficiency on Skeletal Proportions

1. NORMAL DEVELOPMENT

During the development of the skeleton, the relationship between the lengths of the various parts of the skeleton undergoes a change until, with the cessation of growth, constant proportions are reached. Comparison of the graphs of tibia length and metacarpal length (Figs. 2 and 3) gives an example of this shifting relationship. Whereas the growing period of the tibia extends over a 6-month period, the metacarpal completes its growth at about 75 days of age. Clearly, the ratio between the lengths of these two bones is constantly changing during the growth period, for at any given time after birth, the metacarpal is nearer to its definitive length than is the tibia. On the other hand, growth in tibia length and in body length (Fig. 1) proceed at parallel rates, so that a fairly constant ratio is maintained, and at any given time the tibia length is approximately 10% of the body length. Other instances might be offered, the total effect being to illustrate a well-known fact, that the infant skeleton is not a miniature of the adult skeleton but instead must advance to adult dimensions by differential rates of growth in its various parts.

2. HYPOPHYSECTOMY

When growth is retarded by hypophysectomy at 28 days of age, the various bones will have reached different amounts of their definitive length before the removal of growth stimuli. Thus, not only are individual dimensions maintained at those of young rats, but the body proportions (even in old hypophysectomized rats) will be infantile. Table V

TABLE V
SOME DIFFERENCES IN SKELETAL PROPORTIONS OBSERVED IN FEMALE RATS FIFTEEN MONTHS AFTER HYPOPHYSECTOMY AT 28 DAYS OF AGE ^a

Measurement	Length			Per cent of normal adult length	
	Normal young ^b	Hypophysectomized	Normal adult	Normal young	Hypophysectomized
Nose to tail-tip	mm	mm	mm	%	%
Tibia	228.0	279.0	405.0	56	69
Metacarpal III	25.2	30.8	39.6	64	75
	6.3	6.6	7.6	83	87

^a Data from Evans *et al.* (1949)

^b At 28 days of age (the age at hypophysectomy)

illustrates the difference which may be observed. The data from which this table is derived are contained in a more extensive survey of the skeletal proportions of hypophysectomized rats (Evans *et al.*, 1949).

Mortimer (1937) found that the altered proportions of the skull in hypophysectomized rats were so striking and constant as to constitute, in his judgment, sound criteria for the completeness of hypophysectomy. Recent unpublished studies by Frank (1953) with the authors have employed roentgenographic techniques of measuring the skull development of normal and hypophysectomized rats, following the general principles of Broadbent-Bolton cephalometric techniques in human beings. Some of the findings illustrate so well the general principles governing alterations of skeletal proportions in hypophysectomized rats that they merit summary here.

1. In total length (and total width) of the skull, the normal growth curve resembles that of total body length. Dwarfism in these dimensions following hypophysectomy at 28 days remains proportionate to dwarfism in body length.

2. Normal growth in cranial vault length (i.e., length of the brain-case) also resembles growth in body length, in that it is not completed until adulthood, i.e., the ninth month of life. After hypophysectomy the cranial length is stunted.

3. Normal growth in cranial vault width is substantially complete before 28 days of age. No stunting would therefore be expected in rats hypophysectomized at this age.

4. The cranial index (or width-length ratio) normally decreases from a value of 81 to 70 between the first and the ninth month of life; this is due to the early attainment of definitive breadth but continued growth in length. After hypophysectomy, with cessation of growth in length as well as width, the cranial index remains constant at a value of approximately 80.

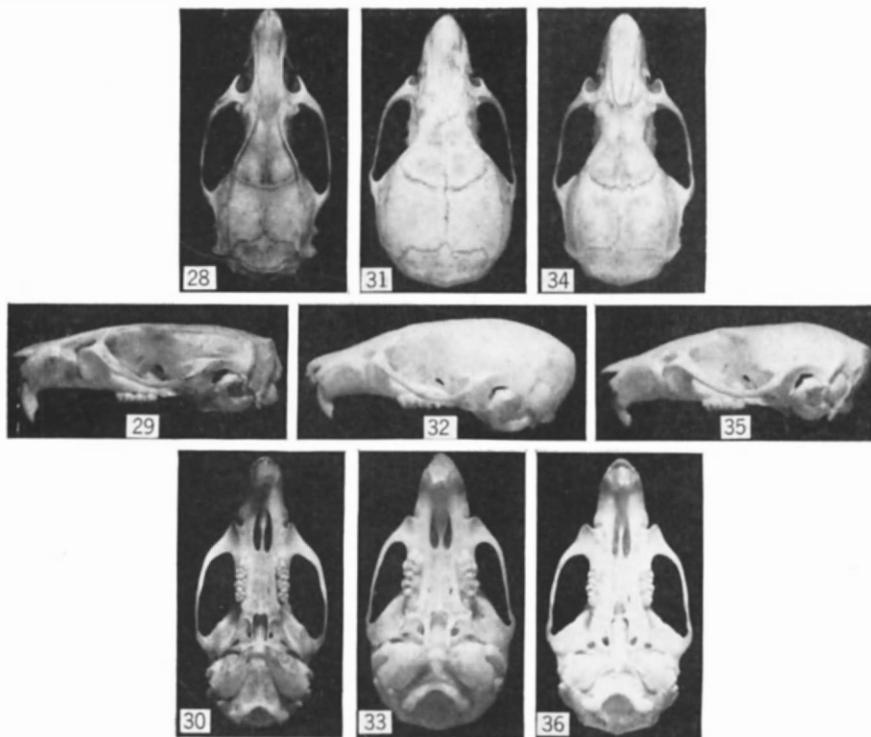
5. Cranial vault height, like cranial breadth, reaches its definitive value before 28 days of age. As with the cranial index, the height-length index also drops (from 50 to 42) during normal development, but in hypophysectomized rats remains at the higher level.

6. Of the two components of skull length—facial length and cranial length—a differential growth normally takes place. The facial length is at first the shorter (17 mm versus 19 mm cranial length) but in adults is longer (24.5 mm versus 23 mm). The curves of growth cross at 60 days of age, when both facial and cranial components are equal at approximately 21 mm. After hypophysectomy both are arrested, and the growth curves never cross. The cranial length remains longer (20 mm) than the facial length (19 mm).

The foregoing measurements and ratios are reflected in the characteristic appearance of the head of hypophysectomized rats during life,

and doubtless represent the deformities which led Mortimer to consider the head-form alterations as pathognomonic of hypophyseal deficiency.

A special case of disproportionate development involving the skull was demonstrated by Walker *et al.* (1952) and Asling *et al.* (1952). When rats were hypophysectomized at 6 days of age, the stunting of the head was extreme. However, the brain continued to grow, in accord with already established principles that its growth is neither retarded by hypophysectomy nor stimulated by growth hormone. The failure of the



Figs. 28 to 36. Dorsal, lateral, and ventral views of skulls of female rats. All photographs projected to same length to show differences in proportions which result from hypophysectomy and growth hormone replacement therapy. (Adapted from Asling *et al.*, 1952.)

28, 29, 30. Normal, 60 days of age. Actual length, 43 mm.

31, 32, 33. Hypophysectomized at 6 days of age, sacrificed at 60 days of age. Actual length, 31 mm.

34, 35, 36. Hypophysectomized at 6 days of age, injected with growth hormone from 30 to 80 days of age. Actual length, 40 mm.

brain case to grow in proportion to the brain resulted in symptoms of cerebral compression (paralysis and convulsions) and medullary damage (respiratory distress and, terminally, Cheyne-Stokes respiration). The

rats invariably died before 60 days of age. The skulls not only showed marked stunting, but malformation was extreme (Figs. 31, 32, 33). For example, cranial indices of over 100 were recorded as a result of excessive breadth, and the cranial vault height was correspondingly increased, the brain case tending to become spherical in form. The foramen magnum was larger than normal, and part of the medulla oblongata had been herniated through it. When growth was re-established in these rats by growth hormone injections starting at 30 days of age, none of the symptoms of neural compression developed, and all rats survived (in some cases, to ages of beyond 200 days). The skull (and the rest of the bones) grew, although the brain remained of normal weight. However, normal proportions of the skull were not re-established, since the growth was not differential but took place in all dimensions (Figs. 34, 35, 36). In shape, therefore, it remained infantile. This was in accord with the general observations next to be described.

3. EFFECT OF GROWTH HORMONE ON SKELETAL PROPORTIONS

The most extensive study of growth hormone's effect on skeletal proportions was that which compared the response of normal and of hypophysectomized rats (28 days old at operation) to injections of large doses of hormone for 14 months (Evans *et al.*, 1949). With a few exceptions, the skeletons of the intact rats maintained normal proportions. The exceptions were chiefly in the paw bones, such as metacarpals, metatarsals, phalanges, and calcaneus. These bones have only one epiphyseal ossification center, whose cartilage plate had been resorbed before the treatment was started. As a result their growth was negligible. The response of hypophysectomized rats to growth hormone was in marked contrast. Although some of the skeletal dimensions were brought to equal those of adult normal rats by this treatment, others exceeded normal. (None fell short of normal.) The skull, the thorax, and the pelvis, in particular, showed disproportionately great growth in width and, in some regions, in thickness of bone. Among other bones, the tibia and scapula were disproportionately long, and the deltoid tuberosity of the humerus was excessively prominent. The causes of these regional variations in growth response were not clear, although it could be shown that some were exaggerations of the differences in proportions between hypophysectomized and adult normal rats. It is clear that different bones may respond differently to growth hormone. For example, both in normal and in hypophysectomized rats injected with the hormone one may see epiphyseal union occurring in the metacarpal bones while body length is increasing actively. Even in the same bone (i.e., the tibia) one epiphyseal plate (the distal) may be resorbed at the same time that the other is responding to growth hormone by vigorous osteogenesis (Asling *et al.*, 1950b).

The observation that disproportions were less frequent in intact rats than in hypophysectomized rats similarly treated directs attention to the likelihood that other hormones participate in maintaining proportionality. There is a need for studies on the skeletal proportions of hypophysectomized rats which have received multiple replacement therapy, particularly considering growth hormone in combination with thyroid hormone, ACTH (or adrenal cortical hormones), or sex hormones.

VI. Other Relationships of the Pituitary Gland to Skeletal Development

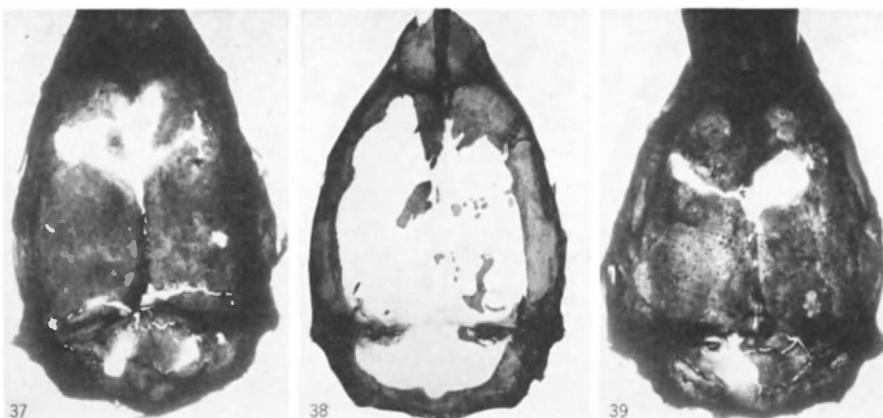
The foregoing sections have been concerned chiefly with demonstrating the roles of growth hormone and thyroid hormone in repairing the defective development of the skeleton which follows removal of the pituitary and the thyroid. Certain topics require discussion which have not been mentioned in these sections. Among them are the relationship of the pituitary to the development of the membrane bones of the skull and to the ossification of the os penis, and the relation of growth hormone to hormones other than thyroxin (and particularly ACTH).

1. THE PITUITARY GLAND AND INTRAMEMBRANOUS OSTEogenesis

Whereas the greatest success in demonstration of the pituitary control of endochondral osteogenesis has been gained by experiments performed on bones during the period when they would normally be undergoing rapid growth, the intramembranous osteogenesis in the calvarium has not been so successfully studied. This is probably due to the early cessation of active growth in this region (it having been demonstrated that cranial width and height attain the greatest part of their adult dimensions during the first month of life). Even with deficiencies induced at very early ages, histologic observations of growth arrest become more difficult in the absence of a conspicuous zone of growth comparable to the epiphyseal cartilage plates of long bones. It has proved possible, however, to establish that intramembranous osteogenesis is, like endochondral osteogenesis, under pituitary control. Simpson *et al.* (1953) devised a technique for removing the calvarium in young (21-day-old) rats, leaving the dura mater in place. Normally, islands of bone appeared promptly (their origin being from small clusters of osteoblasts which adhered to the dura rather than to the extirpated bone at the time of operation). The islands expanded and became coalescent (except at suture lines) in the same manner as in normal intramembranous osteogenesis. The large gap was almost completely filled by 30 days of age (Fig. 37). When rats were hypophysectomized, the day following calvariectomy, no repair took place (Fig. 38). However, when given growth hormone after hypophysectomy, repair proceeded in normal fashion (Fig. 39). Other studies showing stimulation of intramembranous osteo-

genesis by growth hormone are those of Becks *et al.* (1948a), Collins *et al.* (1949), and Asling *et al.* (1955).

The periosteal osteogenesis seen on the shaft of long bones has been



Figs. 37 to 39. Portions of skulls of female rats 30 days after calvarieotomy at 21 days of age. (von Kossa silver nitrate treatment, cleared, $\times 2.1$.) (After Simpson *et al.*, 1953.)

37. Extent of repair in normal rats.
38. Failure of repair following hypophysectomy on the day after calvarieotomy.
39. Extent of repair induced by growth hormone injections started immediately after hypophysectomy on the day after calvarieotomy

demonstrated to be enhanced by growth hormone administration (Asling *et al.*, 1950b). The very interesting restricted region of intramembranous osteogenesis at the tip of the terminal phalanges is now under study in the hope of understanding why the "tufting" seen at this region in acromegalics has not yet been described under experimental conditions.

2. EFFECTS OF SEX HORMONES ON SKELETAL DEVELOPMENT

Since the subject of sex hormones and osteogenesis is being discussed in detail elsewhere in this volume, mention need be made here only of their relationship to pituitary control of skeletal development. Thyberg and Lyons (1948) and Lyons, Abernathy, and Gropper (1950) demonstrated that the os penis was a special case in skeletal development. While it failed to grow and differentiate in hypophysectomized-gonadectomized rats, it was possible to effect almost complete repair by administration of testosterone. Growth hormone had no effect in correcting the atrophy of the bone, although the rest of the skeleton responded excellently to growth hormone and did not respond appreciably to testosterone. Turner, Lachman, and Hellbaum (1941) have shown that the epiphyses of the ischial tuberosities, like the os penis, are responsive

to testosterone, and in this laboratory Caylor (unpublished) showed some increase in pelvic size after testosterone therapy. Although the hormone does not induce general skeletal growth there is some evidence that it augments the effects of growth hormone in hypophysectomized rats (Simpson *et al.*, 1944).

In studies on estrogens, we wish to direct attention here only to the increase in medullary bone observed in estrogen-treated rats.⁷ Urist, Budy, and McLean (1948) found that there were differences between mice and rats in this response, in that while there is an actual stimulation of endosteal osteogenesis in mice (Gardner and Pfeiffer, 1943) the mechanism in rats was through inhibition of bone resorption. Kibrick *et al.* (1942) showed that this increased amount of medullary bone is not found in hypophysectomized rats. Estrinization immediately after operation, and with doses effective in normal rats, did not prevent the usual post-hypophysectomy resorption of bone. This pituitary mediation of the response to estrogen requires further study.

3. RELATIONSHIP OF GROWTH HORMONE AND ACTH

A depressing effect of adrenocorticotrophic hormone on growth in body weight was first demonstrated by Moon (1937) using castrated rats. This was followed by skeletal studies using this hormone in highly purified form. The effect was demonstrated in intact male rats, whose bony growth was markedly impaired by the treatment (Evans, Simpson, and Li, 1943b; Becks *et al.*, 1944a). In hypophysectomized rats a direct antagonism to the effects of pituitary growth hormone was shown by injecting ACTH concurrently with a potent growth hormone preparation (Marx *et al.*, 1943). In the tibia, the proximal epiphyseal cartilage plate was narrow, and possessed irregular cartilage cell columns, while osteogenesis was reduced (Becks *et al.*, 1944b). The effect was not attributable to reduced food intake, for the rats treated with both hormones actually consumed more food than those given only growth hormone. In spite of marked growth inhibition, skeletal maturation has not been found to be delayed by ACTH treatment (Asling, Reinhardt, and Li, 1951) nor was it accelerated. The extent to which ACTH participates in the pituitary gland's regulation of normal skeletal development through its opposition to the stimulating effect of growth hormone is still not clear.

VII. Summary

The roles of the pituitary and thyroid glands in controlling skeletal development of the rat have been described. Growth of the skeleton is

⁷ Mention has already been made of the better response of castrate female rats to growth hormone (Evans and Simpson, 1931). The inhibiting effect of estrogen on skeletal growth which might be deduced from this was demonstrated by Simpson *et al.* (1942), Lippman and Saunders (1944), and others.

retarded following hypophysectomy or thyroidectomy. The extent of the retardation is dependent upon the age at the time of operation. The most marked dwarfism follows hypophysectomy of very young rats, although the retardation develops more slowly than after hypophysectomy of older rats. This suggests that the earliest postnatal growth is relatively less dependent on pituitary hormones. The skeletal growth can be restored to normal in hypophysectomized rats (or even stimulated to excess) by administering growth hormone in adequate amounts. No other hormone tested possesses this ability.

Maturation of the skeleton (as judged by the time of appearance and the progressive differentiation of epiphyseal ossification centers and their fusion to the diaphysis) is also retarded by hypophysectomy or thyroidectomy. The extent of retardation is independent of the age at the time of operation. The equivalent of 2 to 3 weeks of further differentiation follows hypophysectomy or thyroidectomy of immature rats at whatever age. The maturation can be restored to normal by administration of thyroid hormone. In thyroidectomized rats this hormone also stimulates growth, through its action on restoring pituitary function. Growth hormone is without effect on skeletal maturation.

The proportions existing between lengths of the various bones are altered following hypophysectomy of growing rats, so that long after the operation these proportions are found to be more like those of young than of adult rats. Differences in the growth curves of individual bones may be a major factor in accounting for these ultimate disproportions. Administration of growth hormone to hypophysectomized rats tends more to exaggerate than to correct these disproportions, suggesting that other hormones may assist in the control of growth which results in normal adult proportions.

Some of these hormonal interrelations have been demonstrated. Among them, thyroid hormone augments the action of growth hormone, while adrenocorticotropic hormone opposes growth hormone. There is some evidence that testosterone also augments, and estrogen opposes, growth hormone's action.

The extent to which these findings furnish bases for generalizations on the control of mammalian skeletal development, and particularly for man, is not yet clear. This is due in part to some peculiarities of the rodent skeleton, and in part to the lack of extensive comparable studies on other mammals. It can be said that many parallels may be drawn between the findings in these experiments and the clinical observations made on the course of human pituitary dwarfism, cretinic dwarfism and its treatment, pituitary gigantism, and acromegaly.

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CHAPTER XXII

THE PARATHYROID GLANDS AND BONE

FRANKLIN C. McLEAN

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I. Introduction

For more than twenty-five years the physiology of the parathyroid glands has been the subject of many investigations; this chapter will be concerned primarily with contributions to the interrelations of these glands and of bone. During this period there have also been numerous excellent reviews, which may be consulted for a more general and more extensive treatment of the subject (Collip, 1926; Dragstedt, 1927; Thomson and Collip, 1932; Shelling, 1935; Albright, 1941; Campbell and Turner, 1942; Mandl, 1947; Gilmour, 1947; Albright and Reifenstein, 1948; Greep, 1948; Anonymous, 1953; Black, 1953; Bartter, 1954; Escamilla, 1954; Reifenstein and Howard, 1954).

Modern knowledge of the physiology of the parathyroid glands, and more particularly of their relationships to the physiology of bone, dates from the successful isolation of an active extract of the glands, accomplished independently by Hanson (1923, 1924) and by Collip (1925), and from the first parathyroidectomy, performed by Mandl (1925).

The parathyroid glands were first described by Sandström (1880), but little attention was paid to his discovery. Gley (1891) rediscovered the external parathyroids, and Kohn (1895) again described the internal pair. The relationship of the glands to tetany was proved by Vassale and Generali (1900); thyroidectomy without protection of the parathyroids had previously led to attributing tetany to removal of the thyroid gland. The relationship of the parathyroids to calcium metabolism was established by MacCallum and Voegtlín (1909). Experimental work upon the

influence of the parathyroid hormone followed distribution of extracts of the gland, shortly after the publications of Hanson and of Collip.

Among sixteen cases of skeletal disease reported by von Recklinghausen (1891) at least one was certainly the result of hyperparathyroidism, but was not so recognized at the time. Askanazy (1904) reported the association of a parathyroid tumor and generalized osteitis fibrosa. Erdheim (1907) described three cases of osteomalacia in which the parathyroid glands were hyperplastic. Schlaggenhaufer (1915) pointed out that in many cases of skeletal disease only one parathyroid was enlarged. This helped to establish a distinction between primary and secondary hyperparathyroidism, and he suggested that parathyroidectomy might be beneficial in certain instances. Hoffheinz (1925) collected data on 45 cases in which enlargement of the parathyroid glands had been found at necropsy; skeletal disease was associated with the parathyroid enlargement in 27 of the 45 cases; of these 27, seventeen were generalized osteitis fibrosa, eight were osteomalacia, and two were rickets.

Following Mandl's parathyroidectomy (1925) performed almost simultaneously with isolation of an active parathyroid extract; the way was open for a combined experimental and clinical approach to the function of the parathyroid glands, and there have since been many advances. There is now a reasonably complete understanding of the physiology, pathology, and pathologic physiology of these glands. As will appear below, however, there are still areas of uncertainty; moreover the extracts of the parathyroid glands currently available have not proved to be reliable tools in the management of hypoparathyroidism.

II. Anatomy, Physiology, and Pathology of the Parathyroid Glands

In man there are usually, but not always, four parathyroid glands, located in pairs on the posterior aspect of the thyroid gland (Shelling, 1935). One or more of these may be absent, and aberrant glands are not uncommon. The parathyroid glands are ovoid bodies, as a rule 6 to 7 mm long by 2 to 3 mm thick; they weigh about 35 mg each. Microscopically there are two major types of parenchymal cells in the adult (Welsh, 1898)—*chief cells*, containing glycogen; and *oxyphile cells*, free from glycogen. Variations of these cells are described as well as transition forms. A variant of the chief cell is water-clear ("wasserhelle"), i.e., without demonstrable cytoplasm; pale and dark oxyphile cells have been distinguished. Oxyphile cells do not appear until about the age of puberty. Castleman and Mallory (1935) have derived confirmatory evidence, from the histology of parathyroid tumors, for the monophyletic theory of origin of the various cell types, indicating that there is a fundamental cell from which all others are derived.

The pathologic changes in the parathyroid glands in hyperpara-

thyroidism have been divided sharply into two types, hyperplasia and neoplasia (Castleman and Mallory, 1935). Hyperplasia is characterized by diffuse uniform involvement of all the glandular tissue. It occurs in two forms, a water-clear cell type, and a much rarer chief cell type. Localized tumors of a single gland, part of a gland, or rarely parts of two glands, are to be regarded as neoplasms. There is a roughly quantitative relation between the size of the enlarged glands and the degree of hyperfunction. Glands weighing as much as 250 grams have been reported; others described but not weighed appear to have been even larger.

Neoplasms of the parathyroids are commonly adenomas (Castleman and Mallory, 1935; Black and Ackerman, 1950; Woolner, Keating, and Black, 1952). An adenoma, in contradistinction to hyperplasia, commonly involves one gland only. Primary carcinoma, resulting in hyperparathyroidism, and in metastases, is rare (Wray, 1953). Table I, from Black (1953) lists the incidence of the pathologic diagnoses in 112 cases of proved hyperparathyroidism, seen in the Mayo Clinic.

TABLE I
GROSS PATHOLOGY: 112 CASES OF PROVED HYPERPARATHYROIDISM

Single adenoma	92
Multiple adenomas	10
Primary (wasserhelle) hyperplasia	9
Carcinoma with metastasis	1

III. The Parathyroid Hormone

The parathyroid hormone has not been prepared in pure form. Parathyroid Extract USP contains the water soluble fractions of the glands. This is standardized biologically so that 1 cc contains 100 (Hanson) units; each unit is 1/100 of the amount required to raise the calcium level of the blood serum of normal, healthy male dogs, weighing 10 to 12 kg, by an amount of 1 mg/100 cc. Five USP or Hanson units are equivalent to 1 Collip unit; the Collip unit is still in use in some parts of the world.

The calcium-mobilizing principle of the parathyroid glands has been identified as a protein; all protein fractions separated by chromatographic methods or by countercurrent distribution have retained the same ability, per unit of nitrogen, to raise the serum calcium in normal dogs (Handler, 1953). Parathyroid extract is ineffective when administered orally. Exogenous parathyroid hormone also loses its effectiveness on repeated parenteral administration to man or to experimental animals; this is attributed to immune reactions on the part of the recipient (Pugsley, 1932; Thomson and Collip, 1933). Failure of the organism to respond

to its endogenous parathyroid hormone is described, and has been called pseudohypoparathyroidism (Albright *et al.*, 1942).

IV. The Mode of Action of the Parathyroid Hormone

Clarification of the mode of action of the parathyroid hormone, and more particularly of its action upon bone, has been delayed by insistence upon an hypothesis, advanced by Albright and Ellsworth (1929) to the effect that the mobilization of calcium from bones is secondary to an increase in the excretion of phosphate by the kidneys and a consequent fall in the serum phosphate level. The overwhelming evidence for a direct effect of the hormone upon bone will be reviewed in this section; current belief is that there is a second, and perhaps independent effect upon the kidneys, possibly mediated by a second active principle from the parathyroid glands.

Stewart and Bowen (1951) were able to produce characteristic hypercalcemia by administering parathyroid extract intravenously to nephrectomized dogs immediately following the operation. A similar effect was produced in dogs following both nephrectomy and parathyroidectomy. Their results may be accepted as confirmatory of those of others who had reported earlier that parathyroid extract is able to produce elevation of the serum calcium level in animals without renal function, and that consequently this effect could not be attributed to any alteration in excretion of phosphate by the kidneys (Stoerck, 1943). Monahan and Freeman (1944) found that the endogenous parathyroid hormone of the animal's own glands was able to prevent a fall in serum calcium following nephrectomy; when the parathyroid glands were also removed the serum calcium level fell promptly; in both series of animals the serum phosphate level was markedly elevated. Talmage, *et al.*, (1953) demonstrated that administration of exogenous parathyroid hormone also prevents the fall in serum calcium level in the nephrectomized parathyroidectomized rat. On the other hand, failure to produce hypercalcemia by the administration of parathyroid extract in the absence of the kidneys has also been reported (Tweedy, Templeton, and McJunkin, 1937; Neufeld and Collip, 1942).

Selye (1942) and Stoerck (1943) found that parathyroid extract produced osteitis fibrosa in bilaterally nephrectomized rats. Similar results were reported by Ingalls, Donaldson, and Albright (1943). These authors observed resorption of bone following nephrectomy alone, as did Selye (1942); they found, however, that in these animals the bone lesions were qualitatively and quantitatively distinguishable from lesions produced by parathyroid extract, and that they appeared later than did those in the parathyroid-treated animals. They concluded that the effects of parathyroid extract were not due to acidosis, or to the acid of the extract. In

spite of a few dissenting voices, the weight of evidence indicates that the two most characteristic effects of the parathyroid hormone—hypercalcemia and osteitis fibrosa—can be produced in experimental animals in the absence of the kidneys.

McLean *et al.* (1946), making use of a concept they referred to as "biologic solubility," performed an experiment, which they regarded as crucial, to determine whether administration of the parathyroid hormone in some way increases the solubility of the bone salt in the circulating fluids of the body, leading to its solution from bone. They administered a large dose of parathyroid extract to adult dogs, the serum of which before treatment did not produce *in vitro* calcification in rachitic cartilage. The serum calcium rose promptly, with a concurrent but slight rise in the serum phosphate level (Fig. 1).

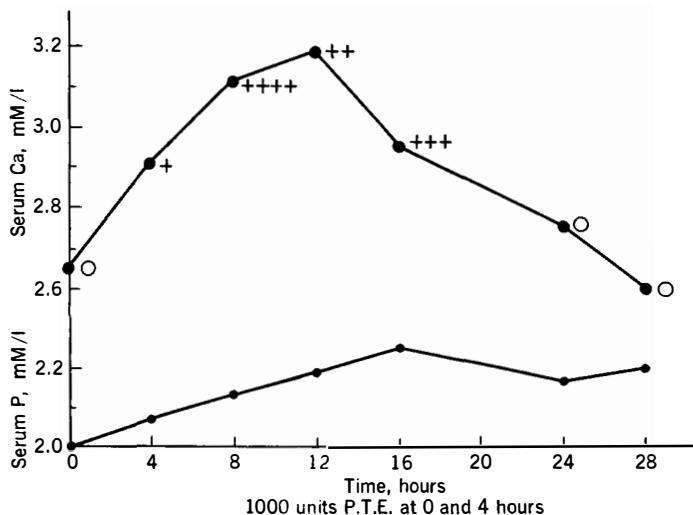


FIG. 1. Serum calcium and serum phosphate of dog, and ability to produce calcification of rachitic cartilage *in vitro*, following administration of parathyroid extract. The animal was given 1000 units of parathyroid extract at 0 hr, and again at 4 hr. Serum withdrawn at 0, 24 and 48 hr produced no calcification of rachitic cartilage *in vitro*; serum withdrawn at 4, 8, 12, and 16 hr produced +, +++, ++, and +++ calcification, respectively. (McLean *et al.*, *Trans. 14th Josiah Macy Jr. Conf. on Metabolic Aspects of Convalescence*, 1946, p. 34. Reproduced by courtesy of the publishers.)

