

THE CELL

Biochemistry, Physiology, Morphology

Edited by

JEAN BRACHET

*Faculté des Sciences, Université libre de Bruxelles
Bruxelles, Belgique*

ALFRED E. MIRSKY

*The Rockefeller Institute
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VOLUME I



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LIST OF CONTRIBUTORS

VINCENT ALLFREY, *The Rockefeller Institute, New York, N. Y.*

ROBERT BRIGGS, *Department of Zoology, Indiana University, Bloomington, Indiana*

JAMES D. EBERT, *Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland*

RALPH O. ERICKSON, *Division of Biology, University of Pennsylvania, Philadelphia, Pennsylvania*

MAURICE ERRERA, *Faculté des Sciences, Université libre de Bruxelles, Auderghem-Bruxelles, Belgique*

A. FICQ, *Faculté des Sciences, Université libre de Bruxelles, Auderghem-Bruxelles, Belgique*

L. GALLIEN, *Laboratoire d'Embryologie, Faculté des Sciences, Université de Paris, Paris, France*

ISIDORE GERSH, *Department of Anatomy, University of Chicago, Chicago, Illinois*

DAVID GLICK, *Histochemistry Laboratory, Department of Physiological Chemistry, University of Minnesota, Minneapolis, Minnesota*

DAVID GODDARD, *Division of Biology, University of Pennsylvania, Philadelphia, Pennsylvania*

CLIFFORD GROBSTEIN, *Department of Biological Sciences, Stanford University, Stanford, California*

B. E. HAGSTRÖM, *The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Stockholm, Sweden*

THOMAS J. KING, *The Institute for Cancer Research, Philadelphia, Pennsylvania*

M. J. KOPAC, *University Department of Biology, New York University, New York, N. Y.*

P. PERLMANN, *The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Stockholm, Sweden*

- B. M. RICHARDS, *Medical Research Council, Biophysics Research Unit and Wheatstone Physics Laboratory, King's College, London, England*
- J. RUNNSTRÖM, *The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Stockholm, Sweden*
- P. M. B. WALKER, *Medical Research Council, Biophysics Research Unit and Wheatstone Physics Laboratory, King's College, London, England*
- PHILIP R. WHITE, *Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine*
- RALPH W. G. WYCKOFF, *National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Department of Health, Education and Welfare, Bethesda, Maryland*

PREFACE

For the past hundred years—from some time before 1855, the year of Virchow's celebrated aphorism *Omnis Cellula e Cellula*—investigation of the cell has been marked by a sequence of revolutionary movements. The most recent of these movements began some fifteen years ago. In each revolutionary change the cell has been viewed dynamically; structure and function have been correlated. From the present movement in cytology there is emerging a picture of the cell that is even more dynamic than heretofore. An important reason for the dynamic quality of the new cytology is that biochemistry is playing a far more significant role in cell studies than it ever did before. The greater role of biochemistry in cytology has been made possible by developments in cytochemistry, by the new methods for isolation and analysis of cell organelles, by techniques for exploiting the potentialities of radioisotopes. At the same time innovations in microscopy, notably in electron microscopy, are bringing structure down to the molecular level. The prospect before us is of a fusion of all cell biology at the molecular level.

It was therefore felt that a comprehensive treatise on the cell, written by an array of specialists might now be useful to many biologists: the present work is the result. It is divided into three volumes, the first one being itself divided into two parts. Volume I, Part 1 includes an introduction and descriptions of the most important methods for the cytologist (optical methods, fixation and staining, autoradiography, quantitative cytochemistry and histochemistry, microdissection, fractionation of cell organelles, tissue and cell culture). In this part devoted to methodology, the aim has been to present critical evaluation of the most important techniques rather than give "cook book recipes" which can easily be found elsewhere.

It is hoped that Volume I, Part 2, which deals with problems of cell biology will prove useful to all interested in general biology. The main topics are: fertilization, sex determination, growth and differentiation, morphogenesis in plant cells, nucleocytoplasmic interaction in morphogenesis, the acquisition of specificity, and the effect of radiation on the cell.

Volume II is devoted to the study of the cell constituents: cell membrane, plant cell walls, cell movements, cilia and flagella, ground substance, mitochondria, Golgi apparatus, secretion granules, chloroplasts, the resting nucleus and its interaction with the cytoplasm, chromosomes, mitosis, meiosis and gametogenesis, cancer cells. The cell constituents

have not been considered from a mere descriptive viewpoint: their biochemical activities and their interactions with other cell organelles have been emphasized as well.

Finally, the object of Volume III is the description of the specialized cells: viruses, bacteria, protozoa, parasites and symbionts, nerve cells, sensory cells, muscle cells, gland cells, kidney cells, blood cells, bone and cartilage, connective tissue, skin and pigment cells, antibody-producing cells are successively studied.

A unified and consistent presentation is, indeed, hardly desirable in a field where knowledge is frequently uncertain and fragmentary. The editors are well aware of the fact that such a large collaborative undertaking, in a complex and enormous field, cannot be perfect, also that differences in point of view reflect the state of knowledge. They are the only ones to blame for the omissions and repetitions which will certainly be found. They are very grateful for the assistance they have received from the contributors and the publishers and for the unfailing help given them by their secretaries, Mme. E. De Saedeleer and Miss Gabrielle Kirsch.

J. BRACHET
A. E. MIRSKY

November, 1958

THE CELL: *Biochemistry, Physiology, Morphology*
COMPLETE IN 5 VOLUMES

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INTRODUCTION

The publication of this collective work under the editorship of J. Brachet and A. E. Mirsky is an important event in biology. It was in 1924 that E. B. Wilson brought out the third edition of his *Cell in Development and Heredity*. His extraordinary book was the work of one man, but the tremendous increase in knowledge and the development of new techniques is such that no single author could now encompass the broad field covered by Wilson thirty-four years ago.