According to the simple solubility hypothesis a rise in serum calcium could only occur if bone salt were being dissolved in a previously unsaturated plasma, which by the same token should be incapable of inducing calcification in bone or cartilage matrix. A fall in serum calcium would indicate that a saturated plasma was depositing a salt of calcium

and phosphate, and serum from such a plasma should induce calcification. Contrary to such predictions, calcification was induced during the rise as well as during the fall of the serum calcium curve (Fig. 1), indicating that if the serum was saturated or supersaturated at 4 hours, when calcification *in vitro* was first observed, it must have been supersaturated at 8 and at 12 hours, with still higher calcium and phosphate levels.

There is no way in which a solution of a salt, in contact with the solid phase, may become supersaturated by simple equilibrium with the solid. Supersaturation requires some sort of active intervention, not merely the dissolving of the salt in the fluid. From the observations in the experiment just described it must be concluded that some mechanism is at work, other than ordinary solution by an undersaturated plasma.

The hypothesis which attributes all of the effects of the parathyroid hormone on bone and on the serum calcium level to a primary effect upon the excretion of phosphate by the kidneys rests in part upon the time relationships between the rise in serum calcium and the fall in phosphate levels. Dent (1953) has found that with parathyroid extract currently available the effect upon phosphate excretion by the kidneys is slight, if present at all. Moreover, Munson (1955) has demonstrated the extreme rapidity with which the serum calcium level falls following parathyroidectomy; this fall occurs before any change in the phosphate level is demonstrable, and can only be explained as the result of a deficiency in the calcium-mobilizing principle. Similar findings have been reported by Talmage and Kraintz (1954). The second major argument in support of the Albright-Ellsworth hypothesis is the fact that in many patients with clinical hyperparathyroidism there are no demonstrable changes in the bones. This subject will be dealt with in the final section of this chapter.

Dent (1953) points out, as have others, that in patients with primary renal disease the changes in the bones, characteristic of hyperparathyroidism, frequently occur in the presence of *elevated* serum phosphate levels. This obvious conflict with the Albright-Ellsworth hypothesis is met (Albright and Reifenstein, 1948) by the assertion that the bone changes in hyperparathyroidism secondary to renal insufficiency (renal rickets) are the result of acidosis, rather than of a direct effect of the parathyroid hormone on bone.

The Albright-Ellsworth hypothesis has not been sufficient to account for certain phenomena, notably the condition of supersaturation of the plasma with the bone salts, similar to that observed experimentally by McLean *et al.* (1946) and by McLean and Bloom (1941). Albright and Reifenstein (1948) accept the view that, under certain conditions, there may be a direct effect of the parathyroid hormone on bone; they regard

this as a form of "parathyroid poisoning," or "hyper-hyperparathyroidism," and do not believe that it reflects the mode of the action of the hormone under less extreme conditions. If this entity were to be established it would afford a better explanation of the bone changes in renal rickets than does the acidosis hypothesis. Once the view that the parathyroid hormone may have characteristic effects directly on bone is accepted, however, it is difficult to draw the line between primary and secondary effects.

Stewart and Bowen (1952) have found that preparations of parathyroid extract inactivated with formaldehyde with respect to their power to raise the serum calcium level may still increase the output of phosphate in the urine of dogs. They incline to the view that the phosphate diuresis is an artifact, since they also observed it when extracts of spleen and of thymus were administered. The weight of evidence, however, is that the effect on phosphate excretion is real (Talmage, Lotz, and Comar, 1953). It could even be argued that such an effect is a physiologic necessity. Resorption of bone results in mobilization of both calcium and phosphate; the effect of the parathyroid hormone on excretion of phosphate enables the organism to dispose of an excess of this element which would otherwise accumulate in the blood.

It seems likely that the effect of parathyroid hormone on excretion of phosphate is upon the kidneys themselves, resulting in an increase in renal clearance of phosphate. Numerous attempts have been made to confirm this view, and to analyze the action in terms of glomerular filtration and tubular reabsorption. A review of the voluminous literature on this subject is beyond the scope of this chapter; an excellent summary of the experimental work will be found in Bartter's review (1954). It has been suggested, but not proved (Handler, Cohn and De Maria, 1951; Handler and Cohn, 1952; Munson, 1955) that the effect upon phosphate excretion depends upon a second active principle secreted by the parathyroid glands.

Further evidence for a direct effect of the parathyroid hormone on bone is derived from the observations of Carnes (1950) and of Engel (1952). Carnes concluded that bone matrix may be destroyed by the action of parathyroid hormone irrespective of its mineral content; Engel found that parathyroid extract causes depolymerization and solution of the glycoprotein ground substance of bone and of cartilage. In neither case does it appear that the effects described could be regarded as secondary to a change in the solubility relationships between blood and bone.

The final and conclusive link in the chain of evidence in favor of a direct action of the parathyroid hormone on bone has been supplied by Barnicot (1948) and by Chang (1951). Barnicot implanted small pieces

of bone, together with parathyroid tissue, intracerebrally in mice, and found that the bone in contact with parathyroids was resorbed. Chang confirmed and extended these observations by transplanting parathyroid tissue to the cranial cavity in mice, in contact with a parietal bone; she found that parathyroid, in contradistinction to other tissues and materials similarly implanted, led to resorption of the living bone of the calvarium. Under the circumstances it is difficult to come to any conclusion other than that the parathyroid hormone exerts a local destructive action on bone. Barnicot (1950) has reported a similar effect from implants of crystalline vitamin A; no other implants have been found to exert this influence upon bone.

V. The Mechanism of Resorption of Bone

Koelliker (1873) wrote a clear description of resorption, to which little has been added. This section will be concerned mainly with advances in the understanding of the resorption process, rather than with its morphologic manifestations.

Resorption is, in essence, the putting into solution of a complicated structure, in such a fashion that it disappears, its end products entering the blood stream. Resorption always progresses inward from the surfaces of bone; it never arises within the deeper layers of the structure. Of the components of bone, a small fraction is already in fluid form, and hence easily disposed of; the remainder is in solid form, for the most part insoluble or soluble with great difficulty in aqueous fluids. In order to resorb bone it is necessary that it be reduced to substances soluble in water, and that these be transferred to the fluids of the body.

Of the solid components of bone, three substances together make up the main bulk. These are (1) the bone mineral; (2) a fibrillar protein, collagen; and (3) an amorphous ground substance, characterized by its content of one or more polysaccharides. The ground substance is small in amount, is closely related to the interstitial fluid, and may be made soluble in water by a change in its state of polymerization (Gersh, 1950). Such changes are accompanied by an increase in the concentration of mucopolysaccharides in the blood (Engel, 1952), indicating that they are readily transferred to the fluid component. Both an enzyme, hyaluronidase, and the parathyroid hormone have been proposed as being concerned in the depolymerization of the ground substance of bone.

Collagen, even at the hydrogen ion concentration of the fluids of the body, can be rendered soluble rather readily. At the temperature and hydrogen ion concentration of these fluids it can be dissolved by digesting or disintegrating its protein structure. This can be accomplished in the test tube by various proteolytic enzymes, including pepsin, but

not trypsin. Koelliker concluded that the osteoclast erodes bone by chemical means, without specifying further the nature of the chemical action required. Later others (Weidenreich, 1930) added the assumption that the action is a combination of that of an acid with that of a proteolytic ferment, but the necessity for the presence of acid to account for solution of the bone mineral has been a difficult stumbling block in the formulation of a theory of resorption. This situation has been radically changed by the introduction of a group of compounds, known as chelating agents, of which the prototype is ethylene diamine tetra-acetic acid (Rubin, Martell and Bersworth, 1954).

Chelating agents are characterized by the formation of very poorly dissociated complexes with metallic ions. Together with this property, which involves the formation of ring structures with coordination linkages, chelate structures exhibit greatly increased stability, lowered solubility in water, and changes in the dielectric constant. These characteristics differentiate chelate structures from such simple complex ions as are formed by calcium with citrate; from the physiologic point of view the important difference is that the ability of a chelated complex to ionize in solution is very much below that of calcium citrate (Schubert, 1954).

It is now a common laboratory procedure to decalcify bones and teeth for histologic purposes with chelating agents, and this is possible even in neutral or strongly alkaline solutions (Sreebny and Nikiforuk, 1951). It may therefore be safely assumed that the bone salt may be dissolved whenever another substance, having a stronger affinity for calcium, is in a solution in contact with bone. This removes one of the obstacles to forming an hypothesis for the resorption of bone. Such an hypothesis may now account for solution of bone salt, collagen, and ground substance, all at the hydrogen ion concentration of the body fluids.

For a long time it has been agreed by most observers that both the organic and inorganic components of bone are resorbed together, leaving smooth surfaces at the loci of resorption (Maximow and Bloom, 1952). If it be assumed that there is continuously applied to the surface of bone a solution which will depolymerize mucopolysaccharides, digest collagen, and hold calcium in a firm combination, this constitutes the basis for a working hypothesis for the mechanism of the resorption of bone (McLean, 1954). Such a mechanism would require only certain enzyme systems and an organic substance to combine with calcium. This does not conflict with any known facts, but there is no positive evidence that it adequately describes the process. Nor is it suggested that any known chelating agent participates in the resorption of bone; the possibility is that substances having similar properties may be formed in the organism.

As phagocytosis has become increasingly well understood in other connections, it has been logical to assume that osteoclasts resorb bone by this process, and to attempt to find evidence supporting this view. Since the osteoclast is a giant cell, resembling in some respects other giant cells known to have phagocytic capabilities, if it could be shown to ingest cellular debris and bone mineral the evidence would be conclusive. But it has not been demonstrated that the osteoclast ever does ingest such debris, or mineral, even under conditions furnishing abundant evidence of phagocytosis of these substances by other cells in the bone marrow in the immediate vicinity of bone undergoing resorption (McLean and Bloom, 1941; Plate I). For the present, then, the possibility that resorption of bone ever occurs by osteoclastic phagocytosis may be excluded.

The part played by phagocytosis by other cells is obscure. Following toxic doses of parathyroid hormone many cells in the bone marrow, but not osteoclasts, participate in the phagocytosis of the debris of necrotic tissue. Necrosis of the marrow elements is readily observed following large doses of the parathyroid hormone; death of osteocytes has also been reported (Heller, McLean, and Bloom, 1950). It seems improbable that either the physiologic action of the parathyroid glands, or the normal turnover of the bone substance, bears any direct relation to these manifestations of the toxic effects of the hormone.

It may still be asked whether there is sufficient evidence to implicate the osteoclast in resorption at all. There is the possibility that this multinucleated giant cell is simply a byproduct of resorption, being formed by aggregation of osteocytes and osteoblasts liberated by resorption effected by other means. An exhaustive review of Hancox (1949) concludes that there is no direct evidence that osteoclasts erode bone, but that their constant occurrence in zones where resorption is taking place is more than incidental. For the present it will be assumed that osteoclasts do bring about resorption of bone, either by some such process as that above suggested, or by some other unknown chemical action at the surface of bone.

Even if the view be accepted that osteoclasts resorb bone, there remains the very important subject of the control of this process; it certainly does not occur spontaneously or at random. Resorption of bone, accompanied by the presence of osteoclasts, begins with the very earliest stages of ossification in the embryo, and continues throughout life, although there are marked differences in the location and rapidity of resorption, according to the physiologic and structural needs of the moment. The influences which evoke and control osteoclastic activity may be found either generally, throughout the organism, or locally, in circumscribed areas. We are here chiefly concerned with the humoral

control of resorption, especially as it is mediated through the parathyroid glands.

VI. The Role of the Parathyroid Glands in Homeostasis

It is firmly established that the parathyroid glands play a decisive part in the homeostatic regulation of the calcium ion concentration of the blood plasma. There is a direct correlation between the calcium levels

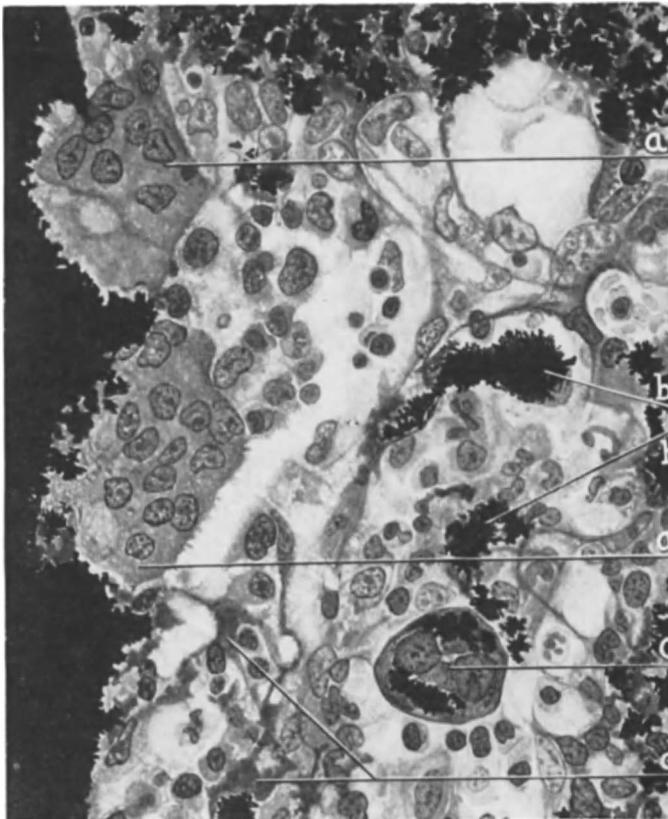


PLATE I

High power view from undecalcified section of a rib of a puppy twelve hours after injection of 200 USP units of parathyroid extract per kilogram of body weight: (a) osteoclasts, free from aggregated bone salt; (b) macrophages, packed with bone salt; (c) megakaryocyte, containing bone salt; (d) basophil network. Formaldehyde fixation; silver nitrate, hematoxylin and eosin; camera lucida; $\times 681$. (McLean and Bloom, *Arch. Pathol.* **32**, 315, 1941. Reproduced by courtesy of the publishers.)

in the body fluids and the state of activity of the parathyroid glands. The relationship between the parathyroids and the control of phosphate concentration in the blood is less clear.

I. THE CALCIUM OF THE FLUIDS OF THE BODY

All or nearly all of the calcium in the blood is in solution in the plasma; the amount in the red blood cells is negligible. In normal man the concentration of calcium in the plasma is usually between 9 and 11 mg per 100 cc, with 10 mg per 100 cc (2.5 mM per liter) a representative figure. This is in constant exchange with the calcium of the extracellular fluid and of the bones; the homeostatic mechanism that maintains the constancy of the concentration in the plasma is the function of the parathyroid glands. In the absence of the parathyroids the plasma calcium may fall to 7 mg per 100 cc or even lower. In pathologic or artificially induced hyperparathyroidism the figure may reach 15 mg per 100 cc or higher.

It has been known since early in the present century (Rona and Takahashi, 1913) that the calcium in the plasma is separable into two major and roughly equal fractions—diffusible and non-diffusible. It has also been known that the non-diffusible fraction is associated in some way with the plasma protein. There was, however, for some years, a considerable difference of opinion concerning the diffusible fraction. It was held by many that a major portion of this fraction is in the form of a complex with the citrate ion, or with some other comparable substance. These uncertainties have been resolved, and the relationship of calcium to the plasma protein has been incorporated in a formulation that describes the plasma as a solution of a weak electrolyte, calcium proteinate, the ionization of which may be represented by the equation (McLean and Hastings, 1935a):



and by the mass-law equation:

$$\frac{[\text{Ca}^{++}] \times [\text{Prot}^{\ominus}]}{[\text{CaProt}]} = K_{\text{CaProt}} = 10^{-2.22} \quad (2)$$

From these two equations, and from the expressions [Total Ca] = [CaProt] + [Ca⁺⁺] and [Total Prot] = [CaProt] + [Prot[⊖]], there has been derived a general equation from which the calcium ion concentration of the plasma or serum may be calculated from analyses for total calcium and total protein, quantities easily determined in the laboratory:

$$[\text{Total Ca}] = \frac{[\text{Ca}^{++}] \times [\text{Total Prot}]}{[\text{Ca}^{++}] + K} + [\text{Ca}^{++}] \quad (3)$$

The calculation of calcium ion concentrations is facilitated by the use of a nomogram, constructed from equation 3, shown in Fig. 2 (McLean

and Hastings, 1935b), which illustrates the relationships of the ionized and un-ionized fractions of calcium at varying protein and calcium levels.

At normal protein levels, about one-half of the plasma calcium is in

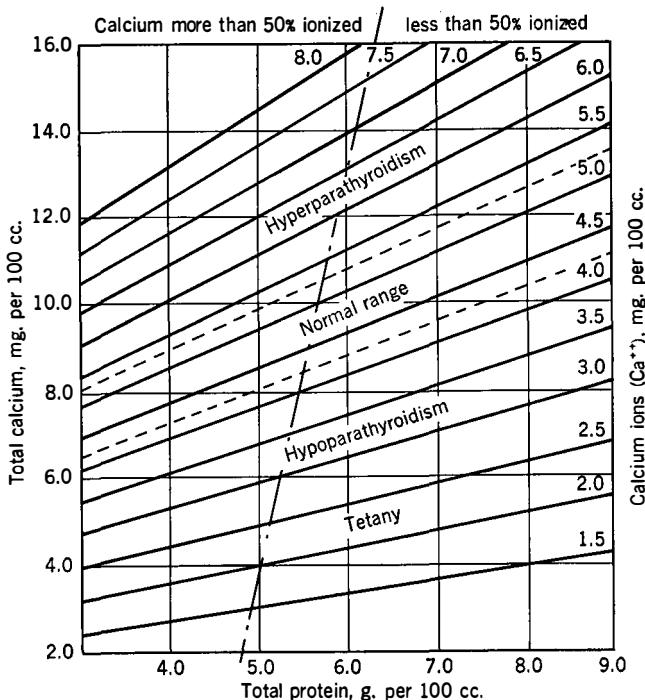


FIG. 2. Chart for calculation of Ca^{++} concentration from total protein and total calcium of serum or plasma. (McLean and Hastings, *Am. J. Med. Sci.* **189**, 601, 1935. Reproduced by courtesy of the publishers.)

the ionized form; the other half is in the undissociated complex with protein. Since this is an ionization phenomenon, shifts from the ionized to the un-ionized form occur instantaneously; the un-ionized calcium is not in firm combination with protein. Moreover, since rapid movement of ions occurs throughout the organism, the formulation does not describe a static condition; it does describe a system in dynamic equilibrium.

The mass-law equation 2 shows at once that the ratio $\text{Ca}^{++}/\text{CaProt}$ is determined by the concentration of protein. When the total calcium falls, as in hypoparathyroidism, or when it rises, as in hyperparathyroidism, the ratio of ionized to un-ionized calcium remains constant, unless the concentration of protein changes.

This description, which takes into account only calcium ions and the un-ionized calcium proteinate fraction, ignores a small amount of calcium in other forms. Of these, the most important is an un-ionized calcium

citrate complex. Calcium also forms small amounts of an un-ionized fraction with the ions of other acids, including phosphate and sulfate, but the aggregate of all un-ionized complexes in the blood, with the exception of proteinate, can in man and most other animals hardly account for more than five to ten per cent of the total calcium. When serum is subjected to diffusion, or to ultrafiltration, these fractions appear in the diffusible portion; for this reason the total diffusible calcium is slightly higher than the calcium ion concentration.

In the protein-poor fluids of the body, such as the cerebrospinal fluid and the intercellular fluids, the state of calcium approximates that in an ultrafiltrate of plasma. In such fluids the concentration of calcium is generally about 5 mg per 100 cc (1.25 mM per liter), virtually all in the diffusible form. Again, a fraction of this may be in the form of un-ionized, diffusible complexes; these have little or no physiologic significance.

In the calcification process, when calcium is to be deposited in the bones it moves first from the plasma to the extracellular fluid, and from this fluid is deposited in locations prepared for calcification. Under such circumstances the plasma requires replenishment, in order to maintain calcium ion concentrations at physiologic levels; such replenishment ordinarily comes from the diet, but the skeleton itself is the principal depot for the storage of reserve supplies in the organism. It frequently comes about that as calcium is being deposited in one or more places in the skeleton, it is being withdrawn from other places; the entire system *plasma—intercellular fluid—bone* remains in a state of dynamic equilibrium.

2. THE PHOSPHATES OF THE FLUIDS OF THE BODY

Inorganic phosphate is present in the fluids of the body in the form of the ions of orthophosphoric acid, H_3PO_4 , chiefly as HPO_4^{2-} (McLean, 1942a). The plasma of the infant, under conditions of active deposition of bone salt, contains approximately 6 mg per 100 cc of total phosphorus as inorganic phosphate (2 mM per liter). In the adult the inorganic phosphate level of the plasma is reduced to approximately one half of that of the infant. It is characteristic of the rachitic infant that the concentration of inorganic phosphate in the plasma is at a level normal for the adult.

Excretion of phosphate through the kidneys varies within wide limits. The amount of phosphate so excreted depends mainly on the intake. Homeostatic control of the plasma phosphate level, mediated by the effect of the parathyroid hormone on renal excretion of phosphate, is also a possibility; that the parathyroid glands respond to fluctuations in the concentration of phosphate ions in the plasma, a necessary step in such control, has been claimed (Helfet, 1940; Törnblom, 1949; Engfeldt, 1950).

3. REGULATION OF THE CALCIUM ION CONCENTRATION BY PARATHYROID ACTIVITY

At all levels of calcium ion concentration in the fluids of the body there is exchange of calcium and phosphate between the blood and the bones; in the absence of the parathyroid glands a relatively constant although low concentration of calcium ions is maintained in the blood. While it is clear that the parathyroid glands are necessary for the maintenance of *normal* calcium ion levels in the blood, the relationship between their function and the ability of the organism to regulate calcium exchange in their absence has not been clearly understood.

There are three methods by which the serum calcium concentration may be lowered experimentally. Hastings and Huggins (1933) withdrew blood repeatedly from dogs, replacing it with serum decalcified by shaking with lead phosphate. When the procedure was interrupted the serum calcium rose rapidly, approaching a normal level in the animals with intact parathyroid glands, but not exceeding a hypocalcemic level in animals previously parathyroidectomized. Patt and Luckhardt (1942) confirmed and extended these observations. They also introduced the second method, that of intravenous administration of sodium oxalate. This caused a sharp fall in the serum calcium level, with a rapid return to normal in the intact animals. When dogs were subjected to parathyroidectomy just prior to administration of the sodium oxalate, injected while the serum calcium was still at a normal level, there was a similar fall in the concentration of serum calcium, followed by a slight increase; the end result was a hypocalcemic level characteristic of hypoparathyroidism. The third method, administration of ethylene diamine tetra-acetic acid intravenously (Popovici, *et al.*, 1950; Holland, Danielson, and Sahagian-Edwards, 1953) has not been explored from the point of view here under consideration.

Stewart and Bowen (1951) extended the observations of Patt and Luckhardt, and developed the *oxalate tolerance curve* as a measure of parathyroid function. Their results differed from those of Hastings and Huggins and of Patt and Luckhardt, in that they observed no appreciable return of the serum calcium toward pre-injection levels in parathyroidectomized animals; they attributed the results of the previous workers to residual hormone activity, owing to the brief interval allowed to elapse following parathyroidectomy.

In spite of the failure of Stewart and Bowen to observe a recovery phase in parathyroidectomized animals, following administration of oxalate, there is good evidence that there is a mechanism, independent of the parathyroid hormone, that will maintain the plasma at a relatively constant but low level, by equilibrium with the mineral of the bones. There is also a second mechanism, mediated by the parathyroid hormone, necessary for the maintenance of a normal plasma calcium level.

In terms of modern concepts of the blood-bone relationship this is interpreted to mean that there is a dual mechanism for control of the level of calcium in the plasma. Of this, one part acts by simple chemical equilibrium with the labile fraction of the bone mineral, variously estimated as from 1 to 5% of the total mineral, and is independent of the parathyroid glands (Fig. 3). This mechanism is adequate to effect

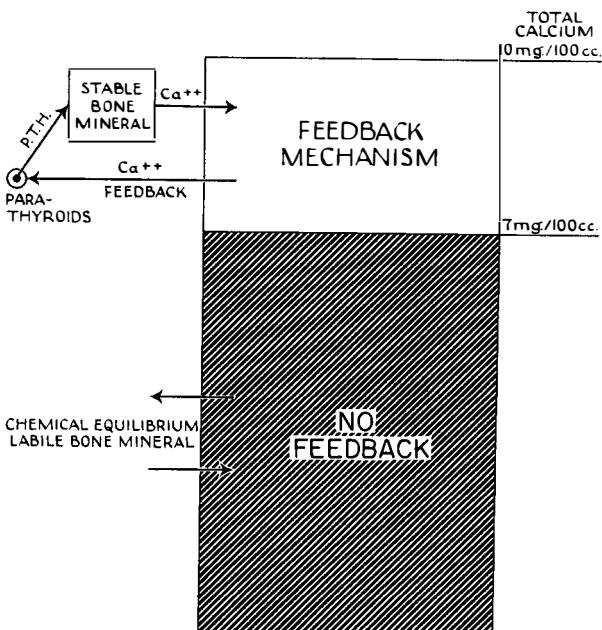


FIG. 3. Diagram to illustrate mechanism of exchange of calcium between blood plasma and bones. Chemical equilibrium with labile fraction of bone mineral is independent of parathyroid glands and is adequate to maintain plasma calcium level at 7 mg per 100 cc. Parathyroid activity is under control of feedback from Ca⁺ concentration in plasma and regulates release of calcium from stable hydroxyapatite crystals of bone mineral. This results in maintenance of plasma calcium at normal level of 10 mg per 100 cc. (McLean and Urist, *Bone: An Introduction to the Physiology of Skeletal Tissue*, University of Chicago Press, 1955. Reproduced by courtesy of the publishers.)

exchange between the blood and the bones, and to keep the plasma calcium level at approximately 7 mg per 100 cc.

The second part of the dual mechanism is required to keep the plasma calcium at the normal level of 10 mg per 100 cc, and is dependent upon mediation by the parathyroid glands. This is best understood in terms of a "feedback" mechanism (Fig. 3). The level of calcium ions in the plasma provides a stimulus to the parathyroid glands, a falling level resulting in increased parathyroid activity (Ham *et al.*, 1940; Patt and Luckhardt,

1942). This leads to an increased output of the parathyroid hormone (PTH), and this in turn brings about an increase in the mobilization of calcium from the bones and a consequent increase in the calcium level of the plasma. The parathyroid hormone causes destruction of both the mineral and organic components of bone, and the feedback mechanism releases calcium from the stable, as well as from the labile fractions of the bone mineral. According to this interpretation, the labile fraction of the calcium of the mineral is easily accessible to ionic exchange with the fluids of the body; the stable fraction of calcium, on the other hand, does not dissolve readily, and requires the action of the parathyroid hormone for its solution.

The summation of the effects of the two components of the dual mechanism is comparable to the maintenance of a constant temperature in a water bath. If it is desired, for example, to operate a water bath at 38°C in a room with a temperature of 20°C it is common practice to supply the greater amount of the heat required from a constant source, adequate to keep the temperature of the bath at, say, 33°C. Above this temperature the additional heat required is under the control of a thermoregulator, itself under the influence of a feedback from the actual temperature of the water. The constant source of heat is comparable to the constant supply of calcium to the blood by chemical equilibrium; the heat under control of the thermoregulator is comparable to the supply of calcium regulated by the parathyroid glands. In both cases accurate regulation is brought about through a feedback mechanism, the effects of which are added to those of a second mechanism that requires no regulation.

Most observers agree that the stimulus to parathyroid activity is a direct effect of the calcium ion level in the blood upon the glands themselves. The possibility of a parathyrotropic function on the part of the anterior lobe of the pituitary gland continues to receive some attention; current opinion is to the effect that the parathyroids are not dependent upon pituitary function for their activity (Carnes, Osebold, and Stoerck, 1943; Engfeldt, 1950). A further possibility, i.e., that the activity of the parathyroid glands may be affected by the serum phosphate level, is given consideration in another place in this chapter (page 718).

VII. Hyperparathyroidism and Bone Disease

The sequence of events in the bones, under the influence of an excess of parathyroid hormone, is best studied in experimental animals, and more particularly in young growing animals. The advanced changes of chronic hyperparathyroidism may be seen only in man, since the rapid development of resistance to exogenous parathyroid hormone prevents experimental production and maintenance of the chronic condition.

The gross skeletal changes in chronic hyperparathyroidism in man

are those of softening of the bones, with consequent deformities and fractures (Compere, 1930; Jaffe, 1933; Hodges, 1936; Snapper, 1949; Anderson, 1953). The diagnosis may be suggested by the deformities and the roentgenographic findings of generalized decrease in the density of the bones, often accompanied by cysts and tumors. The microscopic findings are those of greatly increased resorption of bone, with numerous osteoclasts. These are accompanied by increased osteoblastic activity, indicative of attempts to repair the damaged trabeculae of bone. Attempts at repair also result in an excess of fibrous connective tissue; this has led to the use of the term *osteitis fibrosa* to describe the pathologic changes in the bones. Such bone matrix as is present, old and new, is calcified; it is not correct to refer to the changes in the bones as decalcification. There may be large fibrous scars in areas formerly occupied by spongy bone. Some of these scars are brown, owing to their content of blood pigment, and they may contain multinucleated giant cells; from these characteristics they have been called brown tumors, giant-cell tumors, or osteoclastomas.

If biopsies of bone are performed after the removal of hyperfunctioning parathyroid tissue, it may be seen that reparative changes take place rapidly. Fibrous tissue is removed, and is replaced by new bone, which assumes a normal appearance. Actual cysts in bone may remain unless dealt with surgically.

Much has been made (Albright and Reifenstein, 1948) of the occurrence of severe hyperparathyroidism without bone disease. For the most part this distinction is clinical, rather than pathologic, since few such patients come to autopsy. They are described as being without clinical, roentgenologic, or histologic evidence of bone disease; the negative histologic evidence rests on examination of samples of bone, usually cortical, removed by biopsy. Another distinguishing feature is that unless bone disease is present the serum phosphatase level is within normal limits, whereas when bone changes are in progress the phosphatase level is elevated. Bone changes may be prevented or alleviated by increasing the intake of calcium, and therapy with calcium may lead also to lowering of the serum phosphatase to normal levels.

That the distinction of severe hyperparathyroidism without bone disease is valid remains to be proved by careful microscopic examination of the skeleton; in the meantime it is probably safer to regard differences in the changes in bones as quantitative rather than qualitative, and to refer to certain cases as being without *demonstrable* bone disease, as in fact they were first described by Albright, Sulkowitch, and Bloomberg (1937). In any event there are certainly patients with hypercalcemia resulting from hyperparathyroidism in whom bone changes, if any, are minimal. The situation is comparable to that in adult dogs, treated with doses of parathyroid extract sufficient to bring about maximum elevation

of the serum calcium level; in such animals bone changes are commonly not demonstrable, even on postmortem examination.

Hyperparathyroidism, with or without demonstrable bone disease, is frequently accompanied by pathologic conditions other than those found in the parathyroid glands and in the bones. Metastatic calcification occurs in soft tissues, and is especially harmful when it occurs in the kidneys or in the walls of blood vessels; such calcium deposits are commonly, if not always, associated with severe disease of the bones, and they reflect a condition of mobilization of the bone salt and supersaturation of the blood with this mineral, similar to that described by McLean and Bloom (1941) in experimental animals. Renal calculi are a frequent complication of hyperparathyroidism; they are not dependent upon changes in the bones, and reflect instead the increase in renal excretion of calcium resulting from hypercalcemia.

Experimental hyperparathyroidism has been produced in rats, dogs, cats, guinea pigs, and rabbits with occasional observations on other species (Thomson and Collip, 1932). Damage to and repair of bone have been extensively studied, following administration of single or repeated doses of parathyroid extract, and a coherent picture has emerged which is closely related to the pathologic changes observed in man.

In the young rat administration of single toxic doses of parathyroid extract leads to rapid resorption of bone, with associated histologic changes, followed by a somewhat slower regeneration (Heller *et al.*, 1950). Formation of dense connective tissue in the marrow spaces of spongy bone is especially prominent in rats (Burrows, 1938); other species exhibit this reaction to a much lesser degree. The rapid resorption of bone is also prominent in puppies; it is less so in kittens and in guinea pigs, even when the hormone is administered in doses sufficient to induce systemic poisoning.

Resorption of trabeculae of spongy bone may be very prominent in the first few hours after administration of the parathyroid extract, and before osteoclasts appear in increased numbers. At a slightly later stage there is a marked increase in the numbers of osteoclasts, especially in the areas in which resorption is under way. This stage is noteworthy also for the cellular transformations that can be observed in a rapidly advancing pathologic process. Osteoblasts have been observed to change to reticular cells, phagocytes, osteocytes, and osteoclasts; osteocytes to osteoclasts and reticular cells; reticular cells to osteoblasts; and osteoclasts to reticular cells (Heller *et al.*, 1950). These changes are reversible, and are to be characterized as modulations rather than as differentiation.

Recovery from the effects of large doses of parathyroid extract is accompanied, especially in the rat (Burrows, 1938), by an overgrowth of new bone, sometimes termed hyperostosis, in the areas in which

resorption of bone and deposition of fibrous tissue had occurred. A similar effect has also been produced in rats and in mice by repeated administration of parathyroid extract in doses insufficient to cause extensive resorption (Selye, 1932; Barnicot, 1945).

During the stage of rapid resorption of bone, mobilization of bone salt has been demonstrated in rats and in puppies (McLean and Bloom, 1941). This mineral, brought to visualization by staining with silver nitrate (von Kóssa, 1901) has been seen both diffusely distributed in the bone marrow, and aggregated within the macrophages of the marrow. That distributed diffusely is interpreted as in transit to the venules, in solution during the life of the animal and precipitated during preparation for histologic examination. That found within the macrophages must have been in a particulate form while the cells were still living; it is assumed that it was mobilized from the trabeculae of bone under resorption, and deposited in the form of a colloidal complex of calcium and phosphate while in the bone marrow. Such an interpretation implies that a condition of supersaturation had been brought about by the resorption of bone; it is not compatible with the theory that the bone salt is dissolved by the fluids of the body as a response to undersaturation of these fluids with the ions making up the mineral of bone (Plate I, p. 715).

It is emphasized that the changes just described in experimental animals resulted from the administration of toxic doses of parathyroid extract; doses sufficient, in many instances, to lead to widespread necrosis of the cellular elements of bone and of bone marrow. The degree to which these findings can be extrapolated to physiologic conditions, or even to clinical hyperparathyroidism, remains in doubt. For the most part they correspond to what Albright and Reifenstein (1948) have designated as parathyroid poisoning or hyper-hyperparathyroidism, which they attribute to a mechanism different from that produced by smaller doses of the hormone. A parallel for this position might be found in the differences between the mode of action of vitamin D in prophylactic doses (1,000 I.U. per day) and in toxic or subtoxic doses (50,000 I.U. per day, or more) (McLean, 1942b). While the death of cellular elements of bone and bone marrow cannot be brought directly into relationship with the physiologic action of the parathyroid hormone, both are manifestations of direct effects of the hormone upon bone. The observation of the resorption of bone in contact with transplants of parathyroid tissue supplies a link between strictly physiologic conditions and those in the toxic range.

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CHAPTER XXIII

THE EFFECTS OF RADIATION ON BONE

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I. Introduction

The effects of radiation on bone have both practical and academic interest. The practical interest is dependent upon the injurious effects of radiation. The academic interest lies in the use of radioactive elements as tools in the study of bone physiology and pathology. Injury due to

external radiation may result from the therapeutic or accidental application of beta-, gamma- or X-rays either directly to the skeleton or to adjacent soft tissues. Injury due to internal radiation may result from the ingestion of a large number of radioactive elements which are concentrated selectively in the skeleton where they emit alpha-, beta- or gamma-rays (Hamilton, 1947, 1948). Such radioactive elements may be given for therapeutic purposes or accidentally ingested.

The effect of irradiation on bone has been studied in experimental animals and in humans following therapy or accidental exposure. The experimental and clinical results are not, however, strictly comparable for the following reasons: (1) There is considerable species variation in sensitivity to radiation (Evans, Harris, and Bunker, 1944; Vaughan, Tutt, and Kidman, 1952; Edington, Judd, and Ward, 1953). (2) The experimental animals usually studied are rats, mice, guinea pigs, and rabbits: while the pattern of bone growth in the latter resembles that in the larger mammals and in man to a lesser extent (Amprino, 1952a) the growth pattern in rats and mice differs considerably from that in man. (3) The longer life span in man allows for the development of chronic radiation damage after relatively small doses of either internal or external radiation, which is impossible in short-lived animals. Experimental results in short-lived animals should therefore be used with reservation in interpreting clinical findings in man (Brues, 1949).

II. RADIATION DOSIMETRY WITH SPECIAL RELATION TO BONE

Before discussing the dosimetry and effects of radiation on bone, it is desirable to give a simple and approximate description of the terms used.

1. TERMS USED IN ASSESSING DOSAGE

The radiations which are discussed in the following pages are alpha-, beta-, gamma- and X-rays. These are ionizing radiations, i.e. when they pass through matter their principal means of losing energy is by the ejection of electrons from the atoms of which the matter is composed. The atom is left positively ionized and the result of this is chemical change in the molecules of which the atom is a part. The "specific ionization" thus produced is the number of ionizations per micron of path length of the radiation. This depends on both the energy and type of the ionizing radiation. Alpha-rays, which are helium nuclei emitted by radioactive isotopes, have a short straight range in tissue (about 25 microns for a 4-Mev alpha-ray) and a dense average specific ionization along their path. They are therefore the most dangerous radiations in their effect on biological systems. Beta-rays, which are electrons emitted by radioactive isotopes, have a long irregular range in tissue (about 1,800 microns for a 0.5-Mev beta-ray) and a much lower average specific

ionization than alpha-rays of the same energy. They are therefore less able than alpha-rays to affect chemical change in biological systems. X-rays and gamma-rays are electromagnetic waves, gamma-rays being of shorter wavelength, i.e. of higher energy and therefore more penetrating than X-rays. They both produce their effects in tissue by the projection of high-speed electrons; the range and specific ionization of these are the same as beta-rays of the same energy and depend on the original energy of the gamma- or X-rays (Lea, 1946; Gray, 1953).

a. *Radioactive Decay*

Radioactive isotopes emit their particles at a fairly constant and specific rate and therefore lose their radioactivity at a constant rate. This is usually expressed in terms of the "half-life" of the isotope, i.e. the time in seconds, minutes, hours, days, or years needed for half the original mass to have decayed to the daughter product. Certain of these daughter products are themselves radioactive and will decay with different half lives, until a "stable" nonradioactive isotope results and accumulates as in the radium series. For obvious reasons radioactive isotopes with a long half-life are more dangerous than those with a short half-life.

b. *Energy of Radiation*

Every radiation emission has a fairly specific range of intrinsic energy associated with its liberation. This is expressed in "Mev units" (million electron volts). The energy of the radiation from radioactive isotopes varies from just more than zero up to 10 Mev for alpha particles and up to 15 Mev for beta particles.

c. *Relative Biological Efficiency*

In practice the biological effectiveness of a tissue dose delivered by electrons (beta-rays or secondary electrons in the case of X- or gamma-rays) does not vary much with specific ionization. Therefore, for protection purposes it is assumed that all beta-, X-rays and gamma-rays have the same biological effectiveness. Since most of the directly applicable information on protection has been obtained from the occupational exposure of radiologists to ordinary X-rays up to 250 kv. it is desirable to use this X-ray energy range as a point of reference, especially since the specific ionization in this range is nearly constant. Radiations in this range are assumed to have a biological effectiveness of 1. To calculate the biological effectiveness of other types of radiation having specific ionizations considerably different, it is necessary to relate them to that of X-rays used as the point of reference. This is spoken of as the R.B.E. or relative biological effectiveness.

Amounts of radioactive materials are expressed in curies. *The curie* is a unit of radioactivity defined as the quantity of any radioactive nuclide in which the number of disintegrations per second is 3.700×10^{10} .

2. UNITS OF DOSAGE

The roentgen, introduced in 1928, has been the commonest unit of radiation dose.

It is, in the simplest terms, an ionization unit bearing a constant and known relation to the radiation energy absorbed per unit mass of air. The energy released per gram of air for a dose of 1 roentgen (*r*) is approximately 85 ergs and is independent of the wavelength of the ionizing radiation. The roentgen is still used for expressing the dose of X- and gamma-rays (Spiers, 1946).

Tissues, however, do not have the same average atomic composition or electron density as air and variations occur, therefore, in the amount of energy absorbed per roentgen. What is important about any ionizing radiation is the absorbed dose in the tissue concerned, i.e. the amount of energy imparted by ionizing particles per unit mass of irradiated material at the place of interest. The heterogeneous media encountered by a beam of radiation entering the body make an estimation of dose received at any one point extremely complex.

The rad is the unit of absorbed dose and is 100 ergs per gram of tissue (Recommendations of the International Commission on Radiological Units, 1953). Radiation dose in the literature has hitherto been recorded in roentgens. Allowance has not therefore been made for differences in absorption between air and tissue (see page 732-735).

The rep is a unit which takes into account the factor of absorption in the tissue but attempts to relate itself to the roentgen. Strictly speaking, when tissue is the absorbing medium concerned, the rep is the quantity of radiation such that the energy imparted to the tissue by the radiation concerned is 85 ergs per gram of tissue, i.e. the same as the roentgen (Marinelli, Quimby, and Hine, 1948). In practice the energy imparted to soft tissue by 1 roentgen of gamma-rays is often taken to be 93 ergs, the difference being due to the difference in absorption between air and soft tissue.

The rem is the quantity of any ionizing radiation such that the energy imparted to a biological system per gram of living matter by the ionizing particles present in the locus of interest has the same biological effectiveness as 1 rad of X-radiation with average specific ionization of 100 pairs per micron of water (a dose in rems is equal to the dose in rads multiplied by the appropriate relative biological efficiency).

Permissible dose is a dose of ionizing radiation that in the light of present knowledge is not expected to cause appreciable bodily injury to a person at any time during his life time. It is usually calculated in terms of dosage in rems or rads received by the tissue considered per week. The maximum permissible dose (M.P.D.) of isotopes behaving like radium is calculated in relation to the maximum permissible body burden

of radium, namely, $0.1 \mu\text{c}$. In other cases the mpd is based on a calculation of that burden in the body which will result in an average biological dose of ionizing radiation equivalent to 0.3 rad per week to the organ in which maximum concentration of the isotope occurs.

Radium is used as the common denominator for both experimental animals and man because something is known of its effects in man from the study of individuals who have been accidentally exposed. The maximum permissible skeletal burden of radium in man is at present fixed as $0.1 \mu\text{g}$ (International Committee on Radiological Protection, 1951) since this is one tenth of the amount believed at present in clinical practice to have morbid consequences, i.e. to produce pathological change in bone (Brues, 1949). Theoretical calculations advanced by Spiers (1953) based on the dose rates to soft tissue structures within bone containing radium and its decay products, i.e. the osteocytes which he regards as the critical tissue, agree with this figure as long as the osteocytes are regarded as a limited volume of tissue to which a higher permissible dose rate (of 1.5 rem per week) is allowed and no allowance is made for concentration of the radium within the bone. If the osteocytes are not regarded as a limited tissue and calculations are based on the permissible dose rate allowed for soft tissues as a whole (of 0.3 rem per week) the figure of $0.1 \mu\text{g}$ radium should be reduced to $0.02 \mu\text{g}$. These theoretical considerations suggest that the skeletal radium burden of $0.1 \mu\text{g}$ which in practice appears safe, may prove not unduly conservative.

3. CHARACTERISTICS OF RADIOACTIVE ELEMENTS THAT EFFECT DOSAGE

In the case of ingested radioactive elements, the physical and chemical characteristics of the element itself must be considered in assessing dosage to any particular tissue, namely, (1) the nature of the radiations emitted (see page 730), (for instance the alpha-emitters are five times as effective as beta-emitters, yielding equivalent energy in producing bone tumors), (2) rate of radioactive decay (see page 731), (3) energy of radiation (see page 731), and (4) the metabolic pathway or relation of the element to the normal physiology of the tissue (see page 734).

Assessment of radiation dose may become complicated in clinical practice. For instance, in attempting to assess the dose received by the radium dial painters, it has to be remembered that many of them ingested a mixture of radium and mesothorium. Radium has an extremely long half-life (about 1,600 years), emits alpha-particles and has radioactive decay products. Mesothorium, which has a short half-life (6.7 years), also has decay products which emit high energy alph-, beta-, and gamma-rays, some of which have a long half-life. Further, the metabolic pathway within the bone of the decay products of mesothorium differs from

that of mesothorium itself. The relative part played by radium and mesothorium in the picture of "radium poisoning" is still obscure (Aub *et al.*, 1952) although it is suggested that mesothorium predominated in the early cases described by Martland (Martland, Conlon, and Knef, 1925; Martland, 1929).

III. Relationship of Anatomy and Physiology of Bone to Radiation Pathology and Dosimetry

The behavior of radioactive isotopes once they have gained access to the body depends in part on the route of administration and in part on their chemical relationship to normal metabolites. Radioactive iodine, I^{131} , for instance, behaves like nonradioactive iodine, I^{127} , and is concentrated selectively in the thyroid. Radioactive calcium, Ca^{45} , behaves like normal calcium Ca^{40} , and is concentrated in the skeleton. The metabolic pathway of Ca^{45} in the body differs in no way from the metabolic pathway of Ca^{40} . Elements that are chemically closely related to calcium, strontium, barium, and radium appear to have the same metabolic pathway. They are concentrated in the skeleton, where they behave qualitatively, if not quantitatively like calcium and are associated with mineralized bone (Vaughan, Tutt, and Kidman, 1952). There is a further large group of elements which have been shown experimentally to be concentrated in the skeleton and which are therefore spoken of as bone-seeking isotopes (Hamilton, 1947). These additional bone-seeking radioactive isotopes are yttrium, zirconium, cerium, thorium, plutonium and americium, lanthanum, praseodymium, neodymium, promethium, samarium, europium, actinium, neptunium, and curium (Hamilton, 1947). These isotopes, with the exception of plutonium, are said to accumulate in the osteoid matrix (Hamilton, 1947; Copp *et al.*, 1951). It is more likely that they are bound specifically by the mucopolysaccharides associated with resorbing or quiescent bone surfaces, (see page 741) since they are found concentrated only on such surfaces (Vaughan, unpublished observations.) Plutonium is thought (Scott *et al.*, 1948) to be selectively localized on bone surfaces, as is radiothorium, a decay product of mesothorium (page 733). Autoradiographs, however, prepared from bones retaining plutonium, are difficult to interpret and appear not unlike those from bones retaining curium and yttrium. Detailed experimental studies of the behavior of these bone-seeking isotopes in young animals have hitherto only been made using Sr^{89} , Sr^{90} , and Y^{91} (Kidman, Tutt, and Vaughan, 1950; Kidman *et al.*, 1952; Tutt *et al.*, 1952; Rayner, *et al.*, 1953; Jowsey *et al.*, 1953; Jowsey, Owen and Vaughan, 1953; Copp *et al.*, 1951) and to a lesser extent Ca^{45} (Copp *et al.*, 1951; Lacroix, 1953) and Ce^{144} (Copp *et al.*, 1951). The results are discussed in relation to bone physiology and anatomy in the following pages.

I. NON-HOMOGENEOUS CHARACTER OF BONE

The fundamental mechanism by which radiation, whether external or internal, produces cellular damage, is not for discussion here (Lea, 1946; Gray, 1953). It is generally agreed, however, that the amount of injury produced in any tissue is dependent upon the "ionization" produced by the passage of the radiations through the tissues reached (see page 730). The roentgen, the unit hitherto most commonly used to express radiation dose is based on the ionization produced in air by the passage of X-rays. The dose received by a tissue element, if estimated in roentgens, assumes the body is a homogeneous material, usually water or a water equivalent substance. Bone is not, however, a homogeneous tissue (Spiers, 1949, 1951). It consists of a highly mineralized protein matrix in which are embedded widely separated living cells, the osteocytes, the somewhat larger groups of cells which go to make up the capillaries and blood vessels and the fibroblasts, osteoblasts, and osteoclasts lining these canals. The absorption of X-rays or of any radiation depends on the effective atomic number of the absorbing material. The highly mineralized protein matrix of bone absorbs far more energy than soft tissue (Spiers, 1949). The "ionization" occurring therefore in soft tissue at an interface between it and mineralized bone of inside small cavities within the bone, is very much higher than might be anticipated from the dose originally expressed in roentgens (Spiers, 1950; Munson, 1950). The "permissible" dose in roentgens to bone is about one half of that tolerated by soft tissues for 200-kv X-rays, and may be no more than one third for medium voltage and superficial X-rays (Spiers, 1951). The smaller the cavity the greater the "ionization" per cubic micron (Spiers, 1949). Spiers has suggested that a mean ionization, representative of soft tissue within bone, can be taken as the mean of the average ionization in the small cavities containing the osteocytes and in the larger cavities or Haversian canals, making the simplifying assumption that the total volume of soft tissue in each is the same. In attempting to estimate the dose of radiation received by bone tissue from any source whether internal or external, two factors in bone itself must therefore be remembered: (1) the effective atomic number of the inorganic bone, and (2) the size and location of the soft tissues within it. It is then assumed that the effects of radiation are exerted upon the bone cells (Spiers, 1949, 1953) or possibly upon the small vessels (Aub *et al.*, 1952; Looney and Woodruff, 1953). These cells are isolated, especially in older animals, in extensive areas of calcified matrix, therefore the effects of radiation, although to some extent enhanced by the structure, are also to some extent possibly minimized by the structure. Fewer cells are hit by a dose of radiation than in the same volume of some other tissue such as a lymph gland, although the cell that is hit is hit harder. This may to some extent contribute to the long latent period that is characteristic of

radiation osteodysplasia (see pages 741, 760). The possible effects of radiation on the collagen and ground substance that constitute the bone matrix is still obscure.

2. RATE OF METABOLIC TURNOVER

Recent use of tracer techniques in physiology has demonstrated the extremely rapid metabolic turnover that occurs in the majority of body tissues. The reverse is true of bone. The metabolic turnover under normal conditions appears to be extremely slow (Perrone and Slack, 1951; Neuberger and Slack, 1953; Slack, 1953). The turnover of glycine in the bone of adult rats as compared with that in intracellular proteins is slow, although in young rats it is somewhat faster. These experiments must be repeated using animals whose bone behavior is more akin to that of man (Amprino, 1952b) but they at least suggest that the turnover of the protein matrix constituent of bone is slow. If certain of the radioactive elements, which are selectively retained in the skeleton are bound to the protein matrix, as has been suggested, their long continued retention in the skeleton may well be associated with the slow metabolic turnover of the protein element. If the view (Bloom, Bloom, and McLean, 1941) is accepted that calcium is lost from the skeleton only by osteoclastic resorption removing at the same time the associated protein matrix, a slow turnover of protein should also mean a slow turnover of associated calcium. Radioactive calcium is not an easy tracer to use in metabolic studies. Metabolic investigations have been made using, however, radioactive strontium which appears to behave qualitatively, if not quantitatively, like calcium (Tutt *et al.*, 1952; Jowsey *et al.*, 1953). These studies using both Sr⁸⁹ and Sr⁹⁰ suggest a rapid uptake in certain areas associated with slow loss. This is probably not a continuous process throughout bone but occurs in isolated areas at different times. The slow turnover may then be more apparent than real, depending on the fact that resorption only occurs in a few localities at any one time and is not going on continuously throughout bone.

In young rabbits (5–7 weeks old) 60% of the injected Sr⁸⁹ or Sr⁹⁰ is found in the skeleton within 30 minutes and 70% within 4 hours (Tutt *et al.*, 1952; Jowsey *et al.*, 1953) falling slowly to a retention of 11% eighteen months after injection. In animals a year old, maximum retention is approximately 30% at the end of 4 hours, falling to 3% at the end of 8 months.

3. LOCALIZATION OF AREAS OF BONE GROWTH IN YOUNG AND OLD ANIMALS

As long as bones are growing in length and diameter, active bone formation occurs in the region of the epiphyseal plate, beneath the metaphyseal endosteum and beneath the diaphyseal periosteum. Certain

bone-seeking radioactive isotopes, including calcium, have been demonstrated by both radiochemical and autoradiographic techniques to be concentrated immediately after injection in precisely these areas and to be displaced by new bone subsequently laid down (Figs. 1 and 2). It



FIG. 1. Geographical autoradiograph of longitudinal section of lower end of femur of rabbit injected with Sr^{90} ($80 \mu\text{c}/\text{kg}$) killed 10 minutes later. Note (i) intense localized reaction beneath the epiphyseal plate in the region of the endosteum, in the metaphysis, in the region of the periosteum in the center of the diaphysis; (ii) diffuse reaction throughout mineralized bone. (Reproduced by courtesy of the Editors of the *British Journal of Experimental Pathology*.)

FIG. 2. Geographical autoradiograph of longitudinal section of lower end of femur of rabbit injected with Sr^{90} ($80 \mu\text{c}/\text{kg}$) killed 18 months later. (Note (i) persistence of lines of localized reaction in the diaphysis; (ii) diffuse reaction between the localized lines and in the epiphysis; (iii) extremely faint diffuse reaction extending towards the epiphysis. (Reproduced by courtesy of the Editors of the *British Journal of Experimental Pathology*.)

must be assumed that these isotopes are incorporated in the newly formed bone crystal just as ordinary calcium is incorporated (Leblond *et al.*, 1950; Comar, Lotz, and Boyd, 1952; Rayner *et al.*, 1953). For instance, the retention of strontium in the epiphyseal plate of humerus and tibia 24 hours after injection is 1.45% of the injected dose and in the diaphysis is 0.55%; 9 days later the retention in the epiphysis is only 0.65% of the injected dose while that in the diaphysis is 0.52% (Tutt *et al.*, 1952). In very young animals there is considerable secondary uptake from the blood stream of radioactive material released by the normal process of resorption, which can again be demonstrated both by radiochemical and autoradiographic means (Jowsey *et al.*, 1953).

As well as endosteal, periosteal, and endochondrial bone formation, the normal processes of resorption and apposition go on in cortical bone throughout life (Petersen, 1930; Amprino, 1952a and b). *In vivo* studies using Sr⁹⁰, Ca⁴⁵, and P³² have shown in the long bones of both adult

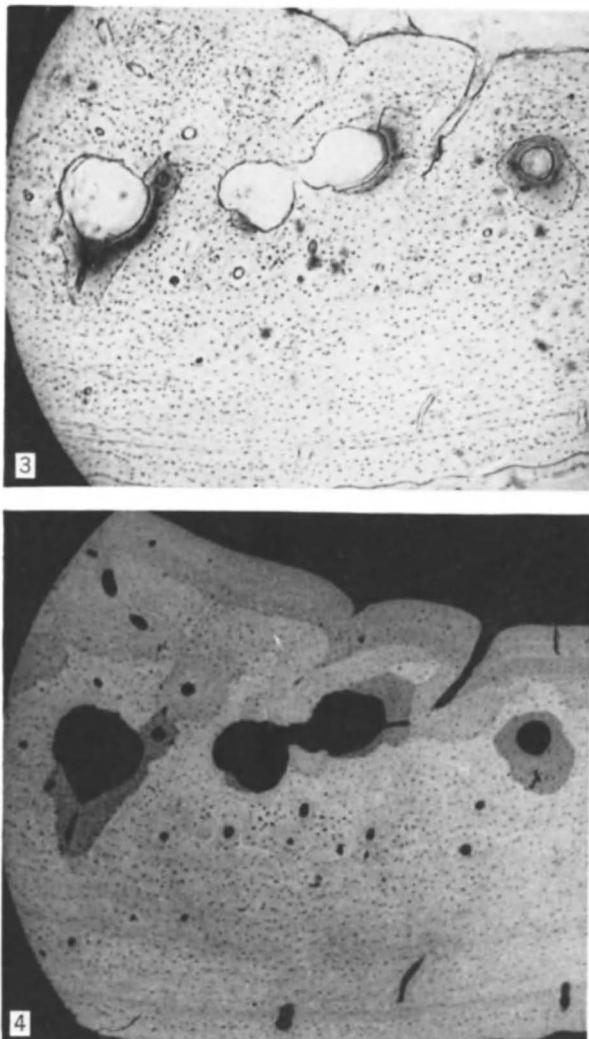


FIG. 3. Microradiograph of section in Fig. 4. Note (i) low calcification of endosteal surface (upper edge); (ii) irregular calcification around building sites and Haversian canals. $\times 60$.

FIG. 4. Autoradiograph left on section from which Fig. 3 was prepared—cross section midshaft of tibia of 6-year-old monkey dying 24–25 days after a single injection of Sr⁹⁰ (1000 $\mu\text{c}/\text{kg}$). Note (i) reaction over areas of low calcification around building sites and Haversian canals; (ii) no significant reaction over low calcification of endosteal bone (upper edge). $\times 60$.

monkeys, dogs, and rabbits (Engfeldt, Engström, and Zetterstrom, 1952; Lacroix, 1953; Jowsey, Owen, and Vaughan, 1953) that the isotope is taken up in active building sites in cortical bone, and in endosteal and periosteal bone. This can be demonstrated by preparing microradiographs and autoradiographs from the same cross section of bone. In old animals retention in areas of low calcification, around building sites, may be considerable (Figs. 3 and 4), but is often irregular. Thus resorption may predominate on one side of a building site and apposition on the other. These building sites occur in a scattered way in cortical bone, in some areas there may be none and in others they are more numerous. Autoradiographs prepared from sections taken immediately after injection of the isotope show reaction at the edge of the building site; those taken 3 weeks after injection show new bone laid down some time after injection containing no isotope at the edge of the building site and bone containing isotope at some distance from the edge of the building site. In animals killed as long as eight weeks after injection the reaction in the autoradiograph appears to be in a circle at some distance from a small Haversian canal, while the microradiograph prepared from the same section shows active building sites with no reaction. These bone-seeking elements, to which radium is closely related chemically, are retained in greater quantities in young people or animals, presumably because the processes of calcification are more active in the young.

Injury in the region of the epiphysis is common in the young because concentration of the dangerous element (page 737), in the case of internal radiation, has been present at one time in the epiphyseal region, although it is not necessarily still present there at the time of death. Concentration of radium in older people, particularly in areas of active calcification in the building sites, possibly explains the localized deposition of radium found in the bones of human beings long after ingestion of radium (see page 738) (Looney, 1951; Hoecker and Roofe, 1949, 1951; Aub *et al.*, 1952). Cortical bone is undergoing resorption and apposition throughout life. Radium, once incorporated in the skeleton, may be repeatedly released to the blood stream by resorption and again deposited by apposition. This is known to occur in the case of both Sr⁹⁰ (Jowsey *et al.*, 1953) and Y⁹¹ (Rayner *et al.*, 1953). A tumor may ultimately develop near the area of bone where primary deposition occurred, but at death this area does not necessarily show radioactivity (Hoecker and Roofe, 1951) since the radium may have been removed and incorporated elsewhere. In fact bone tissue may appear histologically normal at the site of a "hot spot" while at another site necrosis is present without radioactivity (Aub *et al.*, 1952). The mobility of ingested radium is evidenced by the high activity often found in newly formed callus around a spontaneous fracture (Aub *et al.*, 1952).

It is evident that the physiological processes of apposition and resorp-

tion would serve to explain the production of the radium "hot spot," the extremely patchy nature of areas of necrosis and increased calcification of radiation osteodysplasia and the occurrence of tumors in areas without radioactivity at the time of examination. How far the effect is a direct one on the osteocytes and osteoblasts of the bone, or on the enzyme systems associated with the processes of apposition and resorption (Rutishauser and Mäjno, 1951; Woodard and Spiers, 1953), or how far it is secondary to damage to the blood vessels running in the Haversian systems (Spiers, 1949), is not known.

4. DISTRIBUTION OF VASCULAR TISSUE

Combined autoradiographic, microradiographic, and chemical techniques have shown that certain radioactive elements following injection are not located in building sites but around small vessels in cortical bone and in the extremely vascular tissue beneath the epiphyseal plate. Resolution has not yet proved good enough to enable a more precise location to be made. Such a method of deposition is true of yttrium (Fig. 5)

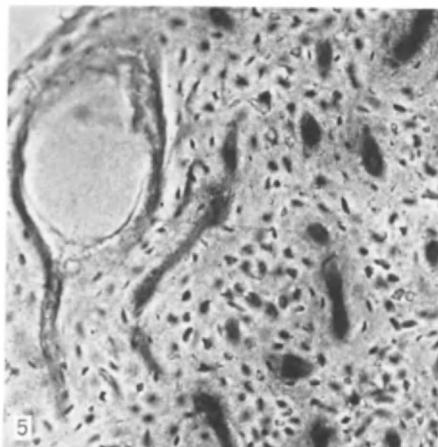


FIG. 5. Autoradiograph left on cross section of midshaft of tibia of rabbit 5-7 weeks old killed 24 hours after injection of Y^{91} ($1000 \mu\text{c}/\text{kg}$). Note (i) heavy reaction around small vessels; (ii) absence of reaction around building site. $\times 180$.

(Rayner *et al.*, 1953), americium (Scott *et al.*, 1948), and curium (Scott, Axelrod, and Hamilton, 1949) and may well prove true of other elements. It must be assumed that the element is combined with some particular tissue component, possibly a mucopolysaccharide, since this has been shown to be concentrated particularly around small vessels (Rogers, 1949). Such a mucopolysaccharide may well be present also in the highly vascular epiphyseal region. The view that yttrium is combined with

osteoid matrix (Hamilton, 1947; Copp *et al.*, 1951) is difficult to understand. Yttrium is never found in areas of endosteal and periosteal bone growth and yet such areas also contain osteoid matrix (Fell, 1931-32; Rayner *et al.*, 1953).

Recent unpublished experiments when ^{91}Y was given to normal puppies and puppies with severe rickets, due to deficiency of Vitamin D, have shown that yttrium is not deposited in osteoid tissue. It is found on actively resorbing bone surfaces and in association with small vessels. (Vaughan, unpublished observations.)

The patchy concentration of all radioactive elements in bone, whether around building sites, around small vessels or in relation to endosteal, periosteal and endochondral bone, is important in assessing radiation hazards. A chemical estimate of the total skeletal content of any element gives no true picture of the hazard to which the individual is exposed since concentration occurs in small isolated areas, enabling prolonged radiation of a small number of cells to take place over a long period of time.

The combination of patchy concentration with slow metabolic turnover and secondary uptake may well account in part at least for the occurrence of tumor development, resulting from retention of minute traces of radium after a long latent period, which appears to be so characteristic of bone damage following radiation.

When the clinical and experimental effects of radiation are described, it becomes apparent that if the skeletal burden of radium is low, the time required for tumor development is within certain limits longer than if the skeletal burden is high. The retention of radium with its long half-life and its highly ionizing alpha particles in strongly concentrated areas of bone where metabolic turnover is slow, would seem to present the ideal conditions for tumor development with low dosage after a long time.

5. THE PRESENCE OF AN EXTENSIVE CRYSTAL SURFACE

It has been suggested, on the basis of chemical experiments largely with dead powdered bone, and from a study of autoradiographs prepared from the bones of animals injected with radioactive isotopes, that the retention of these radioactive elements in the skeleton is accounted for, to some extent, by ionic exchange and adsorption on the surface of the hydroxy appatite crystal of mineralized bone (Hevesy, Levi, and Rebbe, 1940; Hodge, 1949; Neuman, 1950; Neuman and Mulryan, 1951; MacDonald *et al.*, 1952, 1953). Geographical autoradiographs prepared from the bones of animals given Sr^{90} , Ca^{45} , and P^{32} , (Leblond *et al.*, 1950; Jowsey *et al.*, 1953) certainly in young animals, show a diffuse reaction over mineralized bone which is, however, much less than that seen over

the sites of active bone growth. In autoradiographs prepared from thin sections of bone from animals injected with Sr⁹⁰ the reaction is extremely heavy over active building sites and apparent with low power magnification, while the reaction over adjacent bone is, as evidenced by grain counts though considerably above background, only apparent with high power magnification (Fig. 3). Clearly the main sites of retention are in areas of active bone growth, at least in the case of Sr⁹⁰ and Sr⁸⁹, and around vessels in the case of radioactive isotopes behaving like yttrium, although some diffuse uptake, possibly due to extensive exchange on crystal surfaces also occurs.

6. RELATIONSHIP OF BONE AND MYELOID TISSUE

Bone lesions due to radiation characteristically develop slowly for physiological reasons (see page 739). In discussion of dosimetry and of the effects of radiation on bone, it must not be forgotten that within certain parts of the skeleton lies active blood forming marrow, the amount present varying with age. A heavy dose of external radiation or a high skeletal burden of a radioactive element may result in a severe blood dyscrasia. The experimental animal or the patient will die of leukemia or aplastic anemia before bone lesions have had time to develop (Brues, 1949). The radium dial painters with heavy skeletal burdens first reported (Hoffman, 1925; Martland *et al.*, 1925) all died of profound anemia. It was only later that reports of the development of bone sarcoma appeared (Martland and Humphries, 1929). Patients die of radiation-induced osteosarcoma who have had too small a dose to die of a radiation-induced blood dyscrasia.

IV. Pathological Changes Induced in Bone by Radiation

The changes in bone induced by radiation may be grouped under two headings—(1) radiation osteodysplasia, (2) radiation neoplasia.

1. RADIATION OSTEODYSPASIA

Ewing in 1926 introduced the term radiation osteitis to describe the pathological changes in bone following external irradiation. Martland (1931) then applied the same term to the abnormalities found in bone and bone marrow in the radium dial painters dying of severe anemias. The name is a misleading one since there is not necessarily an inflammatory element present, the abnormal bone picture might better be termed radiation osteodysplasia. The changes seen in bone vary according to the age of the bone at the time of irradiation, the severity of the radiation, and the presence or absence of sepsis. In young subjects, when the bone is still growing in length, radiation dysplasia results in retarda-

tion of growth. Severe fracture may result and, if there is associated sepsis, osteomyelitis. In older subjects when growth in length of the bones has stopped, there can be no obvious retardation of growth but fracture and osteomyelitis are not uncommon.

These gross changes in bone are probably dependent upon varying degrees of injury to the bone cells due to the direct effect of radiation upon the cell (Spires, 1949, 1953). The chondroblasts are the most sensitive (Gall, Lingley and Hilcken, 1940; Heller, 1948) followed by the osteoblasts and osteocytes. The osteoclasts are probably the least sensitive (Gates, 1943). Death of the osteocyte contributes to the pathology of dysplasia since the living osteocyte is probably essential to the maintenance of normal collagen and ground substance (Rutishauser and Mäjno, 1951; Wasserman, 1952; Jackson, 1953), while without osteoblasts and osteoclasts the normal processes of apposition and resorption are halted. Similarly, in the region of active calcification occurring at the epiphyseal plate, death of the chondroblasts results in failure of growth in length, due to failure of the normal process of endochondrial calcification.

It has, on the other hand, been suggested that injury to the bone cells is only secondary to injury to either the small vessels directly supplying the bone (Looney, 1951; Looney and Woodruff, 1953; Aub *et al.*, 1952) or possibly in the case of external radiation to injury of the larger vessels in the area irradiated (Baensch, 1927; Dalby, Jacox, and Miller, 1936). No evidence of such gross injury to blood vessels following external irradiation has been recorded in the case, for instance, of fracture of the neck of the femur associated with radiotherapy applied to the pelvic organs. It is, however, known that in experimental animals that have ingested radium (Norris and Evans, 1946; Fink, 1950) excessive calcification of larger vessels is found at post mortem. Theoretically, therefore, internal radiation might be expected to produce damage to the vessels supplying the skeleton. Such damage has not yet been described in human cases or in experimental animals with radiation osteodysplasia following ingestion of radium. Present evidence would suggest that although injury to small vessels may be a contributory factor, injury to bone cells is the primary cause of radiation osteodysplasia. The possible direct effect of radiation on collagen and ground substance must not be forgotten. "Hyalinization" of collagen is characteristic of radiation damage.

There is no fundamental difference in the pathology of the lesions produced in bone by external and internal radiation (Heller, 1948). It is, however, convenient to discuss the clinical picture of osteodysplasia caused by internal and external radiation separately since external radiation is often localized and internal radiation is invariably generalized.

a. Experimental Osteodysplasia

External radiation. Hamsters irradiated to death under a roentgen beam or killed 3 hours after irradiation with 1,500 roentgen show a reduction in the number of osteoblasts lining the primary spongiosa and loss of metachromatic staining (Levy and Rugh, 1952).

It was noted as early as 1903 that therapeutic doses of X-rays given to chickens retarded bone development (Perthes, 1903), the maximum effect being a temporary cessation of growth in length. Subsequent workers have confirmed this finding both in the case of X-rays and radium (Recamier and Tribondeau, 1905; Försterling, 1906; Iselin and Dieterle, 1910-11; Brooks and Hillstrom, 1933; Dahl, 1934; Baunach, 1935; Bisgard and Hunt, 1936; Regen and Wilkins, 1936; Engel, 1938, 1939; Gall, *et al.*, 1940; Hinkel, 1942, 1943; Heller, 1948). It is probably true that periosteal and endosteal bone formation are as sensitive to X-rays as endochondral ossification (Dahl, 1934) but growth in length is always more dramatic than growth in diameter and a check to growth in length is therefore more apparent. With moderate exposure to X-rays growth is temporarily checked; with greater exposure it may be stopped altogether. Generally the degree of retardation is in direct relation to the dose up to the point of maximal response, but the degree of retardation may be greater than the proportional increment of dose (Brooks and Hillstrom, 1933). Fractionation of the total dose and protraction of the interval between exposures greatly lessens the effect (Bisgard and Hunt, 1936). If larger doses are given there may be complete arrest of growth. Similar effects have been noted after placing radium needles alongside the vertebra and close to the epiphysis of long bones of goats, dogs, and rabbits. The side exposed to the radium remains shorter than the other and scoliosis results. Microscopically there is disorganization of the process of ossification of the epiphyseal cartilage on the side exposed to irradiation (Engel, 1938, 1939).

Cessation in growth in length is dependent on degenerative changes occurring in the region of the actively calcifying cartilage cells below the epiphyseal plate (Gall *et al.*, 1940; Heller, 1948). The cartilage cells in rats 3 days after exposure to 600 roentgens of X-rays are abnormally swollen and 9 days later normal interdigititation between cartilage and metaphyseal spongy bone is lost, causing a separation of cartilage and spongy bone (Heller, 1948). This severance may last for some weeks but growth may then be renewed. A new primary spongiosa is laid down against the cartilage plate while the old disconnected spongiosa represented by a plaque of dead bone may be to some extent resorbed or displaced down the shaft by freshly formed bone (Heller, 1948). The renewed growth processes tend to be abnormal and slow, the degree of abnormality and retardation being dependent upon the size of

the dose. A dose of 600 roentgens causes death of some osteocytes in the spongy bone, but not in the dense cortical bone. Osteoblasts disappear after such irradiation; the osteoclasts are less affected and may in fact be increased (Heller, 1948) and with the renewal of growth osteoblasts again become active. A single X-ray dose of 400 roentgens has a less marked effect. A second X-ray dose of 400 roentgens given 6 weeks later has the same effect as the first (Heller, 1948).

Internal Radiation. Similar changes are recorded in the bones of small rodents following the injection of radium and plutonium, both alpha emitters, and Sr⁸⁹, Y⁹¹ (Heller, 1948), and P³² (Kligerman, 1950)

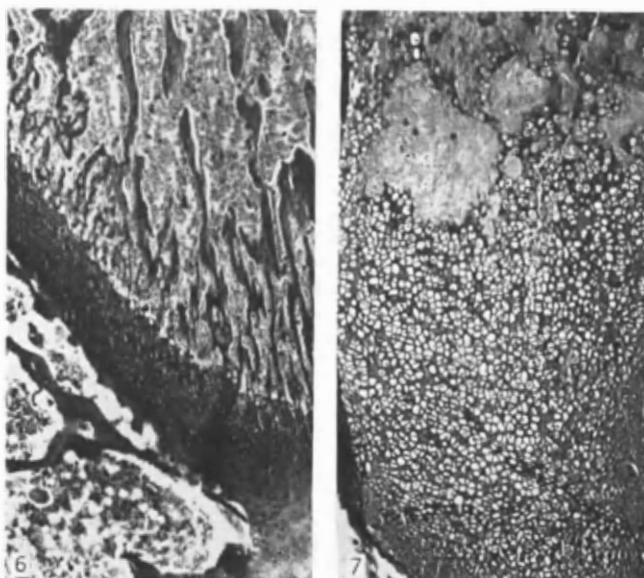


FIG. 6. Photomicrograph ($\times 32$) Control rat aged 56 days. Epiphyseal region. This represents a normal zone of enchondral ossification. Note regular arrangement of columns of cartilage cells, and an orderly transition of these cells from normal cartilage, through the stage of vacuolization, to normal bone formation. (From Kligerman, 1950. Reproduced by courtesy of the author and editors.)

FIG. 7. Photomicrograph ($\times 32$) Rat aged 56 days injected 3,600 $\mu\text{c}/\text{kg}$ P³² in divided doses. First injection when 42 days old. Note widening of the zone of enchondral ossification with increase in number and size of cartilage cells. There is no symmetrical arrangement of these cells in columns. (From Kligerman, 1950. Reproduced by courtesy of the author and editors.)

(Figs. 6 and 7). The only variation is the dose required to bring about the same reaction in a given time (see page 731).

It is impossible to make a precise estimate of the relation of dosage to degrees of bone injury or of the dose required to produce the same

injury in different species, as too many variables are present in the experimental data available (Heller, 1948). In mice 3.6 μc of Sr⁸⁹ per gram weight cause damage so severe that there is no resumption of growth, while a dose of 0.86 μc per gram weight causes only slight damage and has no significant effect on bone growth. In the case of radium 1.0 μc per gram weight checks growth, while 0.5 μc produces less severe changes. Damage, repair, and regeneration consistently follow the same picture: osteoblasts disappear and interdigititation of bone and cartilage at the epiphyseal line is decreased, frequently resulting in complete "severance" of epiphyseal cartilage from the spongiosa and arrest of growth in length. After the lower doses of short-lived isotopes, repair occurs. Growth is sometimes abnormal in character, especially if the longer lived isotopes are used. When low doses of either long- or short-lived isotopes are involved, normal growth is resumed and damaged bone, after displacement by growth in length, is resorbed. The old devitalized spongy bone, which often shows irregular coarsening of connective tissue fibers and increased density of the matrix with an irregular distribution of osteocytes if any are present, is displaced by new bone which in some cases includes an unusual number of calcified cartilage remnants (Heller, 1948).

The picture seen in rat bone following ingestion by mouth of relatively large doses of radium (up to 100 μg) has been well described (Dunlap *et al.*, 1944). Many of these rats developed sarcoma, but radiation osteodysplasia was present in areas where malignant changes had not occurred. There were patchy areas of sterile bone necrosis characterized by an absence of osteocytes in the bone lacunae. Although osteoclasts were rarely found, the necrotic bone usually showed irregular scalloped regions of resorption along its fine margin and sometimes the apposition of new living bone of fibrillar structure.

Fracture. Bones showing the changes of radiation osteodysplasia become brittle and fracture is frequently reported with subsequent healing (Cluzet, 1910; Albee, 1920; Phemister, 1926; Brooks and Hillstrom, 1933; Regen and Wilkins, 1936; Rosenthal and Grace, 1936). The majority of observers suggest that the rate of callus formation is slower than in non-irradiated bone. Phemister (1926) noted that the bone reacted differently if it was involved in weight bearing than when it was not so involved. In the former case the dead bone did not form a sequestrum but was slowly replaced by ingrowing new bone. On the other hand, when non-union of a fracture prevented the bone being used for weight bearing, the necrotic bone formed a sequestrum which was slowly absorbed. Secondary infection, however, led to formation of sequestra regardless of function.

Abscess of the jaw bone associated with necrosis has been reported

in rabbits (Rosenthal and Grace, 1936), and periodontal suppuration associated with necrosis of the jaw bone, in rats, following ingestion of radium (Dunlap *et al.*, 1944).

b. *Clinical Osteodysplasia*

External radiation. Retardation of growth. Therapeutic irradiation over growing bones in clinical practice is known to give rise to retardation of growth. Frantz (1950) described a boy of 16 who showed a shortening of 9½ inches in one femur. At the age of 2½ weeks he received 2,992 roentgens over a short period for treatment of a hemangioma overlying the distal femoral epiphysis. There was also a decrease in shaft diameter and cortical thickness. Transverse dark lines were apparent in metaphyseal bone and two exostoses cartilaginea were also present in the field of irradiation. Murphy and Berens (1952) have recorded shortening of 3 inches in the femur of a child irradiated when 4 months old and measured 12 years later, and shortening of 1 inch in the leg of a child irradiated when 1 year old and seen 14 years later. Retarded growth has been noted in a number of growing bones exposed to irradiation (Bisgard and Hunt, 1936; Murphy and Berens, 1952). A group of 34 children who received external irradiation over the spine for retroperitoneal or paravertebral neoplasm or benign hemangioma and survived for 2–3 years, has recently been studied (Neuhauer *et al.*, 1952). Exposures in excess of 2000 roentgens invariably produced growth disturbances irrespective of the age of the child when treated. The roentgenological findings were:

- (a) Horizontal transverse lines of increased density parallel to the epiphyseal plates resembling the common growth arrest lines.
- (b) Gross irregularity of the vertebral epiphyseal cartilage plates.
- (c) Gross contour abnormalities found only in fields of therapy.
- (d) Scoliosis or lateral curvature causing no functional handicap.

A post mortem on one patient who lived only a short time following therapy showed non-specific growth retardation and irregularity of ossification of the epiphyseal cartilage. In the series benign exostosis cartilaginea appeared to arise with greater frequency than in the normal population. Another group of four children who survived for 10 years or more, showed vertebral damage and unilateral development of the ilium to a varying degree (Whitehouse and Lampe, 1953). Bone growth was also said to have been affected in areas removed from the site of irradiation. The pelvis and the distal femoral epiphysis, it is claimed, were affected by irradiation applied over the proximal portion of the femur only (Stevens, 1935).

Ewing (1926) has described histological changes in the long bones of adults following external irradiation which did not proceed to fracture,

"There is marked thickening of the corticalis, and the new bone is not laid down in lamellae but projects in broad irregular elevations into the marrow cavity which is also partly obliterated by increase in fibrous tissue and bone trabeculae. There is also a thickening of the periosteum, sometimes to as much as 4 mm" (Ewing, 1926). Microscopically such changes pass on to a sterile necrosis associated with the death of osteocytes and osteoblasts and later of osteoclasts. This necrotic bone is characterized by long delay in sequestration and by the resistance of the tissue to solution (Regaud, 1922).

Fracture. Bone showing advanced radiation osteodysplasia becomes increasingly brittle and has a marked tendency to spontaneous fracture. Under favorable conditions such fractures may heal slowly.

The first case of fracture of the femoral neck following external radiation of the generative organs for malignancy was noted in 1927 (Baensch, 1927). McCrorie (1950) collected a further 116 cases recorded in the literature up to 1950 and added 10 of his own. They all occurred in women. Two instances in man irradiated for a carcinoma of the penis are known (Batt and Hampton, 1940; Hight, 1941) and Bonfiglio (1953) mentions three male cases. The interval elapsing between the time of treatment and fracture varies between 4 months and 6½ years. Several instances of bilateral fracture have been noted, although the fractures do not necessarily occur at the same time (Baensch, 1927; Kalayjian, 1938; Strauss and McGoldrick, 1941; Barden, 1943; Gratzek, Holmstrom, and Rigler, 1945; McCrorie, 1950). The left femur is said to be fractured more often than the right (Kok, 1953). The incidence of such fractures in large series of cases receiving irradiation for pelvic carcinoma varies. In 1179 patients, McCrorie (1950) had an incidence of 0.1%. Peck (1939) records an incidence of 2.7% in 1026 cases, Dalby *et al.* (1936) an incidence of 3.2% in 568 patients, Kok (1953) an incidence of 1.45% in 896 patients. Although these figures are variable and there is probably some duplication of cases in the different series, they are higher than the incidence of fracture of the femoral neck in women who have not received irradiation. In a comparable age group this is estimated to be 1 in 4,500 (Dalby *et al.*, 1936). There is no reason to believe that secondary deposits are responsible for the fractures, since in many instances either autopsy (Baensch, 1927; Dalby *et al.*, 1936; Peck, 1939; Strauss and McGoldrick, 1941) or biopsy examination (Peck, 1939; Kok, 1953; Stampfli and Kerr, 1947) showed no trace of tumor tissue, while in others the fractures healed remarkably well (Barden, 1943; Stampfli and Kerr, 1947; Peck, 1939; Bonfiglio, 1953). Nor is it possible to attribute all the fractures to senile osteoporosis. One case at least occurred in a woman of 34 (Stampfli and Kerr, 1947) and many are recorded in women between the ages of 40 and 50, though the average age is about 63 (Kok, 1953). A history of trauma is rare. Further, there are characteristic

features about these post-irradiation femoral fractures not usually found in traumatic fractures.

- (a) There is usually antecedent pain without radiographic evidence of the fracture which takes place later.
- (b) There may be no chronic pain, but sudden acute pain without trauma; a fracture is seen on the radiograph.
- (c) The interval between completion of radiation treatment and the fracture may be considerable.
- (d) If a radiograph is taken before fracture occurs, an irregular area of bone condensation in the femoral neck is often seen.

Fracture following radiation for carcinoma has also been recorded in the ribs and clavicle (Peck, 1939; Slaughter, 1942; Smithers and Rhys-Lewis, 1945) and in the pelvic bones (Peck, 1939; Gratzek *et al.*, 1945) but these sites are much more rarely affected. The fractures may heal well with satisfactory bony union (Barden, 1943; Stampfli and Kerr, 1947) although this is not invariable (Baensch, 1927; Dalby *et al.*, 1936). There has been much discussion as to why the neck of the femur is apparently more liable to radiation osteodysplasia and fracture than bone elsewhere in the body. It is known to be an unsatisfactory anatomical unit liable to fracture. It is possible, therefore, that mild degrees of radiation damage are more likely to result in trauma than in bones less prone to fracture. It has also been suggested that heavy irradiation of the pelvic organs results in occlusion or partial occlusion of the large pelvic blood vessels which are responsible for the blood supply to the femur (Baensch, 1927; Dalby *et al.*, 1936). Diminution in numbers, or absence of osteoblasts and osteocytes and osteoclasts with osteoporosis is recorded in the absence of vascular damage (Stampfli and Kerr, 1947) and may well be a direct effect of radiation. Bonfiglio (1953) who has made an extensive study of the pathological changes in nine cases considers the fundamental lesion is osteoporosis, though he gives no clear account of how the osteoporosis is produced, definitely stating that osteoclastic activity played a minimum role. A "stress" type of fracture then, in his opinion, occurs through an osteoporotic zone. He found adequate callus formation at the site of fracture indicating a good repair response which was in no way different from that seen in non-irradiation fractures, and in adjacent areas he found osteoblastic activity with new bone being laid down on old trabeculae, so accounting for the areas of increased density, seen in radiograms. He found no evidence of damaged blood vessels. Kok (1953) in his study of operation specimens, describes compressed atrophic trabeculae and fragmentation occasionally combined with bone regeneration. Without any definite evidence, he attributes the osteodysplasia to moderate damage to the periosteal vessels and the vessels passing through Volkemann's canals which will interfere with bone nutrition.

The precise pathology and etiology of radiation osteodysplasia is still

obscure. It is, however, important in clinical practice to remember that this condition may occur; it must not be confused with secondary metastases or myelomatosis and treated with further radiation (Dalby *et al.*, 1936; Smithers and Rhys-Lewis, 1945).

Osteomyelitis. Bone devitalized by radiation is susceptible to infection resulting in spreading suppurative periostitis (Ewing, 1926). Regaud (1922) was probably the first to emphasize the relationship of necrosis to the osteomyelitis so often seen in the jaw bones of patients irradiated for oral malignancy. Daland (1949) states dogmatically that teeth and irradiation therapy are not compatible. Teeth that are irradiated show recession of the surrounding mucosa, the blood supply is impaired and pyorrhœa and sepsis follow which will rapidly spread to bone tissue already devitalized by radiation. He quotes the case of a man who had necrosis of the jaw and breakdown of an irradiated wound seven years after successful treatment of cancer of the tongue. No secondaries were found and he explains the disaster on the basis of infection spreading from abscessed teeth which were not removed before irradiation. Whenever deep X-ray therapy is to be directed to lesions of the mouth, teeth through which the rays will pass should first be removed.

In a series of 1,819 patients with intraoral cancer treated with radiation, 235 developed osteoradionecrosis. If the lip cases were excluded, the figure increased to 20%. Early diagnosis should be suspected from the symptoms and the distribution and persistence of the pain. Roentgenograms at this stage rarely show any characteristic lesion (Watson and Scarborough, 1938) but the distribution and persistence of pain should arouse suspicion in patients known to have received irradiation.

External radiation osteodysplasia and dosimetry. Woodard and Coley (1947) have made a special study of the relationship of tissue dose and response in normal bone in a series of patients irradiated for bone growths in which normal bone was also irradiated. The patients were observed over a period of 1-14 years with an average period of 5 years. The authors conclude that a skin dose between 1,700 and 3,000 roentgens affects neither growth nor powers of regeneration, but that above that level effects are noted. The period, however, over which the patients were observed is relatively short. Given a longer period, it may well be that malignant changes will appear. These figures agree well with those of Cowell (1953). Woodard and Coley suggest that the fractures seen in Slaughter's (1942) cases where the dose was estimated to be under 3,000 roentgens may have been due to damage to the deep blood vessels. It must, however, be remembered that it is extremely difficult to give any real idea of the dose delivered to the bone cells from "summatized skin doses."

Internal radiation. Information as to the clinical effects of internal

radiation is almost entirely based on a study of human beings who have ingested radium, mesothorium, or a mixture of the two. Some were given radioactive material for medical purposes (Looney, 1951; Aub *et al.*, 1952), others have ingested it accidentally in the course of their work (Hoffman, 1925; Martland *et al.*, 1925; Martland, 1929).

The clinical picture of the early effects of radium and/or mesothorium poisoning differs markedly from the late effects. The majority of the early cases described occurred in young radium dial painters 4 to 6 years after they had left work, and were characterized by the presence of severe anemias and necrosis of the jaw (Martland, 1929). This was often so serious as almost to completely destroy both jaw bones. It was sometimes associated with bone necrosis elsewhere in the body (Martland, 1929). Such patients had a far heavier skeletal burden of radioactive material than those showing the more chronic lesions (Aub *et al.*, 1952).

It is presumed that this high skeletal content of radium and mesothorium, particularly the latter (see pages 733, 760), affects the marrow cells giving rise to a profound anemia, associated with leucopenia and thrombocytopenia. It is probable (Aub *et al.*, 1952) that the extreme toxicity of mesothorium may be due to the radiothorium produced by radioactive decay which is released to the blood stream and then taken up by the endosteum, where it can more readily irradiate the marrow. Death in such patients results from the blood dyscrasia before bone lesions, other than the jaw necrosis, have time to develop. In individuals with a lower skeletal radioactivity, especially when there is no mesothorium present, the marrow may be unaffected, since the radium is concentrated in a few scattered areas, often deep in cortical bone, and the range of alpha particles is small (see page 730). The cells exposed to bombardment are therefore bone cells with consequent development of abnormalities in bone tissue.

The chronic effects of internal radiation on bone tissue may in their early stages be symptomless and are only found on routine radiological examination of the skeleton (Schwartz, Makepeace, and Dean, 1933; Aub *et al.*, 1952; Looney, 1951). On the other hand, symptoms such as bone pain or spontaneous fracture may draw attention to the skeletal injury (Aub *et al.*, 1952).

The lesions described on radiological examination are:

- (1) Coarsening of bone trabeculae (Aub *et al.*, 1952).
- (2) Areas of increased and decreased density that occur close to one another giving a mottled or moth-eaten appearance. These occur particularly in the head and neck of the femur, in the head of the humerus, the glenoid process of the scapula, and the jaw (Schwartz *et al.*, 1933). Sometimes these areas of

increased calcification are more apparent than real because of their association with adjacent areas of demineralized bone (Looney, 1951).

- (3) Longitudinal areas of decreased density that occur in the shafts of the long bones giving a streaked appearance (Fig. 8) (Looney, 1951).
- (4) Varying degrees of distortion or destruction of normal bone pattern described as "aseptic necrosis" (Looney, 1951).
- (5) Small well-defined areas of decreased density occurring primarily in the skull (Looney, 1951) which may simulate myeloma (Fig. 9) (Aub *et al.*, 1952).

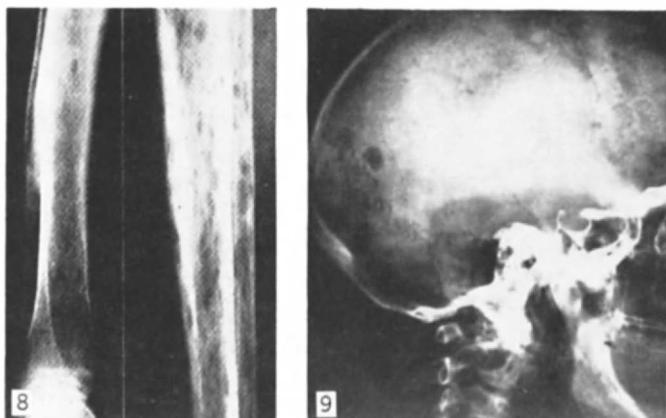


FIG. 8. Radiograph. Radiation osteodysplasia of tibia and fibula. (From Aub *et al.*, 1952. Reproduced by courtesy of the authors and editors.)

FIG. 9. Radiograph. Radiation osteodysplasia of the skull. Note the loss of teeth, atrophy of maxillae and mandible, destruction of temporal bones, and rarified areas in the calvarium. (From Aub *et al.*, 1952. Reproduced by courtesy of the authors and editors.)

Really gross changes of the type described above may probably be accepted as evidence of bone damage. The smaller longitudinal areas of decreased density occurring in the shafts of the long bones giving a streaked appearance, and the mild degree of mottling in the heads of the humerus and glenoid process of the scapula, are certainly far from convincing as seen in some of the published radiographs. It must be remembered that many of the patients described by Looney in 1951 had been in a mental hospital at least since 1930. There is nothing to show that they were not old, bedfast and possibly receiving a far from adequate diet, all of which are factors inducing changes in bone metabolism. The interpretation of the radiographs would be facilitated if control radiographs were available taken from inmates of the same hospital and of the same age who had not received radium therapy.

In addition to these general changes, others of a more specialized nature occur. The damage to the ends of the long bones is especially pronounced in young people who have been exposed to radium while their epiphyses are still active. Closure may always remain incomplete or increased calcification may result giving a so-called "lead line" which is possibly similar in pathology to the growth lines produced by infection or the transverse dark lines following external radiation (Frantz, 1950). Extreme destruction and collapse of vertebrae may occur or collapse of part of a long bone exposed to weight bearing (Aub *et al.*, 1952). Apparent clinical and radiological recovery may occur in patients who have had symptoms which developed after a long latent period (Aub *et al.*, 1952), i.e. bone pain may be lost and the radiographic appearance of the bones become normal. It remains to be seen whether malignant changes occur later. The descriptions of the histological changes in bone tissue in human cases are at present far from satisfactory as inadequate control healthy bone of the same age and from the same site is often not available. The whole relationship of radiation-induced osteodysplasia to neoplasia requires reassessment using the techniques of microradiography, autoradiography, histochemistry, and electron microscopy.

Death of localized groups of osteocytes occurs leaving empty lacunae. There is gross disturbance of lamellar pattern, due to combination of bone destruction and abnormal apposition, associated with irregularity in staining reaction presumably due to changes in collagen and ground substance. Bone necrosis may result which is followed according to Aub (Aub *et al.*, 1952) by resorption of minerals from the dead bone. It is not, however, clear how this mineral resorption is brought about since the osteoclasts are also dead in many areas. In other areas there is increased calcification, possibly due to the fact that osteoblastic function is thought to persist longer than "osteolytic" processes (Warren, 1943). Looney and Woodruff (1953) describe microscopic areas of destruction which are shown in their microradiographs of cross and longitudinal sections. These areas, however, are difficult to distinguish from the normal resorption cavities and building sites always found in bone (Petersen, 1930; Amprino, 1952a; Jowsey, Owen, and Vaughan, 1953). No evidence is given by Looney and Woodruff to enable such a distinction to be made. The fact that the edge of the cavities are in some cases unlined by osteoblasts and osteoclasts may be due to artefact or to damage to the soft tissues. It is essential to compare sections of bone from patients who have had radium with sections taken from exactly the same site in the same bone from a human being of the same age, if misleading conclusions are not to be drawn. The size and distribution of resorption cavities is extremely variable in different parts of the same bone at different ages. (Amprino, 1952a; Vaughan, personal communication).

Hoecker and Roofe (1951) using a combination of autoradiographic and histological techniques showed that radium was found in human bone in numerous microscopic localizations. The radium appeared always to be associated with the optically denser areas of bone and with one particular Haversian system, while in other and adjacent systems there was a negligible amount present (Figs. 10 and 11). This association with

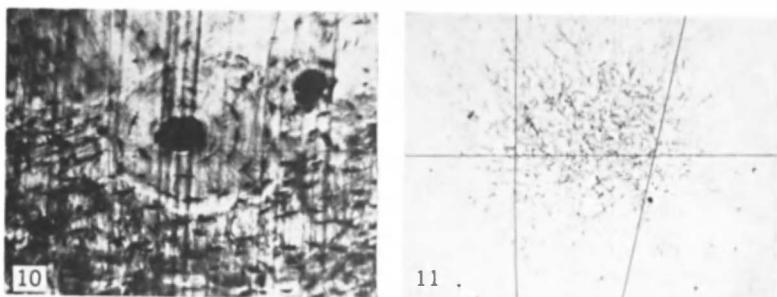


FIG. 10. Photomicrograph of human bone section corresponding to autoradiograph in Fig. 11. (From Hoecker and Roofe, 1951. Reproduced by courtesy of the authors and editors.)

FIG. 11. Photomicrograph of alpha particle track autoradiogram of section of human bone, showing close association of alpha particle tracks with one Haversian system. The distance between points of intersection of horizontal lines with vertical lines is 257 microns. (From Hoecker and Roofe, 1951. Reproduced by courtesy of the authors and editors.)

one system was apparent in both longitudinal and cross sections of bone. The maximum density of radium in these localizations as indicated by the number of alpha tracks per unit area, appeared to vary by a factor of only about 1.5 from one type of bone to another. For instance, in bones removed from one patient it was found that the densities in the periosteal portion of the os pubis was about 1.5 times as great as in the shaft part of the femur. Approximately the same factor applied between the epiphyseal portion of the femur and the shaft. However, in the shaft portion of the femur only about 2% of the Haversian systems contained radium as compared with about 50% in the os pubis. Thus, the average density of radium in the portions of the pubic bone studied is about thirty-five times as great as in the shaft of the femur. In one patient whose total body burden at autopsy was estimated to be 50 μg the concentrations in the Haversian systems in the shaft appeared to be about six times as dense as those in comparable bone from a patient estimated to contain 8 μg at autopsy.

Fracture. The considerable bone necrosis which occurs in advanced radiation osteodysplasia may result in either apparent spontaneous fracture or fracture following minimum trauma. Partial fracture may result

in severe bone pain. The fractures usually heal, sometimes slowly with callus formation and bone deformity. The new callus, in the case of radium poisoning, may be extremely radioactive though adjacent bone may show no radioactivity (Aub *et al.*, 1952).

Osteomyelitis. This occurs most commonly in the jaw bones though it has been described in the temporal bone (Aub *et al.*, 1952). The infective process is probably secondary to radiation effects on teeth and bone in people who already have sepsis present in the gums. Infection spreads readily in necrotic bone. Loosening and loss of teeth is usually the first and sometimes the only symptom (Martland *et al.*, 1925). Once the teeth are removed the jaw usually, although not invariably, heals. The reason for loss of teeth is the destruction of the alveolar crests by alpha and beta radiation following the concentration of radium or radium and mesothorium in the jaw bone with superimposed infection of the bone (Aub *et al.*, 1952). The teeth in patients who show no osteomyelitis of the jaw may have a honeycombed appearance which is possibly non-specific, but finding such appearance in the teeth on routine X-ray—"pink teeth" as they are called—should suggest the possibility of radium poisoning (Looney, 1951). Decreased density of dentin between the red pulp and the outer enamel covering is responsible for the characteristic color.

2. RADIATION NEOPLASIA

The relationship of the bone changes associated with osteodysplasia and neoplasia remains obscure. The former certainly occurs without the latter, but probably neoplastic change is preceded by that of osteodysplasia. The relationship of both to the skeletal burden of radium is discussed later (page 760).

There is a considerable literature covering the production of tumors in rats and mice by both external and internal radiation. Although it must be emphasized that it is extremely unwise to extrapolate results from mice to man, the production of bone tumors following the deposition of radioactive isotopes is of more than academic interest. The bone-seeking radioactive isotopes, other than radium and mesothorium, are the product of uranium fission. In the atomic age they may well be accidentally ingested by human beings following industrial or laboratory accidents or contamination of water and food supplies. Any information, therefore, about their effect upon the skeleton is important, although it must at present be regarded as qualitative rather than quantitative.

a. External Irradiation

Experimental. The first bone tumor produced by X-rays was reported in 1910 (Marie, Clunet, and Raulot-Lapointe, 1910). After a latent period

of 14 months following exposure to soft X-rays, a polymorphous cell sarcoma developed in a rat.

Lacassagne and Vinzent in 1929 produced osteogenic sarcoma in rabbits following exposure to X-rays. The dose was of the order of 1,000 roentgens and the tumors developed after 6–14 weeks. In these experiments associated inflammation was produced since it was thought that the inflammatory process was essential to the production of bone sarcoma (see page 761). Inflammation is, however, not necessary (Ross, 1936; Schurch and Uehlinger, 1931; Lüdin, 1934; Hellner, 1937). Both osteogenic sarcoma and chondrosarcoma have been recorded. In some instances X-rays were used, in others unshielded or shielded radium. Ross (1936) implanted needles containing 0.1 mg of radium shielded with platinum against the spine in rabbits. The latent period before tumors appeared after first exposure to the radium varied from 101 to 135 weeks. The dose is stated to have varied from 1,638 to 2,276 mg-hours of radium. No lesions in the skeleton other than the tumor itself were noted, but there were lung metastases in several rabbits.

Clinical. Thirty-nine cases of sarcoma arising in bone following external radiation have been recorded in the literature. The first sixteen cases followed radiotherapy of tuberculous arthritis (Beck, 1922, 1924; Jaruslawsky, 1922; Marsch, 1922; Baumann, 1927; Backlund, 1930; Denks, 1931; Küttner, 1931; Gruca, 1934; Becker, 1936). The latent period between ending radiotherapy and development of the tumor was 3–11 years. It is not possible to express the dosage in terms of roentgens, but it was clearly high as severe chronic radio dermatitis is described. Norgaard (1939) described a sarcoma arising 9 years after intraarticular injection of 10 µg of radium chloride for chronic arthritis of the knee. Hatcher (1945) mentions three cases. In two, the sarcoma developed in bone adjacent to that previously irradiated for a different non-malignant tumor. Cahan has described eleven cases from the experience of one hospital (Cahan *et al.*, 1948). In all there was microscopic or radiological evidence of the non-malignant nature of the original bone condition, and histological evidence of the sarcomatous character of the tumor which was present in an area included in the radiation beam. The majority died with extensive secondary deposits in the lungs. The latent period varied between 5–22 years with an average of 11.2 years. One case of fibrosarcoma in the mandible 13 years after high radium dosage for gingival epithelioma has been noted (Kaae and Glahn, 1949) and two in the nasal bones 2 and 5 years after treatment for dermatosis (Wolfe and Platt, 1949). Cade (1952) has mentioned two bone sarcomas developing following irradiation of a femoral soft tissue sarcoma and a benign tibial osteoclastoma. Auerbach *et al.* (1951) has reported a case in which an osteogenic sarcoma developed in the ninth dorsal and fourth

lumbar vertebrae four years after abdominal radiation given as a post operation prophylactic measure after removal of a seminoma of the testes (Auerbach *et al.*, 1951). The dose was 4,000 roentgens per field in 70 days given to the abdominal node area. Spitz and Higinbotham (1951) described a somewhat similar case in which the tumor arose in the fourth lumbar vertebra, spreading to the third and fifth lumbar vertebrae. The latent period was again 4 years, the doses of X-ray being calculated at 4,405 roentgens at the level of the eleventh dorsal vertebrae, and 5,289 roentgens at the level of the fourth lumbar vertebrae in 93 days. Jones (1953) has recorded an osteogenic sarcoma arising in the mandible 9 years after radiation for actinomycosis. The bone dose received was calculated to be 6,700 roentgens in 24 months. Of this series of 39 cases, in 13 the tumor arose in previously normal bone, in 17 the irradiation was given for arthritis, usually tuberculous, in 3 for benign giant cell tumor and in 6 for miscellaneous non-neoplastic conditions, such as bone cysts and fibrous dysplasia. The majority occurred in the lower end of the femur and upper end of the tibia.

The ages at which a tumor developed varied from 9 to 62 years, the majority occurring between 15 and 25, while bone sarcoma in the population as a whole has a wider age spread with a double age peak, one between 15 and 25 and one between 45 and 55 (Jones, 1953). Taking the whole series, it is difficult to assess accurately the latent period, as in some of the earlier cases treatment was given over a long period. For instance, in one case (Wolfe and Platt, 1949), treatment was given occasionally over 19 years. Excluding this example, and calculating the latent period as being from the end of treatment to development of tumor, the latent period varied from 3-22 years with a mean interval of 8.6 years. Increasing pain and swelling in the region of a bone previously irradiated should arouse the suspicion of malignant development (Cahan *et al.*, 1948). Radiologically the lesion may not be typical of bone sarcoma although the histological findings differ in no way from those of spontaneous tumors. The tumors in both cases are pleomorphic spindle cell sarcomata with special features that vary from case to case. In many cases described giant cells were prominent, cartilaginous or myxomatous changes were seen and the new bone found was often ill-formed.

The radiation dosage given in the early cases is impossible to calculate satisfactorily. In two of Cahan's cases (Cahan *et al.*, 1948) the dose was as low as 1,500 roentgens. It might, however, be objected that these two particular cases should not be included one followed fibrous dysplasia, a known predecessor of sarcoma, and the other was a chondrosarcoma which followed 17 years after irradiation of a bone cyst. Slow spontaneous development in a benign bone cyst is recorded after 22 years (Jones, 1953). Excluding these two cases, the lowest dose recorded

was 3,000 roentgens, but the majority was higher—4,000–6,000 roentgens—and in one the ethmoids received 25,000 roentgens in 2 years. When the effect of the high atomic number of bone in increasing dose is remembered, it is clear that the bone cells must have been subjected to extremely high dosage. The time of therapy varied from 0.2 to 19 years, but this did not appear to affect sarcoma development.

It is apparent that to some extent at least high dosage is a factor in the development of bone sarcoma following external radiation. The frequent association of sarcoma with the region of the knee joint is interesting. Jones (1953) has pointed out this is the site of maximal post-natal growth. Unfortunately it is impossible to assess the dosage of irradiation given to the patients concerned, since they were treated in the early days of radiotherapy. It is remarkable that so few bone sarcomas have been reported in the thorax and pelvis, both areas which are heavily irradiated prophylactically. The report has been found of one sarcoma only occurring 11 years after prophylactic treatment following a radical mastectomy (Hatcher, 1945). One sarcoma has alone been reported in the head of the femur yet the neck so commonly suffers fracture as the result of radiation osteodysplasia. Secondary metastases to the lung were found in the majority of the cases reported by Cahan (1948) and are reported by other authors (Jaruslawsky, 1929; Beck, 1922; Hellner, 1937).

b. *Internal Irradiation*

Experimental. Sabin and her colleagues in 1932 (Sabin, Doan, and Forkner, 1932) produced osteogenic sarcoma in two rabbits following the injection of radium chloride and mesothorium. The dose given was 5.1 μg of radium and 7.7 cc of mesothorium, and the latent period was 11–19 months.

Alpha emitting isotopes. Transplantable osteogenic sarcoma in rats followed the feeding of 100 μg of radium after an average latent period of 12 months (Dunlap *et al.*, 1944). Bone uninvolved in the tumor process showed radiation osteodysplasia. In some of the rats secondary deposits occurred in the lungs and spleen. Finkel (1953) has studied the effectiveness of radium, plutonium, and uranium-232 in producing tumors in mice following a latent period. The ratios relative to radium of the proportion of mice that developed malignant bone tumors was 7:1 for plutonium and 2:1 for uranium. Two or more tumors per animal were occasionally found following uranium-232 and radium, but were the rule after the most effective doses of plutonium. More tumors are found in the spine than in other parts of the skeleton following injection of plutonium (Lisco, Finkel and Brues, 1947).

Beta emitting radioactive isotopes. Brues (1949) has described radioactive strontium as "par excellence a producer of bone tumors." Tumors in or near bone in mice following injection of Sr⁸⁹, one of the bone-

seeking isotopes, were of several types. Osteogenic sarcomas predominated, but other malignant tumors of mesenchymal and endothelial origin have been observed and for statistical purposes included. Multiple primary tumors occurred with an entirely random frequency, but more frequently with increasing dosage. Another feature of high dosage was the sharpness of the latent period at the end of which tumors developed rapidly. Of the mice receiving 5 $\mu\text{c}/\text{g}$ and surviving 200 days, 35% died without bone tumor and 14 per cent with bone tumors; of the controls, 5 per cent of the 200 days survivors died in the same period. Brues concludes that it is impossible to produce tumors with low doses in short-lived animals, and that the low dosage required to induce radium tumors in man is consistent with the longer period in which tumors may develop than in experimental animals. He suggests, on the basis of his observations, that each quantity of absorbed radiation confers on the tissue absorbing it a probability of tumor formation which is without a limit in time once the latent period is passed; thus the daily tumor morbidity will constantly increase as long as further irradiation occurs. The latent period increases as the dosage decreases.

In an earlier paper presumably dealing with the same mouse population (Lisco *et al.*, 1947) it is stated that the incidence of bone tumors was approximately proportional to the dose administered, and the latent period was in no case less than 200 days. Bone tumors were produced in considerable numbers by doses ranging from 5.0 to 0.05 mc. Sr⁸⁹ per gram body weight, both with single and with monthly repeated injections. Osteogenic sarcoma induced by Sr⁸⁹ and by radioactive yttrium and radioactive cerium are more common in the long bones than in the spine (Lisco *et al.*, 1947).

The relative efficiency of beta emitting isotopes like strontium compared with alpha emitting isotopes like radium in mice is 1:10 on a millicurie basis.

Clinical. In 1929 Martland and Humphries drew attention to the occurrence of osteogenic sarcoma in radium dial painters. Since that time experimental and clinical observations have proved the causal relationship between the presence of radioactive material in the bones and the development of bone tumors (Brues, 1949; Aub *et al.*, 1952). Aub and his colleagues (Aub *et al.*, 1952) who studied thirty patients, the majority of whom sought medical help for the relief of symptoms and who had stored radioactive materials over a long period of time, record eight malignant tumors, five of these bone tumors. Looney and his colleagues on the other hand, who followed up a group of individuals known to have been given radium chloride by intravenous injection, but who had not necessarily sought medical advice, found only one case of malignancy.

The majority of tumors following injection or ingestion of radium are

described as osteogenic sarcomas. One case of giant cell tumor diagnosed by roentgenogram, but not yet confirmed by histology, has been recorded (Aub *et al.*, 1952), and one of fibrosarcoma arising in the knee joint capsule (Aub *et al.*, 1952). In many but not all reported cases, extensive secondary metastases are found in the lungs (Martland, 1931; Aub *et al.*, 1952).

Tumors have been reported in the following bones: the femur and pelvis most frequently, but also in the scapula, ribs, humerus, tibia, jaw, metacarpal, and orbit. Martland (1931) recorded one instance of two primary tumors in the same patient, one in the orbit and one in the pelvis.

The majority of patients with tumors due to internal radiation show radiation osteodysplasia elsewhere in the skeleton. Osteodysplasia is not recorded in association with bone sarcoma associated with external radiation (page 765).

The relationship of neoplasia to dosimetry. The relationship between dose, latent period and tumor development in man following the ingestion of radioactive materials remains extremely obscure (Aub *et al.*, 1951). It is complicated by the fact that some patients, apart from the radium dial painters, have been found to contain mesothorium as well as radium, and mesothorium is probably more toxic than radium because of the character of its decay products (page 733). It must not be assumed that because radium injections were given, contamination with mesothorium was not present. If there is an extremely high skeletal burden of radium, death from blood dyscrasia is likely to occur before there is time for the bone tumor to develop (page 751). Estimations of skeletal burden in Martland's early cases is probably only approximate. One patient estimated at the time of death to have a skeletal radium content of 50 μg died with a tumor 6 years after first exposure—another estimated to have 6 μg died with a tumor after 8 years. Excluding tumors of the jaw, where the previous occurrence of osteomyelitis may have been a complicating factor in Aub's series, the body burden of radium in the patients developing bone tumors varied from 9 μg to 0.8 μg and the latent period from 26 years to 12 years. Mesothorium was also present in some patients. The latent period in the patient with the highest burden was 25 years. On the other hand, all patients in this series had radiation osteodysplasia, though only a few developed bone tumor. Eight patients had a radium content of 8–23 μg ; mesothorium being also present in some patients, the average latent period before symptoms developed due to some form of bone disorder was thirteen years. Twenty-five per cent of patients in this group developed tumors. Nine patients had a radium content of 2–7 μg ; mesothorium being also present in some cases, the average latent period before symptoms developed was 20 years. Thirty-three per cent of patients in this group developed tumors.

Nine patients had a radium content of 0.7–2 μg ; but mesothorium again was present; the average latent period before symptoms developed was 19 years and again 33% developed tumors (Aub *et al.*, 1952). In Looney's series the patient who developed a tumor contained 1.9 μg of radium while a patient of 67 containing 11.4 μg of radium had no tumor.

It is remarkable that osteodysplasia does not necessarily seem to have preceded tumor development following external irradiation (Jones, 1953). One tumor only is reported in the region of the head of the femur where osteodysplasia occurs not uncommonly following external radiation (page 748). The part played by preceding inflammation has in the past been controversial. It was at one time thought to be important since it often followed irradiation for tuberculosis of a joint. But in clinical practice and in experimental animals (page 756) tumors have often arisen with no source of infection and this theory has been abandoned.

3. DISCUSSION

It is clear from both the clinical and experimental data discussed that sarcoma may develop in bone following both external and internal irradiation. In the case of external radiation, the latent period hitherto recorded has been on the average shorter than the latent period recorded as developing after internal irradiation. Tumors following external irradiation have developed in the majority of cases after a dose greater than 3,000 roentgens. As already mentioned (page 757), it is extremely difficult to estimate the dose received by the bone cells from "summed skin doses." The dose of internal radiation necessary for tumor development appears to be variable, the lower limit being extremely small. The extreme variation in the susceptibility of individuals to a given dose of administered drug or chemical in the field of toxicology is well known. The same appears to hold good of the response to irradiation and must be remembered in assessing the risks it is justifiable to run, both in the field of therapy and of experimental and industrial investigation.

V. Conclusions

Both external and internal radiation may have remarkable and sometimes disastrous, although long-delayed, effects on normal bone.

In the treatment of malignant conditions of both the bone itself and adjacent tissues, the risk of osteodysplasia developing later is a risk that must clearly be taken, though minimized as far as possible by careful technique.

How far external radiation should be used for non-malignant conditions in the skeleton, except in old people, is questionable. Long-lived bone-seeking radioactive isotopes should not be administered for therapeutic purposes.

The increasing use of radioactive isotopes in all fields of research may well result in accidental ingestion. The possibility of injury due to radiation must not be forgotten in investigation of obscure bone lesions.

This account of the effects of radiation on bone raises more questions than it answers. Until more is known of the normal physiology of bone, there can be little understanding of its pathology. Perhaps the outstanding challenge is to find an explanation of the enormously delayed "latent period" in the development both of radiation osteodysplasia and of malignancy following the retention of minute amounts of radioactive elements.

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CHAPTER XXIV

PATHOLOGICAL CALCIFICATION *

GEORGE M. HASS

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I. Introduction

Calcification in biological systems is a complex process by which calcium salts are maintained at higher concentrations in non-circulating matrices than in regional circulating humoral or other mobile fluids. The principal result of normal calcification is the concentration of calcium and associated inorganic salts in crystalline patterns of similar arrangement and chemical composition in specialized intercellular matrices, all of which may vary among the different species. The net effect of pathological calcification is the concentration of calcium and associated inorganic salts with a greater than normal range in chemical composition or diversity of pattern, not only in these specialized matrices but also in other intercellular, extracellular, and cellular materials.

A discussion of pathological calcification of the specialized matrices in man would require lengthy consideration of diseases of bone, cartilage, dentin, enamel and other normally mineralized structures (Albright and Reifenstein, 1948; Barr, 1932; Bennett, 1953; Reifenstein, 1951-1953). For this reason, the present comments will be restricted principally to pathological deposits of calcium salts in abnormal or unusual locations (Barr, 1932; Wells, 1914). Systemic influences will be purposely minimized and emphasis placed upon the influence on calcification of local factors, especially the local composition of circulating fluids, the local structure of intercellular matrices, and the local activity of cells upon which matrices depend for their formation and maintenance. These

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factors, alone or in combination, may produce a local positive or negative calcium balance in one matrix which need not be connected with the direction of the balance in another matrix, nor need the sum of the balances necessarily be reflected in the more distant metabolic measurements of dietary ionic intake versus output or ionic levels in the blood plasma.

II. Sequences in Pathological Calcification

Under proper conditions an abnormal concentration of calcium may occur in almost any biological matrix. Normally, some matrices readily undergo calcification while others are very resistant. Properties of matrices, which determine their susceptibility to calcification, are elementary in the sequences of the process, whether it is normal or pathological. Perhaps, the most important initial change which favors pathological calcification is the modification of a matrix in the direction of reduced viability without an undue disintegration of the structural pattern of the matrix or enhancement of its vascularity. This applies not only to intercellular matrices but also to those matrices composed largely of cellular structure. The choice of the term "reduced viability" to encompass deteriorative sequences preliminary to increased orders of calcifiability of matrices and cells will be discussed more fully later on.

Once the appropriate matrix is created, the sequences in calcification require a supply of minerals, ordinarily made available through humoral transport and local mechanisms governed by cellular activity (McLean, 1943; McLean *et al.*, 1946). The conditions which are a resultant of the properties of the matrix, the supply of minerals and local cellular activity, may favor rapid concentration of calcium with maximal deposition in the matrix in a few hours. Under slightly different conditions the maximal concentration of calcium within each unit volume of the matrix may require many years. Whether the rate of calcification is rapid or slow, there is probably a continuous exchange of ions between the calcific deposits and the environment (Armstrong, 1952; Reifenstein, 1951-1953). The rate of exchange may be closely related to the rate of deposition so that, other things being equal, the reversal of conditions which favor the rapid deposition of calcium may lead to rapid resorption. An extension of this idea indicates that the continuous ion exchange between calcified matrices and circulating bodily fluids may be active, slow or negligible in accordance with the life history of the mineral deposit. A study of pathological calcification offers evidence to support this concept, but only insofar as relative stabilities of the mineralized matrices are concerned. For instance, certain matrices may undergo maximal pathological calcification in a few days and within a similar time interval the matrices, together with the minerals, may be resorbed.

During this time it may be assumed that ion exchange is very active. Under other conditions maximal pathological calcification of matrices may progress slowly over a period of many months. Ion exchange at the periphery of matrices in close relation to mobile fluids of the extracellular compartment may still be fairly active even though there is no quantitative change in mineralization. With increasing distance from the periphery, it seems likely that there is decreasing ion exchange and, for reasons such as this, the aged secluded pathological deposits of calcium do not participate to a significant degree in mineral metabolism. Neither do the matrices. It seems clear that the probability of these sequestered deposits re-engaging in systemic mineral metabolism will depend upon the stability of the matrices. This situation is different from that of normally calcified matrices but probably not greatly different if due consideration is given to conditions which govern the variable ion exchange rates of normally calcified matrices. Certainly some ions such as radium and lead may remain in a permanent position, incorporated in normal bone matrices throughout the span of a human life. On the other hand, there is likely to be very active ion exchange at surfaces where mineralized matrix is in contact with the mobile intercellular fluids. It may be assumed that, at least under normal conditions, factors operating at these skeletal surfaces will be the major determinants of the level of serum calcium and phosphorus, as well as other ions such as serum lead, if the principal available source of the ion is in relation to the skeletal surfaces (Armstrong, 1952; Reifenstein, 1951, 1952, 1953). Evidence indicates that many grams of calcium can be accepted or released at the extensive surfaces without building up or breaking down any matrix-held apatite crystals. This evidence in relation to osseous matrices does not apply in any established way to pathological deposits of calcium with which we are concerned. It is an easy matter to demonstrate the accumulation of calcium in a matrix but it has not yet been possible to demonstrate resorption of pathological deposits of calcium without other significant local changes in matrices from which the calcium is being removed.

A second sequence in pathological calcification is the attraction of other than calcium or phosphate ions to the matrices in much the same way as in normal calcification. In abnormal calcification, however, the variation in the type, quantity and distribution of the attracted ions and molecules is much greater than in normal calcification. At times, the calcium deposits are almost pure calcium phosphate, urate, carbonate or oxalate. At other times they contain various mixtures of these and other calcium compounds but usually phosphate and carbonate predominate in ratios similar to those in osteoid matrix or bone. Some calcium deposits contain considerable amounts of iron, lead, magnesium, or

silica (Bunting, 1951; Wells, 1914). Rare minerals, such as radium, strontium, and beryllium are occasionally detectable (Reifenstein, 1951-1953). Traces of minerals may influence pathological calcification, though the exploration of this subject beyond the demonstration of effects of beryllium, several heavy metals and radioactive materials in skeletal matrices has been neglected. It is now known that several metals may not only be deposited in skeletal matrices but also that the deposited metals may either interfere with or compete with calcium in the sequences of local mineralization by mechanisms apparently unrelated to those which regulate calcium deposition and resorption. Large amounts of some other minerals such as silver and bismuth may, however, be concentrated in the same matrices as calcium without influencing the precipitation of calcium. The meager evidence indicates that other trace elements, as yet undisclosed in amounts and location, may be responsible for the unexplained variations in the life history, particularly the pathogenesis, stability, and patterns of organization of pathological deposits of calcium.

A third sequence in pathological calcification is the sequestration of the mineralized matrices by mechanisms which treat them as foreign bodies. One mechanism is that of extrusion by which tissues operate in a complex way to guide the calcium deposit to some surface and to extrude it in some secretory or excretory channel from which, with good fortune, it may be evacuated or otherwise disposed of. Without good fortune, it may persist as a nidus for an enlarging concretion. A second mechanism is that of encapsulation which may be accomplished by proliferation of fibrous tissue, by surface epithelialization, or by marginal mobilization of mononuclear or multinuclear phagocytes. Encapsulation by dense avascular and relative acellular fibrous tissue tends to insure permanence of the deposits and eliminate them from systemic influences which govern calcium balance between matrices and their environment. The process of surface epithelialization may assist extrusion, encapsulation, or imposition of special influences of the epithelial cells on the calcified matrices. This is of greatest interest in the collecting tubules of the kidney wherein calcified intratubular casts may be extruded to an interstitial position by growth of epithelium around the cast in such a way as to isolate it and restore continuity of the obstructed lumen. By reversal of this mechanism, calcified interstitial matrices may be extruded to an intratubular position or to the exterior of the body. Encapsulation by endothelial leukocytes or multinucleated foreign body giant cells is ordinarily accepted as evidence of removal of either the mineral deposits or the matrix, or both, by the local enzymatic and phagocytic action of these cells. In some instances, however, these cells perform no detectable function, other than marginal encapsulation. This form of reaction is of

interest because of its resemblance to the osteoclastic reaction which is so conspicuous in rapid resorption of calcified chondroid and osseous matrices. I believe that the reactions are comparable because the mobilization, polarization, and multinucleation of these mesenchymal cells seem to me to be governed principally by the properties of the accessible surfaces of the mineralized matrices. This belief does not exclude the possibility that the action of mononuclear phagocytes may create surface properties which favor the formation of multinucleated cells. It is very likely indeed that the dissolution of intercrystalline protein matrices would expose sufficient crystal surface in breadth and depth to induce multinucleation. The same argument may be used in defending the idea of primary dissolution of minerals, leaving extensive porous protein surfaces to induce multinucleation. In general, however, phagocytic cells do not attack autologous proteins unless they have been extensively damaged.

The fourth sequence in pathological calcification is related to the processes of organization and conversion of normal calcium deposits. One fascinating biological device in skeletal growth is the insurance of the continued formation of bone by the sequence of proliferation, reduced viability, and calcification of cartilage. In this instance "dying" calcified cartilage matrix depleted of fully viable cells is invaded by vascularized mesenchyme which displays an osteogenic function where it comes into contact with the matrix (Bennett, 1953; Follis, 1950). The differentiation of the mesenchyme to osteogenic tissue may be partly due to the inductive influence of calcified cartilaginous matrix which appears to be a local stimulus and source of materials for the construction of bone. Pathologic deposits of calcium in diverse locations, not necessarily related to the skeletal framework, often stimulate the operation of similar mechanisms of conversion and organization. The final product of this sequence which involves mesenchymal penetration, vascularization, resorption of the calcified matrix, occasional chondrogenesis, and osteogenesis is often mature bone, which at times incorporates a marrow filled with adult fat tissue and groups of hematopoietic cells. This heterotopic bone may promptly be resorbed or possess a prolonged life span, during which time it does not ordinarily react to local or systemic conditions in quite the same way as normal skeletal bone and bone marrow.

It is not implied that organization of pathological deposits of calcium by mesenchyme, whether vascularized or not, results in formation of bone. Actually, the processes are very complex and in their totality encompass all observed sequences in the reactivity of stroma in formation and dissolution of any tissue which either normally calcifies or any tissue which undergoes modification in the presence of matrices which

normally calcify. Therefore, the accumulation of mineral deposits, rich in calcium, in extraskeletal locations may instigate such remarkable sequences of reaction that one is inclined to suggest that similar reactive sequences in skeletal locations may be primarily related to local mechanical-matrix-mineral balances and secondarily to activities of highly specialized cells in the neighborhood. Furthermore, these complex sequences, wherever they occur, follow stromal penetration which without vascularizing penetration has limited capacities beyond the sequence of chondrogenesis. The stimulus, whatever it may be, to vascularizing penetration of calcified matrices is the key to the general result. If vascularization is conspicuous and rapid, the calcified matrices are usually resorbed. If vascularization is absent, the calcified matrices tend to persist. If vascularization is in just the proper amount, rate, and location, the calcified matrices resorb as osteogenesis proceeds nearby.

The preceding comments have indicated that there are some curious differences in the sequences by which the tissues react to normal and pathological deposits of calcium. It would seem that these differences are largely due to similarity of composition and location of normally calcified matrices and the dissimilarities in composition or location of abnormally calcified matrices. In each instance the composition or location of the matrix is probably no less important than the chemical structure and orientation of the mineral deposits, especially in determining the pathogenesis of stromal and vascularizing sequences.

III. Relations Between Electrolyte Imbalance and Pathological Calcification

There is good evidence that pathological and normal calcification may be regulated in many instances by the same fundamental factors. Sites undergoing calcification must be adequately supplied by the necessary ions, so that any consideration of electrolyte imbalance in its relation to pathological calcification should include an appraisal of the concepts of solubility products of the involved ions (McLean, 1943; McLean *et al.*, 1946; Reifenstein, 1951-1953). This appraisal should account for at least three accepted observations. One is that all normal tissues are not equally calcifiable in the presence of the same plasma for if this were so, we would all be converted to lithopedions *in utero*. The second is that tissues which do not normally calcify may undergo calcification, while fully calcified normal tissues in the same region are undergoing resorption. The third is that a tissue which does not normally calcify may undergo changes which favor the local concentration of calcium salts, unassociated with any recognized changes in the ionic composition of the plasma. Once the calcium salts have been deposited, they may or may not respond to those influences in the plasma which affect normally

calcified tissues. These observations do not exclude the possible participation of ion products or solubility products in the mechanisms of calcification. They do support the idea that, if calcification is regulated by solubility products of calcium, phosphate, and other involved ions, the values for plasma should be less significant than the values for the matrices in which the minerals are being deposited. In any case, a clear distinction should be made between hypothetical solubility products and true solubility products, the latter probably being indeterminate in the complex systems under consideration.

Many factors are involved in the formation and maintenance of a mineralized matrix. Whether the factors are humoral or local is a matter of some importance but in general a better understanding of the processes will depend upon an eventual analysis of local factors. Therefore, in a consideration of the relations between electrolyte imbalance and pathological calcification it may be convenient to look beyond the general over-all metabolic treatment of positive and negative balance (Albright and Reifenstein, 1948). The general concepts of positive and negative balance should be reduced if possible to local concepts, for local factors may not only create the imbalance but they in turn may also be responsive to it. This local view of regulation of ionic movements and response to imbalances does not by all means exclude the importance of over-all ionic balance, circulating hormones, and other more general or distant matters. On the other hand, it accepts the importance of these matters insofar as they exert influence on local conditions and emphasizes the local conditions receptive to the influence.

Conditions which lead to a positive calcium or phosphate balance between the blood and its normal sources for these ions may lead to a disease known as metastatic calcification (Barr, 1932; Mulligan, 1947). The most important conditions are those which tend to increase the amount of either calcium or phosphate or both in the serum by resorption of bones and retardation of excretion or enhancement of absorption of the ions. The most important elective sites of deposition of calcium salts are in certain extraskeletal tissues which have a "tendency toward alkalinity" (Wells, 1914).

The absorption of bone matrix and minerals in different cases of metastatic calcification may be due to wholly unrelated pathological processes (Barr, 1932). It may occur as the result of locally destructive diseases, such as metastatic carcinoma, multiple myeloma, leukemia, or widespread osteomyelitis. It may occur as the result of conditions which do not directly invade and replace the structure of bone. Among these, sarcoidosis with or without osseous lesions, immobilization of the skeleton, idiopathic osteoporosis, chronic renal disease, hypervitaminosis D, or hyperparathyroidism may be important. Finally, combinations of these

different conditions may operate toward the same end. In this situation metastatic calcification is most likely to be conspicuous and certain vicious cycles become significant. For instance, primary hyperparathyroidism, due ordinarily to an excess of parathormone secreted by a tumor of a parathyroid gland, is usually characterized by a positive calcium balance in the direction of the plasma and urine, a negative balance in the direction of bone, and essentially normal balances in the direction of other fully calcified tissues such as enamel. The positive balance in the direction of the plasma often but not necessarily leads to a hypercalcemia and this in turn to a hypercalcinuria. The increased calcium concentration in the urine enhances the tendency for calcium deposits to occur in the peritubular stroma and protein matrices within the tubules or renal pelvis. This formation of calculi in the excretory channels predisposes to hydronephrosis or pyelonephritis and decreased renal function. The reduced renal function may lead through phosphate retention to hyperphosphatemia and a depression of the serum calcium. This imbalance may stimulate the secretion of more parathormone and eventually induce an hyperplasia of the parathyroid glands. The secondary hyperparathyroidism may further aggravate the primary disease. The fatigue, bone pain, pathological fractures, and other debilitating aspects of the illness may lead to skeletal immobilization, which further favors resorption of bone with accompanying intensification of electrolyte imbalance. Other secondary factors, such as hyperchloremic acidosis, hypochloremic alkalosis, administration of certain vitamins, and electrolyte imbalances created by an excess or deficiency of dietary electrolytes may intensify or minimize the tendency to local or disseminated metastatic calcification.

The resorption of bone associated with local destructive diseases or primary hyperparathyroidism is the most important common factor in most human cases of metastatic calcification but it is to be remembered that most of these conditions frequently lead to an impairment of renal function. Ordinarily, impairment of renal function alone does not lead to metastatic calcification but in some cases, the only important recognized factor is a disturbance in renal function (Follis, 1950). The type of renal disease varies and is usually encountered in children or young adults. It is a chronic disease characterized by progressive renal failure, high NPN, low CO₂ combining power, anemia, hypo- to hypercalcemia (6.7–13.6 mgm %) and hyperphosphatemia (5–16 mgm %). Most patients have enlargement of all parathyroid glands and osseous lesions described as osteitis fibrosa cystica, renal rickets, or osteoporosis. It is generally believed that the renal disease is primary in these cases. The renal disturbance leads to a decreased phosphate excretion. The elevation of phosphate in the blood favors a reduction in the level of the blood

calcium. This leads to stimulation of the parathyroid glands, increased excretion of hormone, hyperplasia of the glands, and absorption of calcified bone as well as interference with mineralization of osteoid tissue. Disseminated metastatic calcification accompanies these processes. There is some evidence that hyperchloremic acidosis or hypochloremic alkalosis may bring about similar trends but, though renal calcification may be conspicuous, extrarenal mineral deposits so characteristic of disseminated metastatic calcification are seldom found.

Another condition which may be responsible for metastatic calcification is hypervitaminosis D (Reifenstein, 1951-1953; Smith and Elvolve, 1929). This has been described in adults though it is more common in children. The mechanism apparently involves a positive plasma and urine balance with a negative bone balance of calcium and phosphorus due to an increased absorption from the intestine, increased urinary excretion, and perhaps some osteoclastic bone resorption but the exact action of vitamin D in excessive amounts is unknown.

Typical metastatic calcification due to dietary factors alone has not been described in man. In animals, however, the syndrome has been produced by an acid diet followed by an alternating acid and alkaline diet supplemented with adequate amounts of calcium and especially phosphorus (Dreyfuss, 1927). The mechanism involves the opposing effects of acid and alkaline diets. Acid diets favor a positive plasma calcium balance by increasing the absorption of calcium from the intestine and mobilization of calcium from the bones. Alkaline diets have opposite effects.

The pattern of distribution of the deposits of calcium in metastatic calcification is about the same, irrespective of the differences in conditions responsible for the syndrome (Mulligan, 1947). The first and often the most conspicuous deposits are in the kidneys (Anderson, 1939). Here, the cells and basement membranes of the convoluted tubules, Henle's loop, and collecting tubules are the principal elective structural sites for calcification. Less often the intertubular stroma, basement membranes of glomeruli, and walls of small blood vessels are involved. The formation of calcified casts in the lumina of tubules and the extrusion of these casts or calcified intertubular stromal elements into the renal pelvis and calices create conditions which favor the development of calculi of much larger dimensions in the major excretory channels of the kidney.

A second location of the deposits is in the lungs. Here, the principal elective site of calcification is in elastic tissue of alveolar walls and pulmonary veins. The elastic tissue of the bronchi, pulmonary arteries, and interlobular stroma is seldom affected.

A third common location of calcific deposits is in the cardiovascular

system. The principal site of cardiac calcification is in the elastic tissue of the endocardium of the left auricle. The systemic arterial system, usually not seriously affected, has a variable amount of calcium deposited ordinarily in the internal elastic membrane and subjacent mural elastica. Occasionally, there is calcification of the entire media of muscular arteries and arterioles.

The most unusual location of calcium deposits is in the connective tissue stroma which supports the acid-secreting glands of the fundus of the stomach.

Calcific deposits occasionally are found in other rare and unusual locations but without sufficient regularity to justify comment.

Wherever the deposits have occurred, chemical analyses have indicated that they are usually composed of mixtures of calcium phosphate and calcium carbonate in variable proportions far beyond those encountered in normally calcified matrices. At times the deposits are almost entirely calcium phosphate and at other times, especially in the lungs, calcium carbonate is predominant. Often, iron is conspicuous, being easily demonstrated either focally or diffusely distributed throughout the deposits.

The distribution of calcific deposits in metastatic calcification associated with different conditions is similar but not identical. The similarities are greatest when the pathogenesis involves destructive diseases of bone or chronic renal disease. In cases with hyperparathyroidism calcific deposits in unusual locations are more commonly encountered. For instance, calcification in the heart is more conspicuous at times in the left ventricular muscle than in the left auricular endocardium. Also, in hypervitaminosis D, arterial calcification exceeding that encountered in other forms of metastatic calcifications, occasionally occurs. Some other differences in amounts, as well as distribution, might be enumerated but they are so inconstant that obscure multiple systemic and local factors involving tissue viability are probably involved.

Experimentally, the syndrome of metastatic calcification has been produced in several animal species. The syndrome produced by excessive doses of parathormone is similar to that encountered in human hyperparathyroidism. Similar syndromes have been produced by administration of large doses of vitamin D and by experiments in which alternating acid and alkaline diets containing adequate amounts of calcium and especially phosphorus were used. The principal difference between the distribution and sequences of calcification in the various experiments is related to variable interpretations of the factor of tissue viability. The consensus is that degenerative and at times inflammatory changes precede calcification in experiments with parathormone and vitamin D. In the dietary experiments, however, the evidence indicates that calcium is deposited in fully viable tissues which do not normally calcify.

This syndrome, therefore, illustrates the general relations between disseminated pathological calcification and systemic electrolyte imbalances which tend to increase the plasma concentration of one or more ions directly implicated in the formation of calcium deposits. Among these ions calcium and phosphate are most important, the latter probably more so than the former. The increase in the plasma concentration may be due to one or more factors, the most important of which are increased intestinal absorption, decreased urinary excretion, and increased mobilization from the bones of one or both of the ions. The increase in plasma concentration of these ions should, according to physicochemical rules, favor the mineralization of bones. On the contrary, the condition favors the simultaneous demineralization of bones, retarded mineralization of tissues which normally calcify, and accelerated mineralization of certain tissues which do not normally calcify. Though the state of viability of the tissues undergoing pathological calcification remains undetermined, it is apparent that the distribution of some calcium deposits corresponds to the region of certain acid-secreting or acid-excreting activities. Furthermore, certain but not all matrices between or adjacent to cells involved in acid secretion rather than the cells themselves seem to have an undue affinity for the minerals. For this reason, it has been proposed that local alkalinity produced by local acid secretion favors precipitation of calcium salts, especially in the lungs, pulmonary veins, left auricle, kidneys, and gastric mucosa. In this connection, even in the local areas, each type of tissue, illustrated by the calcifiability of elastic tissue, basement membranes, and degenerate cells or matrices has its own patricular level of susceptibility to calcification. This serves to illustrate once more that the problem of calcification eventually must be reduced to an analysis of strictly local factors. How, otherwise can the paradox of simultaneous extra-osseous mineralization and osseous resorptive demineralization in metastatic calcification be understood?

Metastatic calcification is more closely related to the systemic regulation of calcium metabolism than most forms of pathological calcification. A careful analysis of this relationship discloses some curious departures from predicted trends in calcification based upon a knowledge of the levels and balances of the plasma electrolytes. Though the levels and ratios among the pertinent inorganic ions may be normal or disturbed, the usual compositions, even when disturbed, would be regarded as favorable in terms of ions and ion products to deposition of calcium salts in normally calcifiable matrices. The reverse is true and there is no better illustration of the fact that the plasma is a two-way street for ionic traffic in which the direction and volume of traffic have different meanings to different matrices in the body.

The first attempt to escape from the dilemma was to propose that some plasma compound was masking calcium or phosphorus so that

they were in effect removed from the metabolic pool of ionic reactions (Reifenstein, 1951-1953). This suppression of ionization in the plasma would naturally stimulate the migration from the skeleton of the required ions so as to re-establish ionic equilibria. A search for abnormal or excessive amounts of appropriate normal materials which might sequester these ions in the plasma was not fruitful. There was no consistent change in the types and amounts of plasma proteins or less conspicuous organic masking compounds such as citrate. Also, it was clear that the percent of ultrafiltrable calcium was not reduced as calcium levels in the plasma were increased to very high levels. The filtrability of phosphate likewise remained unchanged. It still seemed unwise to make exception to the non-biological concept that passive chemical equilibria between the enormous surfaces of skeletal crystalline mineral deposits and interstitial fluids governs the level of serum calcium and phosphorus. It seems true that normal skeletal calcified matrices have many empty or partly filled compartments into which many grams of excess calcium in the serum can pass and from which an equal amount can be promptly withdrawn to support falling serum calcium values. It is assumed that this labile storehouse for calcium is on the surfaces of apatite crystals or in the matrix between crystals where it may possibly reside as a carbonate or citrate inasmuch as these matrix anions will not fit into the crystal lattice of apatite. Presumably, this store of calcium has long since been exhausted in the general case of metastatic calcification, for this syndrome does not ordinarily develop until considerable amounts of bone matrix have been demineralized and resorbed. This involves mobilization of apatite crystals as well as other inorganic and organic constituents of the matrix, so that at least skeletal mineral metabolism must be extended beyond the labile exchange stage before metastatic calcification becomes apparent.

There seems to be no fundamental correlation in the mode of action by which metastatic calcification is produced by so many unrelated conditions. The means by which the positive balance in the direction of the plasma is maintained is unknown. It seems likely that action of parathyroid hormone is mediated through activation of osteoclasts. At the moment parathyroid hormone is a chemically undefined material and the action of parathyroid extract may be due to more than one substance (Stewart and Bowen, 1951). There is good evidence that the substance in the extract which produces phosphaturia is allied to foreign proteins and separable from the substance responsible for elevation of the serum calcium. Whatever the basic mechanism of action, the variable type and magnitude of lesions from place to place in the skeleton in hyperparathyroidism should direct attention to mediation of action by local osseous factors which are not uniform even in structurally identical skeletal areas.

The method of action of physiological amounts of vitamin D is also unknown and the relation of any observed physiological action to the effects of excessive dosage in hypervitaminosis D is speculative. The effects of vitamins in excess or "pathological effects" may bear little relation to their "physiological effects" in proper doses. It is curious that vitamin D is supposed to facilitate normal calcification of cartilage by raising levels of serum calcium and especially phosphorus through increased intestinal absorption. At the same time it increases renal excretion of calcium and increases the tubular reabsorption of phosphate even though serum calcium may be at low levels. Dihydrotachysterol has a similar action and both can be used in the effective treatment of hypocalcemia due to hypoparathyroidism. Also, as the serum calcium rises with vitamin D, the serum citrate also rises (Reifenstein, 1951-1953). If citrate is given, however, to cure human rickets instead of vitamin D, the healing of the rickets proceeds with a depression rather than elevation of the serum calcium. In hypervitaminosis D, however, as serum calcium and phosphorus rise above normal levels, there is no exaggerated normal calcification. On the contrary, a large amount of uncalcified osteoid tissue appears in the metaphysis and osteoclastic activity with resorptive demineralization of fully calcified bone becomes conspicuous. As this proceeds, calcium is deposited in extra-osseous matrices which do not normally calcify and metastatic calcification is the result.

The mechanism by which certain forms of renal disease lead to metastatic calcification is still obscure. As a rule, there is phosphate retention and hypercalcinuria in connection with a hyperchlloremic acidosis. The end result, perhaps mediated through the depressed serum calcium and subsequent secondary hyperparathyroidism, leads to resorption of bones and deficient mineralization of osteoid matrices. In this condition, metastatic calcification is favored, especially by acidosis and hyperphosphatemia, for the serum calcium is often below normal.

Experimentally, the syndrome may be produced by acid diets containing adequate amounts of calcium and especially phosphorus. The acid diets favor increased intestinal absorption of calcium, bone resorption, and decreased mineralization of osteoid matrices. Alkaline diets have opposite effects. Renal disease is not required.

There is no explanation for the high serum calcium and metastatic calcification in sarcoidosis. Though there may be destruction of bone by the lesions of sarcoidosis, this can not be correlated with the disturbances in electrolyte or calcium metabolism. Nor is there any constant endocrine or renal disease. It seems likely that serum proteins may have a bearing on the problem for the partition of calcium in the serum depends to some extent upon the partition of serum proteins and in sarcoidosis this is usually abnormal.

The inconsistencies which disturb attempts to coordinate observed electrolyte imbalances with the behavior of minerals in the various bodily matrices are numerous. It is to be re-emphasized that conditions which favor metastatic calcification tend to increase the amount of calcium and/or phosphorus in the plasma at the expense of bones and retarded mineralization of normally calcifiable matrices. As the levels of calcium and especially phosphate rise in the plasma, minerals accumulate in matrices which do not normally calcify. The matrices which undergo mineralization either stand high in the order of calcifiability under miscellaneous pathological conditions or are related to regions where there is active secretion of acids. Chemists seem to agree that any changes in pH which are liable to occur in the interstitial tissues are unlikely to have any influence on the deposition of calcium phosphate (Reifenstein, 1951-1953). This is substantiated by *in vitro* studies of calcification of cartilage, where reduction or elevation of pH beyond the normal for plasma reduces calcium deposition. The facts, however, are as given in human and experimental metastatic calcification. The distribution indicates that the participation of a local alkalosis in the mechanism of this form of calcification should not be dismissed lightly. The observed calcium deposition is *in vivo*, not *in vitro*, and final conclusions concerning the effects of pH and shifting levels of CO₂ should await clarification of the tolerable ranges of pH in tissues, especially on surfaces of cells and fibrils or at laminar interfaces so conspicuous in renal tubular casts, crystals, and miscellaneous types of matrices in concretions.

The failure to find explanations for normal or metastatic calcification in levels or ratios of inorganic ions in the plasma indicates that other factors must be considered. The question arises as to whether organic ions might be important. Among these, proteins deserve primary consideration. There seems to be no special plasma protein concerned with the binding of the fraction of serum calcium which is not ultrafiltrable or ionized. It is believed that this fraction of serum calcium is normally associated principally with serum albumin. In this regard it is of interest that the serum calcium of egg-laying birds may rise above 500 mg % during calcification of the egg shell. This is a reflection of a conspicuous movement of calcium from either the intestinal tract or bones or both into the plasma. The increasing plasma level of calcium, presumably under hormonal control, seems to be associated with plasma proteins and despite the profound electrolyte shift, "metastatic calcification" is restricted to the specialized matrix of the developing egg shell. Movement of calcium into matrices which calcify under somewhat similar electrolyte imbalances in the human does not occur in the bird. Whether the amounts and types of plasma proteins serve to regulate in some unknown way the events in metastatic calcification remains an intriguing

problem. Often, the levels, types and ratios of different plasma proteins are very abnormal in many human diseases associated with metastatic calcification. This is especially true in chronic renal disease, sarcoidosis and multiple myeloma or other destructive diseases of bone. Perhaps the relation between calcium and cryoglobulinemia, hyperglobulinemia and hyperglycoproteinemia deserves study. Pathological serum proteins may react with calcium in a fashion different from normal serum proteins of the same general class. Apparently, normal plasma proteins continue to bind calcium so that the ratio between the bound and the ultrafiltrable fraction of calcium remains constant as the total serum calcium approaches 20 mgm %. This indicates that plasma proteins can bind a great deal more calcium than they are called upon normally to do. More pertinent data concerning the calcium protein compounds in pathological sera, the reversible limits within which these compounds may form and dissociate and the permeability of capillaries to them are not available. Most of us have long since given up the view that capillaries have the permeability of Cellophane membranes, but the relation between abnormal calcification of matrices and the mobility of normal or abnormal calcium-protein compounds through capillaries need not be discussed here.

Despite the possible role of proteins in the pathological sera of patients with metastatic calcification, chemists have concluded that this direction of study would not be profitable (Reifenstein, 1951-1953). Other organic ions or compounds which might have a strongly reactive relation to calcium in the plasma have displaced the interest in proteins. Of these, citrate has been studied more than any other naturally occurring metabolite in its relation to calcium metabolism. There is a close relation between the capacities of proteins, citrate, polysulfates, natural amino acids, and synthetic amino acids such as ethylene diamine tetraacetate to sequester calcium or in other words to limit its participation in what are ordinarily called ionic reactions. This is accomplished by binding the primary and secondary valences of the calcium ion. This does not necessarily require an organic chelating mechanism but can be accomplished by appropriate inorganic ion interactions, for the theory of chelation was initially developed to coordinate the loosely applied but convenient chemical terminology involving inorganic complexes. However distant the theory of organic and inorganic calcium chelate compounds may be from the position of our current knowledge of pathological calcification, the strict application of the theory will eventually have an important and proper place.

Citrate, tartrate, oxalate, and many other organic compounds may effectively deprive calcium of some or all of its customary ionic reactions. Those organic calcium compounds which suppress these reactions with-

out precipitation can act as carriers of un-ionized calcium quite as well as proteins. Some may differ from proteins in their small molecular dimensions, ultrafiltrability and ready diffusibility. Others may be macromolecular and not filtrable or diffusible even to the same degree as some proteins. These comments should not be taken to imply that metal chelate compounds are permanent and inert. Actually they may be very reactive and unstable. The nature of the organic radical may confer upon the metal very special properties which it wholly lacks as a metal ion and the reverse may be equally true.

There is very little citrate in the plasma but, as in many instances, the quantity at one time in plasma need not be great in order for the compound to be important in reactions requiring a large quantity. Citrate is also present in bone but has not to my knowledge been described in pathological deposits of calcium. There is a tendency for plasma values for citrate to rise and fall with plasma values for calcium. The parallel rise is well-demonstrated following use of vitamin D. The administration of citrate to patients with rickets depresses the serum calcium and healing of the rickets with calcium deposition proceeds despite the movement of the plasma $\text{Ca} \times \text{P}$ value in the wrong direction. Tartrate has a similar effect in human rickets. It has even been suggested that vitamin D is useful in treating rickets because its administration leads to a rise in the level of plasma citrate. The inference here with respect to metastatic calcification is that the distribution of pathological deposits of calcium may follow the distribution of some diffusible organic calcium compound which secondarily attracts phosphate and other metals or ions. It would be of interest to investigate this matter, especially in hypervitaminosis D. In such a study or in consideration of diverse conditions which lead to the systemic pattern of metastatic calcification, it is to be remembered that the rules for absorption, deposition, mobilization, transport and excretion of metal chelates have little or no relation to the rules for corresponding metal ions. Thereby, the spectrum of mineral metabolism is increased immeasurably by this broadened scope of potential metabolic pathways.

We have noted that iron and magnesium may often be demonstrated in large amounts in connection with calcium in metastatic calcification (Bunting, 1951; Mulligan, 1947; Wells, 1914). The incompleteness of analytical data leads to the conclusion that the likelihood of other minerals being present has not been excluded for the mineral ashes have never been systematically analyzed. The amount of iron in some pathological deposits has been so conspicuous that some have concluded that the deposition of calcium depended upon the presence of iron. This subject of mixed mineral deposits whether encountered in matrices which normally calcify or in pathological calcification is of considerable interest, not only from the standpoint of ionic mechanism but also of

matrix affinities and the properties of metal chelates. The chelated metal, whether it is calcium, iron, magnesium or some other metal, may not only undergo active exchange reactions with another metal ion of the same kind but it may also undergo displacement reactions so that its position in the chelate will be occupied by a different metal. It may even retain its position in the chelate and attract or hold another metal ion. A common chelating compound, versene, which is a synthetic amino acid, will bind many metals and most of the metal versene chelates are soluble in water at neutrality. Calcium will displace magnesium, strontium, barium, and radium from their respective versene chelates. Intravenous versene magnesium chelate will promptly reduce plasma "ionic" calcium and increase plasma "ionic" magnesium demonstrable by oxalate (Reifenstein, 1951-1953). In this way, even though the total plasma calcium may not be materially changed, when "ionic" calcium is sufficiently reduced, a cessation in mineralization of osteoid tissue and withdrawal of calcium from the skeleton may occur at once. On the other hand chromium, copper, nickel, lead and cobalt will displace calcium from calcium versene (regular) chelate. Advantage has been taken of the properties of these and other chelates to remove mercury, plutonium, cobalt, lead, iron and nickel from the body. It should be no surprise, therefore, that if metal chelates participate in the movements of calcium in metastatic calcification, they might also implicate other metals so that there may be, at least in theory, some heretofore unrecognized diseases such as metastatic plumbification, ferrification, and so on. The principle of chelation in the strictly inorganic sense is also potentially applicable to the incorporation of multiple metals in variable ratios in the mineralization of matrices in metastatic calcification.

It is to be pointed out that these reactions are not restricted to components in solution but may apply equally to reactions between an insoluble component of a matrix with another insoluble component or with a substance in solution. The rigidity of a structural system or even its dryness does not necessarily preclude ion or atomic movement, displacement and exchange. Thus, the concepts of metal chelates and those of ion exchange, as exemplified especially by reactions of insoluble anion and cation exchange resins, should assist in the eventual description of many events in mineralization and demineralization which have defied description in terms of ion products and supersaturation of plasma with calcium phosphate or other appropriate inorganic compounds.

IV. Relations Between Matrices and Pathological Calcification

The body may be divided for discussion into compartments. The primary compartments are the intracellular and extracellular. The extra-

cellular compartment may be further subdivided into the fluid, colloidal, and fibrillar compartments. The major fluid compartments contain the blood and lymph. The major colloidal compartments contain various concentrations of materials derived from the blood and lymph mixed with non-fibrillary substances, usually of local cellular origin. The major fibrillar compartments contain various types of fiber structures surrounded by the other cellular and extracellular compartments of the region.

The matrices of the body consist principally of the colloidal and fibrillar compartments so that they represent regions of complex admixture of varying proportions of material derived from plasma, lymph, and local cellular activity. Hence, the matrices are subject to modification through changes in systemic or local factors which regulate the composition of the plasma, the lymph or the activity of the cells concerned with the formation and maintenance of the matrix.

Normally, matrices which undergo calcification seem at first glance to have very little in common though it is true that the matrices of cartilage, bone, dentin and cementum are basically collagenous and fibrillar. Furthermore, the fibrillar collagenous matrices are imbedded in a homogeneous colloidal protein complex of considerable density. Also, each matrix contains variable amounts of acid mucopolysaccharides often emphasized as important components of matrices which readily calcify. There are other similarities, especially between osteoid and chondroid matrices. Basically, however, the principal difference is that a normal chondroid matrix is, with due consideration for its distance from a blood supply, *not* readily calcifiable, while a normal osteoid matrix is very readily calcified. This may be surprising to some who have used normal hyalin or fibrous cartilage in non-vital physicochemical studies of calcification. It is to be emphasized, therefore, that calcification under biological conditions occurs readily only in abnormal cartilage (Wolbach, 1937). The abnormality in the cartilage is manifest either by changes indicating reduced viability of the cells or disturbance in the integrity of the matrix. More often than not, these changes in cells and matrix occur together so that it is difficult to determine which is primary or secondary.

This behavior of cartilage directs attention to the factor of viability which is fundamental in so many aspects of pathological calcification. Pathologists have recognized for many years that calcification often occurs in "dead" tissues, whether the tissues are autologous, homologous or heterologous. Some of the best examples of calcification may be noted in homologous tissue transplants or heterologous intracellular Trichinae which have become non-viable. On the other hand, "dead" tissue may not calcify at all even over a period of many weeks or months. There-

fore, the concept of viability, not only of cells but also of matrices, must be examined if we are to gain an adequate insight into the calcification of cartilaginous and other abnormal matrices. As yet, no one has proposed an acceptable definition of what is meant by a viable or dead cell and a viable or dead matrix. Until such a definition is made, a search for factors which regulate the calcification of matrices might properly include attempts to determine the mutual changing relationships between matrices and the cells in connection with them. As a beginning, the point of view may be taken that matrices tend to deteriorate and disappear, with or without mineralization, if the cells concerned with their formation and maintenance also deteriorate and disappear. Under these conditions the disappearance of the matrix is ordinarily retarded by and may be entirely prevented by the process of mineralization. Demineralization of the matrix is ordinarily then followed by a continuation of the process of resorption previously retarded or prevented by mineralization. In other instances, resorption of the minerals follows or accompanies the disappearance of the matrix which is apparently responsible for attracting and holding them. This point of view does not account for the common situation in which matrices tend to deteriorate and disappear, with or without mineralization, even though cells concerned with formation of the matrices retain full viability. In this situation the matrix usually has acquired an essentially independent status, having differentiated beyond the point of dependence upon the cell for its maintenance. Deterioration of matrices of this kind may have no effect upon the cell or only fortuitous effects. More frequently the deterioration tends to stimulate the cell to produce an entirely new supply of matrix to take the place of that which is undergoing deterioration and mineralization or resorption.

The principal question to be answered is concerned with the distinctions between matrices which are elective sites for calcification and those which do not attract calcium salts during deterioration. A cursory inspection of the differences in these matrices leads to the tentative conclusion that the preservation of surfaces is important. This is quite the opposite of the usual viewpoint in which hyalinization and homogenization of structure are emphasized as favorable to calcification. The best examples of pathological hyalinization and homogenization of structure occur in amyloidosis and arteriolarsclerosis. Calcification is not characteristic of either of these conditions so that the homogenization of matrices either by fusion of fibrillar structure or obliteration of interfibrillar fluid compartments by the accumulation of occluding materials is not, in itself, a specific physical determinant of calcification.

Hyalinization and amyloid degeneration are generally interpreted as evidence of deterioration of matrices. In each instance the principal con-

stituents responsible for the physical change in the direction of homogenization are proteins. Other elements also may be encountered in increased amounts in matrices which are undergoing these or other forms of deterioration. Emphasis has often been placed on the accumulation of mucinous substances as significant in the course of deterioration and calcification of matrices. As a rule, it is assumed that the mucinous substances are combinations of proteins and acid mucopolysaccharides of the type normally found in connection with the matrices of bone, cartilage, and connective tissues in general (Gersh and Catchpole, 1949). Some have therefore concluded that acid mucopolysaccharides of the glycoproteins which may accumulate in matrices during deterioration, lend to these matrices some significant chemical relationship to chondroid or osteoid matrices—and calcification is thereby favored. This idea has some histochemical support and some indirect support from *in vitro* studies of the ion exchange properties of cartilage (Boyd and Neuman, 1951; Reifenstein, 1951-1953). The argument is as follows. Calcifiable tissues are readily calcifiable even in the apparent absence of enzymes. According to interpretation of metachromatic staining reactions, acid mucopolysaccharides in the matrices of cartilage and bone are related temporally and spatially to calcification. Columns of calcium-free costal cartilage *in vitro* bind equivalent quantities of calcium, barium and sodium. Furthermore, the principal cation-binding constituent in these columns is the chondroitin sulfate of the matrix. The binding of phosphate is conditioned by the amount of calcium already bound. As the amount of phosphate is increased, calcium phosphate is apparently precipitated in the matrix and the chondroitin sulfate will again bind calcium and the process repeated. Studies of this kind lend theoretical support to the significance of acidic tissue polysaccharides in calcification. However, generalizations on the basis of such impressive data are not in accord with the pathological observations of the regulation of calcification by mucins. Some tissues which normally have a high content of mucopolysaccharides have no greater tendency to calcify under pathological conditions than tissues which have little or no content of these substances. Actually the evidence that calcified bone matrix contains a significant amount of acidic mucopolysaccharides of the polysulfate variety is very meager (Logan, 1935). Also the dependence upon metachromatic staining to define the temporal and spatial connections between calcium deposition and chondroitin sulfate in osteoid tissue is unjustified. Further quantitative analytical data in these areas are badly needed. There is evidence that normal and pathological calcification of cartilage is accompanied by some major changes in the composition of chondroitin sulfate or its displacement from the matrix (Hass, 1943). In this connection, the chondroitin sulfate

content of ageing costal cartilage is depleted in rough proportion to the accumulation of calcium. The mucinous products elaborated by epithelial or mesenchymal tumors do not readily calcify unless the cells engaged in the elaboration of these products are rather well differentiated in the direction of types of cells, normally productive of matrices that calcify. Amyloid, with due regard for its properties, its common low cellularity and its limited vascularity, may actually be regarded as refractory to calcification even though certain forms of amyloid contain according to my analyses 1-3% of sulfate-bearing acidic polysaccharides similar in composition to chondroitin sulfate of osteoid and chondroid matrices.

The second homogenizing pathological process defined by the descriptive term, hyalinization, is a chemically nondescript process. Some forms of hyalinization occurring in certain locations and affecting certain tissues under certain conditions favor calcification. But it is wrong to take the position that hyalinized matrices are in general readily calcifiable. Some hyalinization is due apparently to intrinsic chemical changes in normal matrices, especially fibrillar matrices. These changes may affect the fibrils themselves, the substance between fibrils, or both. In general, when the predominant change is in the microscopic appearance and staining properties of fibrils or other similar structural elements, calcification is favored. Also, when the substances which accumulate between the fibrils are relatively rich in lipids, calcification is favored.

It is generally believed that lipids in one form or another may be among the several possible determinants of the location of calcific deposits and even participate in the mechanism of precipitation (Wells, 1914). This idea arose originally because of the tendency for calcium salts to be concentrated in areas of necrosis of adipose tissue. It was assumed that calcium and magnesium ions were attracted to the fatty acids liberated by hydrolysis of the glycerides and thus formed local concentrations of insoluble calcium and magnesium salts of fatty acids. Actually, calcium is seldom concentrated in significant quantities for prolonged periods in areas of fat necrosis, except under unusual conditions resembling those which favor persistent calcification in other types of tissues. Other evidence against the proposed mechanism is the demonstration that calcium salts of fatty acids are absorbed rather than converted into local deposits of inorganic calcium salts in the interstitial tissues (Wells and Mitchell, 1910). Our experiments in which numerous simple and complex, natural and unnatural oils and esters of fatty acids were injected into the subcutaneous tissues failed to disclose any tendency for calcification to occur. Many derivatives of these compounds accumulated locally in the tissues as the result of hydrolysis, oxidation, and polymerization. Even the most complex and persistent derivatives, which often combined locally with tissue proteins to form continuous

membranes at tissue-lipid interfaces, did not attract calcium in recognizable amounts. Nor did the incorporation of iron in these membranes stimulate the subsequent or simultaneous deposition of calcium salts.

The best evidence for the participation of lipids in calcification is related to the frequency with which calcification occurs in the neighborhood of lipids which either complicate or participate in the process of hyalinization. Our studies have indicated that lipids may be directly connected with the process of hyalinization and lose their histochemical or perhaps even chemical identity as they become locally incorporated in the interstitial tissue. It is in this kind of a process where lipids derived either from the cells or the plasma seem to favor calcification. Within the range of microscopic resolution, the deposition of the calcium salts under these conditions seems to occur, first and foremost, at the interface between small lipoid droplets and the regional hyalinized fibril or interfibrillary colloidal substrate at a distance from viable cells. Perhaps, the principal role of the lipids under these conditions is purely structural, that is to create a surface which serves as an elective locus for calcium deposition in the same way as other surfaces such as those fibrils. On the other hand, the interface may be the locus of concentration of a phosphatide component. If so, calcium ions might be attracted thereto, and a local disturbance in equilibrium in favor of calcification created by hydrolysis of the phosphate ester bond. Enzymes capable of splitting this bond are present in many tissues.

The association between hyalinization, lipid accumulation and calcification is most conspicuous in man in the walls of arteries undergoing athero-arteriosclerotic changes. Similar changes may be produced experimentally. If the aortic wall of the rabbit is devitalized by freezing, the concentration of lipids and hyalinization of new or old collagenous matrices are not associated with any appreciable degree of calcium deposition. Therefore, though the participation of lipids in a process of hyalinization may be demonstrated, there is no consistent relation between these events and calcification. Wherever calcification occurred in these experiments, it was restricted principally to surfaces as previously discussed.

It is possible that surfaces of this kind may play an undisclosed part in the calcification of cartilaginous matrices. In this regard, more attention should be given to fibrils and to the refractile granules in chondroid matrix at the zone of provisional calcification. Metachromasia which represents the assumed location of chondroitin sulfate and histochemical phosphatase is a property of the fibrils of the matrix. Some unpublished work has indicated to me that the granules between the fibrils in the calcifying zone may possess a lipoid component, despite the fact that the total lipoid content of cartilage is very small and probably largely intracellular.

It may be that accumulation of calcium salts in some hyalinized and otherwise condensed matrices is to be attributed to non-specific effects accompanying an occluding concentration of protein in the colloidal interfibrillar or fluid compartment of the matrix. Proteins will bind calcium as well as other ions, especially on the alkaline side of the iso-electric point and the complexes formed are not necessarily reversible. Still, the evidence for protein fixation of calcium followed by prompt release to react locally with phosphate and carbonate as an important mechanism in progressive calcification is lacking.

Although we ordinarily think of enzymes in terms of intracellular activities, there is plenty of evidence that enzymes may be secreted by cells and act only under extracellular conditions. The search for enzymes in normal matrices has been confined largely to histochemical attempts. If histochemical interpretations are accepted, phosphatase at least may be demonstrated in matrices especially in connection with collagenous fibrils. This enzyme is especially conspicuous in the matrix of cartilage and it is regarded by some as important in calcification. The enzyme, however, is difficult to demonstrate in the matrix of osteoid tissue except in connection with the osteocytes and osteoblasts or their cell processes.

There is still a great deal to be learned about the specific localization of enzymes in matrices. Certainly, either normally through synthesis and secretion or pathologically through death and disintegration, a cell may contribute active enzymes to the matrix into which it disintegrates. Whether enzymes, so acquired, might undergo autocatalytic regeneration outside of cells may be doubtful. A few pathologists, however, strongly suspect that many matrices probably contain as many if not more enzymes than blood plasma. The analytical difficulty lies in the segregation of the matrices from cells or cell particulates so that most conclusions must eventually be based upon topographical histochemical work at a microscopic level.

There are reasons for believing that local concentration of inorganic ions, other than calcium, may favor the accumulation of calcium. This belief has arisen because of interpretations placed upon histochemical findings, following the use of methods which are, at best, only qualitative and usually not specific. Pathologists have depended upon certain staining reactions to define the distribution of microscopic deposits of calcium (Bunting, 1951). The most widely used staining reaction, namely the reaction between a matrix and oxidized hematoxylin, does not reveal calcium. The reaction reveals areas which favor the deposition of calcium. It depends partly on the presence of iron but mostly on the properties of a peculiar matrix, normally present in bone and cartilage or in matrices undergoing pathological calcification. A second staining reaction, generally used for demonstrating microscopic deposits of calcium is the Von Kossa stain. The positive reactions of the Von Kossa

method are attributable to anions rather than to cations such as iron, calcium, or other metals in the "calcified" area. There is no proof that iron is essential or important in calcification. Iron is frequently demonstrable in pathological deposits of calcium carbonate and phosphates (Bunting, 1951). The iron may be in ferrous and ferric forms, being much more soluble than the iron of hemosiderin. At times iron is predominant and calcium hardly detectable. At other times, calcium is predominant and only a faint trace of iron is demonstrable at the periphery of the calcium deposits. The evidence indicates that certain matrices which have an affinity for calcium may also have an affinity for iron. There is no proof that iron catalyzes calcification. It seems more likely that iron competes with calcium in the mineralization of matrices, or that matrices devitalized by mineralization with iron become thereby converted to materials with a higher order of calcifiability. A similar view may be taken with respect to other heavy metals. Among these which may fortuitously be incorporated during normal mineralization of matrices, lead, radium, and thorium are perhaps the best examples in human disease. It is doubtful that these scarce or trace elements participate in a significant way in the pathogenesis of extra-skeletal pathological calcification. Nevertheless, the incorporation of these substances in the mineral lattice of calcifying or fully calcified matrices may lead to imperfections which become apparent only after the passage of many years. Despite the lack of positive evidence, there are good reasons for assuming that matrices which attract calcium in pathological calcification may also attract minerals such as lead and radium.

Further consideration of the matrices which attract calcium salts as they undergo changes, loosely defined as due to reduced viability, requires some attention to changes in hydrogen ion concentration. Theoretically, what is generally termed tissue alkalinity should favor calcification and tissue acidity should favor decalcification. Actually, there is no reliable information concerning the acidity or alkalinity of normal matrices, nor are there any critical data which connect calcifiability to a presumed acidity or alkalinity of a matrix. The theory that the distribution of the calcium deposits in metastatic calcification is determined by local alkalinity secondary to acid-excreting or acid-secreting functions of the regional cells may be attractive but it remains unproved. The idea that arterial blood favors calcification and venous blood favors decalcification due to the relative alkalinity of arterial blood may have some merit. There are very conspicuous differences between the apparent order of calcifiability of similar matrices in arterial and venous walls. The matrices of veins or of tissues supplied principally by venous blood have very little tendency to calcify under pathological conditions which

often lead to conspicuous calcification of similar matrices of arteries and of tissues richly supplied by arterial blood. A good example is the refractoriness of the parenchyma of the liver and the walls of pulmonary arteries to pathological calcification.

The influence of the hydrogen ion concentration of matrices is closely linked to concepts of matrices functioning as anion or cation exchange systems. These concepts have not been fully explored but there can be little doubt that some matrices operate as ion exchange systems. The reactive centers in these systems may have relatively fixed position in the matrices and may be influenced by fixed or mobile enzyme systems in the immediate region. Reference has already been made to the study of powdered costal cartilage and the function of the matrix polysaccharides as ion exchangers (Boyd and Neuman, 1951; Reifenstein, 1951-1953). Studies of this type, though non-biological, direct attention to the increasing significance of the chemistry of surfaces of large complex molecular aggregates. These aggregates make up the principal part of structure and, more often than not, a part regarded as structure also participates in function. It seems likely that the understanding of the role of matrices in calcification must await further development of the chemistry of structure (Gersh and Catchpole, 1949).

Pathologists have recognized for a long time that essentially all matrices in the body are calcifiable under appropriate conditions. Furthermore, observations in various pathological conditions indicate that one matrix may have a greater or lesser susceptibility to calcification than another matrix under essentially the same conditions. Our present knowledge will not justify the systematic arrangement of matrices in any specific order of calcifiability because the conditions vary so much in unknown and uncontrolled ways in the body. Nevertheless, the essentials of a specific order exist and no harm can come from an attempt to bring attention to the subject. A few examples may be used. If the wall of a musculo-elastic artery is acutely frozen without anatomical disruption of the general structural pattern, calcium salts are progressively deposited. The first recognizable deposits are in the structural residues of dead smooth muscle cells. Dead fibrocytes, monocytes, histiocytes, leukocytes, and red blood cells are relatively refractory to calcification. Collagen and reticulum are likewise refractory while elastic tissue progressively binds calcium in large amounts. The calcium deposition in the matrices of the structural residues of smooth muscle cells seems to occur first and foremost in the intracytoplasmic granules and along the distribution of myofilaments. Cytoplasmic and nuclear membranes have a lesser affinity for calcium. There is a sort of competition thereafter between the forces of resolution and permanent mineralization of matrices. The forces of resolution of matrices of mineralized cell structure ordinarily

succeed in resorbing much or all of the matrix with a tendency for cytoplasmic and nuclear membranes to persist. As this resolution proceeds there seems to be what I regard as a sort of transfer of calcium from its primary site of deposition to decreasing orders of elective depositional sites in the immediate vicinity. These sites are: first, elastic tissue; second, collagenous tissue and, third, osteoid matrix, if it happens to have been formed by mesenchymal penetration. We find therefore, evidence of a curious and fascinating sequence in a discrete locale in pathological calcification. This sequence calls to mind the possibility that each matrix may possess a characteristic calcification potential and when these matrices are in close union calcium may move from one matrix to another in accordance with their respective potentials.

A second example may be given. In this instance a piece of fascia with attached skeletal muscles is resected and transplanted. The denervated avascular muscle undergoes deterioration. Only those muscle cells adjacent to the fascia accumulate significant deposits of calcium. This is not attributable entirely to preservation of myofibrils in the dead cells because cells elsewhere in the transplant and at its margins retain myofibril structure. Rather, it seems to be attributable to the delay of mesenchymal vascularizing penetration and proteolytic resorption of the subfascial muscle cells. In this connection the myofibrils seem to electively adsorb calcium from the diffusing interstitial fluids, so that a boundary zone of calcification moves slowly through the tissue toward the more distal subfascial cells. The calcium here is bound to intracellular fibrillar matrices but as mineralization proceeds there is no evidence of transfer to sarcolemma or intercellular collagen, neither of which calcify. Competition between resorption and permanent mineralization of a persistent matrix proceeds. In this instance, mesenchymal penetration and vascularization ordinarily supervene so that progressive resorption aided by cells resembling foreign body giant cells is the usual result. Here, we note a very specific localization of calcium in only one of several available matrices undergoing deterioration.

A third example may be given. If cortical necrosis of the kidney is locally produced by measures which do not lead to rapid disintegration of structure, a very large amount of calcium may accumulate in the area of injury. This is particularly conspicuous in the earliest stages in intracytoplasmic matrices of cells which lie in the zone of transition between fully viable well-vascularized tissue and non-viable avascular damaged tissue. In this transition zone there are different degrees of reduced viability of cells as well as matrices. This twilight zone is the zone of the most active mineralization. Presumably, some of the calcium is being supplied by glomerular filtrate which may continue to pass down the lumina of the damaged tubules. The larger part, however,

seems to be supplied by diffusion from the regional capillaries. Here, it is noted that the order of calcifiability of matrices is generally as follows: first, intracytoplasmic mitochondria of convoluted tubular epithelial cells; second, basement membranes of tubular epithelial cells; third, cytoplasmic membranes of tubular epithelial cells; fourth, glomerular basement membranes; fifth, elastic tissue and, sixth, collagenous interstitial stroma. These are not to be regarded as absolute potentials of matrices but rather as calcification potentials characteristic of the sequences of deterioration following a special form of injury. Other types of injury need not necessarily lead to the same result, as indicated in the discussion of mercurial poisoning and metastatic calcification. The principal point is that in an essentially similar environment, matrices display degrees of affinity for calcium which become conspicuous only when some change in the direction of reduced viability is locally produced. These degrees of affinity may be regarded as lending some support to an hypothesis of calcification potentials characteristic for each matrix. Furthermore, the transfer of minerals from one calcified matrix to another in the immediate vicinity is not too difficult to envisage in the final resolution and organization of the initial deposits. The ultimate transfer in this process as in most other locations is into any available adjoining osteoid matrix which, we may assume has the highest calcification potential of all matrices with the possible exception of dentinoid and enameloid matrices. In connection with dental matrices, further analysis is necessary but some data indicate that enamel will not form in the absence of dentin and that the calcification of enamel depends upon the transfer of calcium from dentin (Thoma and Goldman, 1946). Hence, it may be conceded that the enameloid matrix has a higher calcification potential than dentin. If these proposals will bear up under critical investigation, the transfer of minerals among mixed or neighboring matrices in accordance with their respective calcification potentials would remove one more function from among the "vital processes" of regional cells.

V. Relations Between Cells and Pathological Calcification

It is not correct to consider cells apart from intercellular matrices, even for our present purposes. They are usually interdependent. However, it may be proper to focus attention to one or the other major compartment of the body, keeping in mind that they are closely related.

The connection between "viability" of matrices and calcification has been discussed briefly. In this discussion mention was made of the relation between viability of cells and viability of the intercellular compartment dependent upon regional cells for its formation and mainte-

nance. Though we are no more able to define a dead cell or a dead matrix than a live cell or a live matrix, we may approach a definition by postulating that the loss of the viability of a cell is the loss of the sum of its integrated functions and forms. If we view viability in this way, it becomes easier to discuss the vagaries of pathological calcification. Such a view of viability implies that enzymatic activity, normally concentrated in the intracellular compartment and less conspicuous in the matrices of the extracellular compartment, may participate in pathological calcification of tissues.

Phosphatases have been emphasized as important in normal and pathological calcification (Armstrong, 1952). Interest in the role of phosphatases has been stimulated by the demonstration of enzymes of this class in cells which apparently participate in the local mechanism of calcification. It has been proposed that one function of the phosphatases in chondrocytes, osteocytes, and osteoblasts is concerned with reversible conversion of organic to inorganic phosphate. The increased local concentration of inorganic phosphate resulting from this enzymatic action, thereby becomes the important local factor in calcification because of its influence on the solubility product of calcium and phosphate ions. The principal objection to this idea seems to be the discrepancy between the generalized distribution of phosphatases of diverse kinds and the specific distribution of calcium deposits under normal conditions. There is also no well-defined relation in quantitative terms between the biological functions of the phosphatases demonstrated by histochemical methods and those demonstrated by chemical methods. Despite these and other objections, the role of phosphatases in increasing the local concentrations of phosphate ion to a degree sufficient to induce precipitation of calcium phosphate should not be dismissed. Evidence acquired from studies of pathological calcification supports this conclusion to a limited extent. The evidence is strengthened if the idea is accepted that even though a cell may be "dead," the phosphatases which are retained in the residual "dead structure" may continue to perform not only certain synthetic but also degradative functions. These may be bizarre and unregulated functions but nevertheless meaningful in terms of favoring calcium deposition. Though the local concentration of phosphate ions may be the principal function which favors calcium deposition, phosphatases may also participate indirectly in the continued synthesis of matrices which may be quite abnormal. It is possible that activities of this latter kind, though limited in scope and usually in duration under most pathological conditions, may favor calcification. It is generally agreed that energy derived from splitting certain high energy phosphate bonds is necessary in many syntheses concerned with growth and replacement. There is often a concentration of phosphatase

wherever these activities are conspicuous and the concentration of phosphatase is more closely related to the synthesis of matrices that calcify rather than provision of inorganic PO_4 to facilitate calcification. Indeed, the mineral deposit in the presence of a great deal of phosphatase in molluscs is calcium carbonate. It has been suggested that ester phosphate may bind calcium and thereby inhibit calcification. If the esters are degraded by phosphatase, this inhibitory influence is removed. Another idea is that ester phosphate may occlude the motes responsible for crystallization so that phosphatase action may enhance the potential for crystallization of apatite. Whatever the final explanation, it now seems clear that adenosine triphosphate does not directly participate in supplying inorganic phosphate for calcification. Other phosphate esters may be more logically implicated.

There are also reasons for believing that sulfatases may participate in calcification. The action of esterases of this class on the sulfate-bearing acidic mucopolysaccharides such as chondroitin sulfate of cartilage and bone deserves further scrutiny because the correlation between matrix changes in acid mucopolysaccharides and calcification in some instances is very convincing. This correlation seems to be more convincing under conditions which are at least lightly detrimental to the persistence and continued synthesis of the mucopolysaccharides. These compounds, at least in cartilaginous matrices, are very stable chemically so that enzymes, such as sulfatases, which might participate in the degradation of the matrix polysaccharides deserve more attention in the study of calcification. Apparently, however, sulfatases which split sulfate from chondroitin sulfate esters are not conspicuous in mammalian tissues. The absence of enzymes of this class should not divert attention from the problem of removal of the acidic matrix mucopolysaccharides. There is no evidence that they pass from the calcified matrix of cartilage to adjacent osteoid matrix at the epiphyseal line. These compounds must either be degraded, resorbed, or converted to something else prior to, during, or after calcification. There is good evidence that they are not resorbed as such so that the local mechanisms which prepare them for resorption are probably important.

The possible participation of lipases and esterases in the process of normal calcification has not been seriously considered. Certain types of pathological calcification have been attributed to the enzymatic liberation of fatty acids and the formation of calcium soaps, which because of low solubility accumulate locally. Though this mechanism may operate in the formation of calcific deposits at sites of necrosis of fat cells, there is no proof of it. Nor is there any evidence that calcium soaps, if formed locally, will favor the local precipitation of calcium phosphate. The results of all experimental studies on the role of lipases, esterases, and

local accumulation of calcium salts of fatty acids in other than transient pathological calcification are negative.

The formation and disappearance of most matrices which undergo calcification under normal and pathological conditions presumably are governed primarily by mechanisms concerned with the local synthesis and degradation of proteins. These proteins may be fibrillar or without any particular recognizable macromolecular orientation. They may be admixed or conjugated with innumerable substances of wholly unrelated chemical or cellular origins and compositions. They may possess from time to time variable activities conferred upon them by contributions of coenzymes, enzymes, hormones, and other "functional" substances from regional cells or the circulating phase of the extracellular or intercellular matrix. These complexities defy comprehensive analysis but the best evidence indicates that there are some very interesting relationships among the local conditions concerned with synthesis, maintenance, and degradation of certain protein matrices presumably through the action of enzymes which perform these functions. These functions may all be proceeding at the same time in the neighborhood of a single cell. And in the same neighborhood, the matrix may be accumulating, exchanging, and losing bone minerals. In general, however, the view is accepted that demineralization of a calcified matrix does not occur under biological conditions. The minerals and the matrix either are supposed to disappear simultaneously or the disappearance of the matrix is supposed to precede the disappearance of the minerals. The third possibility is that the matrix may disappear, leaving practically no organic residue in connection with persistent calcium deposits. In any event emphasis currently is placed upon the local synthesis and degradation of matrices by local enzymatic cellular action which is responsive to several local and systemic regulations.

Pathological calcification is a reflection of the operation of these mechanisms but not always in the same way or to the same degree. In general, proteolytic enzymatic action which proceeds to the stage wherein structure, even in outline, becomes unrecognizable, is a deterrent to calcium deposition. In other words, liquefactive necrosis of tissues is not conducive to concurrent or subsequent pathological calcification. The type of necrosis most favorable to pathological calcification is that in which minimal overall structural changes in the cellular and extracellular compartments accompany the reduction in tissue viability. This is particularly true in the pathological calcification of cells wherein criteria for viability are usually more definite than in the case of matrices and in many respects the structural components of cells at some still undefined stage of devitalization may be regarded as a part of the extracellular matrices.

Normal calcification requires that intercellular matrices be constructed principally by the conspicuous synthetic activity of presumably normal cells. In the instance of cartilage, the onset of calcification of the matrix coincides with a state of maturation in which activity in matrix formation is reduced and morphological signs of reduced viability of the original cartilage cells become apparent. In the instance of bone, however, the sequences are quite different. The active calcification of osteoid matrix proceeds in the presence of continued active formation of the matrix by cells which show no morphological signs of decreased viability. The fact that these two sequences in calcification are accompanied by two extremes of viability of cells and matrices does not necessarily indicate that the elementary factors concerned in the mechanism of calcium deposition are different. This will become more apparent in the following discussion of the participation of cells in pathological calcification.

There is doubt whether pathologic calcification ever occurs in entirely normal cells or intercellular materials though calcium may accumulate very slowly, as in the ageing of tissues, without any other significant change. This means that pathological calcification is ordinarily preceded by some degree or type of deterioration of cells or intercellular materials. The deterioration may occur in a number of different conditions and calcific deposits may thereby accumulate in the cellular or extracellular compartments.

Traumatic injury to tissues may lead to a state of devitalization conducive to calcification. Among the many forms of trauma, those which do not unduly interfere with retention of cell structure tend to create conditions more favorable to calcification. An excellent example of pathological calcification due to trauma is often observed in muscle, especially striated muscle. This form of calcification is of great interest because of the structural determination of the pattern of mineralization. Other things being equal, the intercellular compartment of partly devitalized muscle is refractory to calcification, and the calcium is electively deposited in the intracellular compartment. Here, it is oriented along the myofibrils principally in relation to the A disks. The elective deposition of calcium salts in these locations may be directed by physical factors alone but it must not be forgotten that the myofibril, as a part of the "devitalized cell," need not have been unduly affected by the process of devitalization. Under these conditions it may not only retain certain enzymes within its structure but also acquire new functional and enzymatic properties which it did not possess in the fully viable cell. Furthermore, certain regions in the myofibril may normally contain calcium or phosphate in such chemical combinations that their ionic interactions are prevented or minimized by equilibria maintained by

integrated vital processes. The same is true of certain enzymes for at least ATP-ase and myokinase are presumably a part of the structure of the myofibril. A degree of loss of viability may involve nothing more than a disturbance in these equilibria and miscellaneous enzymatic functions without any significant modification of either the implicated vital processes or their integration.

In this regard it is still more important to remember that analytical data which direct attention to the presence of much more phosphorus and magnesium in certain cells than in intercellular compartments of the region need amplification. Knowledge of the specific distribution and concentration of these and other elements concerned in pathological calcification of cell structure is probably required before mechanisms and distribution of the process can be understood. This is especially so because of the dependence of so many enzymatic functions upon a particular type and amount of metal "catalyst."

Infections may produce changes which favor calcification. As indicated previously, however, infectious agents which produce a form of necrosis in which the structural outlines of degenerating tissues tend to persist, are responsible for many lesions which calcify. Examples of these diseases are tuberculosis, coccidiomycosis, histoplasmosis, and toxoplasmosis. In general the pyogenic infections and other forms of infections in which liquefactive necrosis is followed by repair with highly vascularized granulation tissue are not favorable to calcification.

There are no important hereditary diseases in which pathological calcification is conspicuous. Among the congenital disorders, there are a few conditions in which the forms of pathological calcification discussed here may be encountered. Rarely, infants for unknown reasons may have calcific deposits in the walls of arteries. Coronary arteries are usually involved and the calcification is usually most pronounced in the region of the internal elastic membrane. In the absence of any metabolic or other etiologic disturbance, it has been suggested that the calcification occurs as the result of defective formation of elastic tissue. So far as is known, this condition is unrelated to fibroelastosis of the heart. In this latter disease, usually occurring in newborn infants or in the first few months of life, the deposits of calcium are usually found in the fibrous or fibroelastic tissue of the thickened endocardium of the left side of the heart. The so-called congenital calcification of the brain is usually due to toxoplasmosis or as an aftermath of intracranial hemorrhage. Perhaps the most common congenital diseases characterized by pathological calcification are related to the blood dyscrasias. In these conditions the deposits of calcium are in the form of congenital cataracts or locally related to iron deposits derived from the destruction of red blood cells. In hemophilia, the pathological calcification occurs usually in the interstitial tissues in locations where there have been repeated massive

hemorrhages which were slowly absorbed. Among the hemolytic anemias, sickle-cell anemia favors calcium deposition in relation to the deposits of iron, which are usually most conspicuous in the fibroelastic framework of the spleen. The calcium and iron deposits together delineate the fiber structure of the splenic stroma, converting many collagenous and elastic fibrils into long fragmented refractile structures which often resemble crystals. Iron, which has the properties of hemosiderin whether it occurs in the hemolytic anemias or in the course of other diseases such as hemochromatosis and transfusion hemosiderosis, is usually not accompanied by calcium in significant amounts. These facts indicate that deposits of iron probably do not, as such, attract calcium. On the contrary, it is more likely that matrices which attract iron may similarly attract calcium, especially when their viability has been reduced by ferrification, and that the iron bound to these matrices is in a different form than that encountered in the usual sequences of hemoglobin deterioration through hemosiderin or other pathways.

The study of neoplasms offers an opportunity to consider the mechanisms of calcification in various ways. Though many hormones exert a direct or indirect influence upon calcification, these influences primarily bear upon special problems of skeletal growth and maturation. These hormonal actions may be accentuated in the presence of benign or malignant tumors which may not only lead to excess secretion but in certain instances to a diminished secretion of the hormones. In this discussion we have elected to restrict our consideration to extra-skeletal pathological calcification. However, a brief enumeration of functional tumors of endocrine glands in relation to pathological calcification may be permissible. Adenomas and the much less common carcinomas of the parathyroid glands may lead to pathological calcification of the type already discussed as metastatic calcification. Basophilic adenomas of the pituitary and its functional counterparts in the form of adrenal, ovarian, or multiple endocrine tumors may be associated with osteoporosis and nephrolithiasis. There is, however, little tendency for the fully-developed syndrome of metastatic calcification to occur. Chromophilic adenomas of the pituitary may profoundly affect the skeletal growth patterns but pathological calcification is not a characteristic result of the function of these tumors. Dwarfism, cretinism, and similar effects due to hormonal imbalances and deficiencies are again principally problems of growth and not of pathological calcification in the usual sense.

A complete understanding of calcifying neoplasms would require something more than a complete understanding of normal or pathological calcification. Neoplasms may not only produce viable matrices which are readily calcifiable but neoplastic cells and matrices may also undergo a variety of deteriorative changes favoring pathological calcification. As

a rule, the deposition of calcium salts in the fully viable matrices of neoplasms occurs in the neighborhood of neoplastic cells arising from or related to cells that normally produce readily calcifiable matrices. The majority of these tumors, therefore, arise in connection with the skeleton, teeth, or pluripotential tissues of embryonic character (Thoma and Goldman, 1946). These tumors present many complex problems, not only with respect to matrix formation, but also in relation to calcifiability of the matrices, once they have been formed. Unsolved problems dealing with differentiation, induction, evocation, maturation, and organization complicate this situation. In general, however, the calcifiability of matrices produced by neoplastic cells is closely related to the origin and degree of differentiation of the cells. For instance, there is usually a greater tendency for the osteoid matrix of slowly-growing well-differentiated osteogenic tumors to calcify and be converted to bone than the osteoid matrix of rapidly growing highly undifferentiated osteogenic tumors. This is also true of tumors characterized by fibrous, cartilaginous, dentinoid, or enameloid matrices. There is therefore a curious parallelism between the sequences in the life history of a neoplastic matrix and the extent to which cells concerned in the formation and maintenance of the matrix deviate from the normal. These generalizations should not be taken as an indication that all problems of matrix formation are related to cellular differentiation. Normal cells as well as neoplastic cells do not necessarily exercise complete control over the production or composition of the matrices. In this regard, the variability of calcification of similar osteogenic or odontogenic matrices, which in any given group of tumors should theoretically calcify equally is at present wholly mysterious. The best conclusion to be reached is that local conditions primarily regulate the deposition of calcium, that the cells may have the capacity to form matrices but lack the capacity to participate in the mechanism of calcification, or that the matrices produced by the cells are defective in those properties and materials which normally are responsible for attracting and binding calcium salts. Suffice it to say that in osteogenic tumors we encounter all variations from no osteoid matrix to osteoid matrices which, if calcified, may or may not be converted to bone. Similar statements hold for the variations in chondroid, dentinoid, and enameloid matrices of neoplasms.

Pathological calcification of cellular or intercellular matrices of reduced viability in tumors follows the same general rules as described for non-neoplastic tissues undergoing degenerative changes. Calcium deposits in general are more conspicuous in degenerative neoplasms which retain a considerable degree of structure while undergoing devitalization. This is especially notable in slowly growing well-differentiated tumors in which the process of devitalization is slow, progressive, and unaccompanied by diffuse repair, by vascular organization, or

liquefactive necrosis with or without conspicuous participation of polymorphonuclear leukocytes. Furthermore, tumors rich in matrices are more liable to calcify during deterioration than those composed almost entirely of cells.

Most of the metabolic diseases which are significant in pathological calcification have already been discussed briefly. The most important ones favor a form of pathological calcification known as metastatic calcification. Hyperparathyroidism, chronic renal insufficiency, chronic destruction of bone and hypervitaminosis D are conditions in human disease, most often implicated in the pathogenesis of this syndrome. All conditions are characterized by an ionic imbalance in which either calcium or phosphate or both moves in the direction of the blood plasma. In general, these ions probably are in negative balance with respect to the matrix of bone and in positive balance with certain extraskeletal matrices which do not normally calcify. There is evidence that this syndrome may be aggravated though not produced in the human by skeletal immobilization, excess dietary intake of salts especially rich in calcium and phosphate, and special ionic imbalances created by different forms of renal insufficiency. It is probable that, in most instances, the fully developed pattern of metastatic calcification requires the synergistic action of at least two of the possible etiologic factors.

Diabetes is another metabolic disease in which pathological calcification is common. The calcium deposits, however, follow the pattern of those encountered in the degenerative diseases of the ageing process which will be considered in a separate section.

Many nutritional diseases may interfere with proper skeletal growth but there is little established relationship between dietary factors and the forms of pathological calcification discussed here. There is no evidence in man that acid or alkaline diets, alone or in alternation, will produce the forms of metastatic calcification encountered in animals on such diets. Proteins, fats, or carbohydrates in excess or deficiency seem to have no bearing on pathological calcification in man. Among the vitamins, vitamin A, C, and D are implicated in the production, maturation, and calcification of skeletal matrices. There is no proof that any vitamin other than vitamin D is implicated to any significant degree in pathological calcification. This matter has been discussed previously. When the group of human diseases, generally classified as either degenerative or related in some way to toxins or abstract ageing processes, is brought up for consideration, the occurrence of pathological calcification becomes frequent and significant.

The simplest example of the special effects of a toxin is the nephrosis of acute mercurial poisoning. The acute degenerative changes in the convoluted tubular epithelial cells are, in the more advanced cases, associated with the cessation of renal function. The prompt and con-

spicuous deposition of calcium salts in the structural framework of these degenerating cells is remarkable, for in many cases, the degree and distribution of the mineralization defines in a very specific way, the etiology of the necrosis.

The selective action of toxins of different types may have a bearing upon the curious distributions of calcium deposits in cells or tissues. I have commented upon the lack of any valid reasons for the presence or absence of calcium deposits in infarcts. Certainly, the infarcts in the lungs which commonly calcify and then undergo conversion into bone occur most often in patients with mitral stenosis. Likewise, the calcification of nerve cells and axones, singly or in groups, often has no ready explanation unless it is regarded as following some special form of deterioration, in which case a special kind of toxin might be an etiologic factor.

In Raynaud's Disease, dermatomyositis, scleroderma, calcinosis circumscripta, and other clinically related conditions the calcium deposits occur in the subcutaneous tissues. It would seem that the deposits occur in the subcutaneous tissues. It would seem that the deposits are secondary to degenerative changes in matrices but the vagaries of deposition may indicate more specific effects prior to calcification. I doubt whether the mechanisms here are different from those which obtain in subcutaneous tissues following radiation injury, thermal burns, or freezing.

In calcinosis universalis the deposits of calcium may be widely disseminated and despite the frequent relation between this syndrome and scleroderma, there may be no evidence of tissue necrosis preceding calcification (Barr, 1932; Bauer, Marble, and Bennett, 1931). These conclusions are similar to those reached by some workers in studies of metastatic calcification. In calcinosis universalis, the only metabolic anomaly seems to be an undue retention of absorbed calcium and phosphate, presumably in the matrices undergoing calcification. There is indirect evidence that the organic components which attract the calcium deposits, being metachromatic after decalcification, are protein-acid mucopolysaccharide complexes.

The remaining diseases in which pathological calcification is commonly encountered belong principally to the class of senile degenerative diseases. These are discussed in the following paragraphs.

VI. Relations Between the Ageing Process and Pathological Calcification

Pathologists have studied for many years the frequent association of pathological calcification in degenerative diseases which seem to be connected in some way with the ageing processes. Most of these diseases

are accompanied by slowly progressive deposition of calcium salts in relatively avascular matrices subject to mechanical stress. The affected matrices are, as a rule, either a part of the vascular or skeletal system. Likewise, they are types of matrices which have, under normal or appropriate diverse pathological conditions, a tendency to calcify readily. Among these matrices, chondroid matrices, elastic tissue matrices, and miscellaneous fibrous or cellular matrices modified by degenerative processes which favor calcification are most important.

The present author has suggested, elsewhere, that the ageing of the cartilaginous matrix at provisional zones of ossification is associated with degenerative changes, among which diminished viability of regional cartilage cells with pigmentation and calcification of the matrix serve as preludes to the mesenchymal sequences of organization, culminating in osteogenesis. I have also indicated that, in the absence of these changes in the direction of diminished viability, chondroid matrices remain youthful, resilient, and relatively refractory to calcification. Hence, upon cessation of skeletal growth by conversion of cartilage to bone and, in other locations unrelated to the skeleton, the growth of cartilage becomes almost stationary in early adult life. In the succeeding years these adult tissues undergo deteriorative changes, perhaps favored by the limited regenerative capacity of the cells. All chondroid matrices are not equally affected nor do similar chondroid matrices in different people at the same age show identical changes but there are unmistakable trends connected with advancing age in everyone.

These changes are most readily studied in the costal cartilages where, with increasing age especially notable after the third decade, there is a progression of pathological changes (Hass, 1943). These begin in the center of the cartilage and as a rule progress radially from the central axis toward the perichondrium. The cells become swollen and exhibit some changes reminiscent of those encountered in swollen cartilage cells at normal provisional zones of calcification. The matrix acquires pigment of unknown nature. Calcium salts accumulate especially in the perilacunar zones. Fibrillation of the matrix and a depletion of the chondroitin sulfate content determined by analysis become conspicuous. In the absence of effective vascularization these trends persist so that the changes are usually very pronounced in advanced age. If vascularization is effective, the degenerate calcified matrices are often resorbed and organized by mechanisms of mesenchymal penetration and osteogenesis similar to those occurring in normal growth. The changes, which have been described, represent the general trends which affect all ageing cartilages of the body. The ultimate modifications in terms of calcification are, as a rule, more conspicuous in types of cartilaginous matrix which are more readily calcified under normal conditions. The mucocollagenous and

semi-tendonous forms of fibrocartilage are perhaps the least involved. Elastic cartilage is less commonly affected by the complete sequences than the fibrohyalin extraskeletal cartilage. This latter type of cartilage along the respiratory tract commonly shows in the aged all sequences up through conversion to mature bone with marrow.

A second common location of deterioration and calcification of matrices with age is in the annuli fibrosi at the bases of valves of the heart. In some species, a cartilaginous or bony ring normally develops in this location. In man, the tissue to which the valves are attached is fibrous and poorly vascularized. With advancing age, this fibrous tissue becomes less cellular and more rigid and compact. At the base of the leaflets of the mitral valve, especially the lateral leaflet, and less commonly at the bases of the cusps of the aortic valve, calcium is deposited in the avascular degenerate fibrous tissue. As the locale of calcification spreads progressively toward the margins of the annulus, mesenchymal reactions are excited. Vascularized stroma penetrates the irregularly calcified collagenous matrices, which at times have acquired a fibrocartilaginous appearance. The customary sequences of mesenchymal organization ensue and frequently mature bone with marrow appears among the degenerate calcified matrices which are being resorbed or sequestered by fibrocellular encapsulation. It is to be noted that the tissues which undergo these changes are subject to considerable mechanical stress. It is interesting that the sequences of repair of the degenerate aged tissue often terminate in the formation of a new osseous structure which, though irregular and imperfect, resembles the bony ring which normally develops in this position in some species.

The most common matrix to acquire an increased amount of calcium in connection with senile degenerative diseases is elastic tissue (Hass, 1939). It is doubtful that this is a general process which affects all aged elastic tissue. If so, the elastic tissue is not affected to the same extent in all locations (Lansing, 1951; Lansing, Blumenthal, and Gray, 1950). The principal deterioration-calcification sequences affecting elastic tissue occur in the systemic arterial system. These changes may begin very early in life but they are usually not conspicuous until the fourth decade, which is at about the time when changes in costal cartilage become conspicuous. From this time on they proceed at different rates in different locations in different people. These modifications are, therefore, probably not simply due to age or for that matter to any single process, irrespective of age.

It is perhaps better to take the point of view that reduced viability of elastic tissue renders it more readily calcifiable, if the basic minute fibrillar matrix of the elastic tissue is not destroyed. It is advisable therefore not to emphasize the age of elastic tissue as such but rather the

conditions imposed on the tissue in the aged individual in one or another location. There are at least three modifications which may for the moment be regarded as signs of reduced viability of elastic tissue matrices. One is a change in optical properties in the direction of homogeneity. The second is concerned with changes in staining reactions. The third is evidence of increased rigidity and transverse fragility. Elasticity in the common sense remains unchanged while resistance to stretch and decrease in tensile strength become conspicuous. Though any or all of these changes may be demonstrated in many anatomical locations, they are most conspicuous in the elastic tissue framework of the systemic arterial system. Though they favor calcification, they may become progressively worse in the absence of calcification and may even favor ferrification, if iron is available in proper concentrations. The portion of the elastic tissue framework which ordinarily undergoes the earliest and most easily recognized changes is known as the internal elastic membrane. These changes may occur in the absence of any other consistent pathological change in any regional component of the arterial wall. There is a tendency for these changes to affect the remaining elastic networks of the arterial wall but the progression of the disseminated changes is ordinarily preceded or accompanied by signs of deterioration of other constituents of the media. This deterioration of the ordinarily avascular fibroelastic-muscular media provides the necessary conditions for extensive mineralization of vascular walls. The fibrous, elastic, or smooth muscle cells of the media may undergo mineralization at different rates and to different degrees. This should serve to emphasize that in the ordinary course of atherosclerosis, calcium deposition is not at all restricted to elastic tissue. Nor is this true even in the absence of fibrinous-fibroelastic-atheromatous calcific changes in the intima. The order of calcifiability of medial matrices undergoing appropriate simultaneous degeneration is essentially as follows: first, smooth muscle; second, elastic tissue; third, collagen. As smooth muscle cells calcify the residual cell matrix tends to disappear and, if my interpretation is correct, the minerals which are released tend to pass to the next adjacent readily calcifiable matrix. This is usually elastic tissue in the aortic media and fibrous tissue in the media of many peripheral arteries. Finally, the sequences of organization and resorption may intervene. The penetrating stromal reactions may provide entirely new matrices with their respective orders of calcifiability and quite frequently in the calcified media of musculoelastic arteries, new bone and bone marrow are formed. There are many variants of this simplified concept of arterial calcification in senile arteriosclerosis. It may, however, assist in understanding the principal sequences and serve to point out that arterial calcification is not a specific problem of calcification of elastic tissue

matrices. In the usual case many matrices are implicated and must be so considered in the interpretation of quantitative analytical work. Standard procedures for separation of mixed normal matrices on the basis of solubility properties may need revision to cope with the problems introduced by the effects of mineralization upon the solubility properties of the matrices in question. Our experience with the reversible interference of dyes upon the solubility of the protein-acid mucopolysaccharide matrices of cartilage and amyloid discloses one aspect of this difficulty.

There are many other places where calcium tends to accumulate with advancing age. These need not be enumerated or described in detail. Though attention has been directed primarily to deteriorative changes in cells and intercellular matrices as preludes to calcification, it should be pointed out that calcium deposition also tends to occur in some more distal matrices. This tendency provides a fertile ground for the progressive formation of concretions and calculi in the excretory or secretory passages of the body. This large subject lies beyond the limits set for the present discussion.

Finally, we may propose that pathological calcification would not be a problem of ageing, if factors connected with advancing age did not bring about deteriorative changes which increase the order of calcifiability of the implicated cells and matrices. The causes of the deteriorative changes may not be the same in every aged person. One may suspect, however, that they achieve the observed result through interference with the same mechanism. This mechanism is that which governs the youthful flux of nourishment and metabolites through the intercellular medium. The interference with this mechanism in young and old is achieved principally by occlusion of the paths of transfer and diffusion through the intercellular matrices. In either case, the matrices and cells are bound to suffer though not necessarily to the same degree or in all instances even to an irreversible degree.

VII. An Exchange-Replacement Theory of Calcification of Organic Matrices

Pathologists are continuously confronted with the mysterious complexities of normal and pathological calcification. Perhaps, for this reason, they have maintained a cautious silence while chemical associates have proposed and defended one theory after another. Not so long ago, all that was needed to predict calcification, or the lack of it according to prevailing chemical theory, was an analysis of the total serum calcium and phosphorus. Later, to improve the accuracy of predictions it became desirable to know the amount of protein in the serum because there was a fraction of serum calcium which was not ultrafiltrable and this seemed to be associated with the serum proteins. Next, it became desirable to

know how much of the serum calcium was ionic and how much was "non-ionic." Now and then, calcium seemed to be forgotten as interest shifted to serum phosphorus which was principally ionic, ultrafiltrable, and a more dependable criterion for predicting vagaries of calcification in diverse diseases. Undue deviations from prediction, however, stimulated a search for other inorganic and organic ions in the plasma with the hope of finding special carriers or chelating agents for calcium. Thus, the study of calcium and phosphate partition in the plasma became complicated as facts belied theory and, rather than increasing our knowledge of calcification, served largely to direct attention elsewhere. It soon became apparent that, though the amounts of calcium and phosphorus in the serum were important, it was still more important to know from whence they came, where they were going, and why. This aroused a host of metabolic studies concerned with overall calcium and phosphorus balance with recent refinements made possible by elegant tracer studies with radiocalcium and radiophosphorus (Reifenstein, 1951-1953; Albright and Reifenstein, 1948; Armstrong, 1952). These confirmed an important fact, long overlooked as quantitative data were compiled in impressive tabular form on balance sheets. This fact is that most systemic metabolic data are a sum of a welter of activities and usually do not give even a hint of the many critical events which may either balance out against one another or contribute little to the final figure. However, it gradually became established that variable rates of deposition, resorption, exchange, displacement, transfer, crystallization, recrystallization, and so on of calcium salts could all occur at the same time in the presence of the same plasma in a very tiny microscopic field of tissue. As confirming evidence accumulated, the old classical views of the histologist began to excite the curiosity of chemists and interest shifted gradually to the locale of calcification. Thus, cells and matrices implicated locally in calcification were finally brought under the scrutiny of modern physicochemical and biochemical analysis (Meyer and Rapport, 1951). As the analysis proceeded, concepts of ionic inorganic chemistry had to be extended into the more difficult areas of organic-inorganic interactions among cells, enzymes, hormones, vitamins, matrices, ions, minerals, and crystals. At the moment, the integration of research on calcification in diverse special fields is being undertaken with a better understanding of the problem by the chemist, who has begun to regard ideas of the morphologist with something less than suspicion. In this "histological" approach the chemist has finally become interested in the possible role of cells and the complex organic structure of calcifiable matrices. Extensive investigation of enzymes of the cells and matrices has been undertaken with the idea of disclosing some mechanism for locally concentrating either the calcium or phosphate ions. In the latter instance, phosphatases

and phosphate esters have assumed first importance. In the instance of the calcium ion, much attention has been given to the non-enzymatic properties of matrices. Because of the calcifiability of cartilage and the richness of the protein-acid mucopolysaccharide composition of the matrix, the study of the polysaccharide compounds as ion exchangers which may regulate local calcium ion concentration or calcium phosphate precipitation have been revealing. From this, it may be inferred that all matrices rich in protein-acid mucopolysaccharides are readily calcifiable. Actually, the reverse is true, and there are good grounds for defense of the thesis that acid mucopolysaccharides protect normal matrices against undue calcification. These contrary views serve a good purpose for they direct attention to a fairly sound observation—that there is an order of calcifiability among matrices and that under proper conditions any protein matrix may calcify, whether it possesses acid mucopolysaccharides or not.

This raises the question of the basis for the order of calcifiability or calcification potential of matrices. Two logical assumptions are: first, that the matrix may have a structure which minimizes the probability of calcification and, second, that the matrix may have a structure which favors calcification. The conversion of the former type of matrix into the latter type seems elementary to my understanding of sequences of local events in pathological calcification. The evidence indicates that at least three events participate in establishing a high order of calcifiability as matrices undergo this conversion. First, the main structural protein lattice of the matrix should be preserved. Second, pre-existing surfaces should be maintained and new surfaces created. Third, the matrix should degrade or dissociate in such a way that "prosthetic" groups, usually polymeric compounds, become separated from the main structural lattice. Polypeptides, polycarboxylic acids, polysulfuric acids, and polyphosphoric acids may be regarded as common forms of polymeric prosthetic groups implicated in this process. It is curious that the nature of the bonds between the structural matrix proteins and polymers commonly affixed to them has not been established but there are reasons for believing that the bonds, though similar, are not identical in all cases. In any event new reactive centers are created when the bonds are broken. For example, basic reactive groups of the matrix structural lattice may fix phosphate ions while the more acidic or chelating groups of the dissociating polymeric compounds fix calcium ions. In this way temporary or permanent bridges may also be formed between the matrix lattice and the dissociated polymer. In any event the concentration of these dissociating compounds as well as the rate of their dissociation in relation to the supply of implicated metals or ions in the region should be of primary significance. For optimal reaction, the separation of the

prosthetic groups from the persistent mosaic of the structural residue of the matrix should not occur at a rate in excess of the transport of minerals destined to occupy the reaction centers. Otherwise, disturbing secondary reactions, diffusion and progressive degradation involving either the prosthetic group or the matrix residue will minimize the probability of mineral concentration and deposition. In a sense, therefore, calcium and phosphate ions take up the positions just vacated by organic polymers often in the form of polyelectrolytes of diverse kinds. The reactive surfaces of residual matrix may also possess or acquire enzymes and together with the dissociating polymer may actually serve as dual anion and cation exchange systems to bring about the final orientation and concentration of ions for interaction, precipitation and formation of motes for crystal growth as well as lattices for limitation of crystal growth. These intimate associations should allow for the formation of weak or secondary valence bonds by which the mineral deposits are joined to the matrix. The strength and nature of these bonds will principally determine the "migration" of minerals among mixed or contiguous matrices which have different calcification potentials. Basic disintegration of the matrix by continued dissociation or enzymatic action, also should release the minerals to return, other things being equal, to the original source. Other things being unequal, the minerals may persist in unbound form or undergo the sequences of mesenchymal resolution and organization.

The ideas presented here may be vague, unorthodox, and even indefensible from a chemical viewpoint. They are, however, not entirely without foundation in descriptive pathology and, what is more important, are subject to experimental attack. Should they fail to survive the attack, they will have at least served the useful purpose of drawing attention to the unquestionable significance of the changing composition of matrices in sequences of pathological calcification and other forms of tissue mineralization. The present author is fairly certain through long apprenticeship that Professor S. Burt Wolbach, who has stimulated a good deal of thinking by pathologists about intercellular matrices, might, in view of this one useful purpose, have tempered justifiable criticism of this incomplete and unproved exchange-replacement concept of calcification and calcification potentials of organic matrices (Wolbach, 1937).

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