The study of the cell occupies a central position in biology. Many aspects of development and function of higher plants and animals are interpreted in terms of cellular activities. In addition, there is the varied world of unicellular life, and many fundamental biological problems are investigated at the cellular level.

Initially, interest in the cell was largely centered on its position as a structural unit of higher organisms, even though Leeuwenhoek's discovery of unicellular life and human sperm occurred within twenty years of Robert Hooke's first description of the cell in 1665. After the first studies of the seventeenth century, little interest was taken in the cell until the opening years of the nineteenth century. The reason for this time lag has never been particularly clear, but an explanation may lie in the fact that the eighteenth century was largely a period of botanical and zoological exploration, and biologists were primarily concerned with taxonomic problems. In addition, from the time of Leeuwenhoek's discovery of the sperm through the eighteenth century, biologists were preoccupied with the concept of preformation, to the exclusion of the consideration of the cell itself. However, the nineteenth century, particularly from 1840 on, was a period of great activity in describing the role of the cell in embryology, fertilization, pathology, and physiology. The major structures, including mitochondria and chromosomes, had been described, as had mitosis and meiosis. The fact that metabolism was basically a cellular phenomenon became accepted. Pflüger in 1872 made the pronouncement that respiration was a cellular process. In fact, the role of the cell as a functional unit in muscular contraction, nerve conduction, osmotic behavior, and glandular secretion now gained general acceptance. This was also the period of the rapid extension of our knowledge of unicellular life, and of the diversity of structure and behavior of bacteria, algae, and protozoa. By the end of the century, the basic knowledge was at hand to interpret genetic phenomena in terms of chromosome behavior.

Although the cell theory is one of the great unifying principles in

biology, the study of the cell itself has largely been a precise description of the observations of diverse cells. The cell theory has not been followed by a series of derived generalizations, and it has not played a role in establishing theoretical biology comparable to the role of the kinetic theory of gases or the atomic theory in physics and chemistry. There are perhaps three basic generalizations included in the cell theory: (1) the existence of life only in cells or multicellular organisms; (2) the genetic continuity of life; (3) the reciprocal relation of structure and function.

The first generalization includes the statement that organisms are made of cells and that the activity of the organism is dependent upon cellular activity. The nineteenth century clearly established the observational basis of this statement. Another corollary of this generalization is that there is no life below the limit of the cell. This must not be too readily accepted, for we recognize that the isolated virus carries within it the genetic information which determines that, when the virus invades a susceptible host cell, the virus will be multiplied. We also recognize that such cellular components as cell nuclei, mitochondria, and chloroplasts may be isolated and still retain metabolic and synthetic activities which are normally associated with life. Whether the virus particle or the isolated cell structure should be considered alive is uncertain. Perhaps we had best accept life as an undefined term, recognizing that no single property of living organisms uniquely distinguishes life from the non-living.

The continuity of life is based upon the single cell, and the spore or fertilized egg contains all the information necessary for a complex pattern of biosynthetic pathways, the precise duplication of intricate structure including multiplication of the cell itself, and the potentiality of differentiation into the varied cellular forms of the multicellular organism. Genetic continuity is a concept that applies not only to cells but also to chromosomes and genes, chloroplasts, and probably mitochondria.

The last quarter of the nineteenth century saw the recognition of chromosomes and of mitosis and meiosis. The concept of genetic continuity not only of the cell but also of subcellular parts had been established. Perhaps the greatest achievement of twentieth-century biology has been genetics and the chromosomal basis of the transmission of genetic information. Inherited internal information does not exclude the cell or organism receiving external (non-genetic) information, for the actual fate of any cell is the result of the interaction of the internal information and the cellular environment. However, the ability of the cell or organism to receive the external information, and the pattern and limits of its response, are genetically determined.

A corollary of the concept of genetic continuity is the cellular basis

of developmental biology. In large part, developmental biology has been a precise description of cellular divisions, migrations, and differentiation that lead one from the zygote to the organism. The biologists of the late nineteenth and early twentieth centuries have left us a magnificent heritage of beautiful studies of cell lineage. The interactions of gene and cell, and of cell and neighboring cell, are problems largely for future study.

The third generalization, of the reciprocal relation of structure and function, is rarely stated, although it is implicit in many modern studies. This generalization might be described as follows: the metabolism of cells occurs in an orderly and regulated manner, since the chemical processes occur within, and are determined by, organized structures; these same processes, however, result in a duplication of the specific structures. This I would name the principle of complementarity.

There are many examples of the relation between structure and process. In extracts of cells one may carry out many individual biochemical reactions. In some of these extracts we may observe the coupled reactions involving the storage of energy in chemical bonds; the organizing structure here is the soluble enzyme. More complex metabolic processes are studied, not in solution, but in such cell organelles as mitochondria and chloroplasts. We find that not only may pyruvic acid be oxidized over a Krebs cycle coupled to a cytochrome system but part of the energy is conserved in oxidative phosphorylation. Such a system, isolated from a cell, retains its regulatory mechanism, since the metabolism idles at low level when all of the phosphate acceptor is exhausted, and responds to the release of the phosphate acceptor by a racing of the oxidative processes. This regulatory mechanism depends upon the intact structure, and control of the metabolic rate disappears in a poisoned or injured mitochondrion. In the chloroplast water is photochemically reduced to OH and H radicals (though the radicals are probably bound). Such a process requires two quanta of visible light, and is an apparent exception to Einstein's law of photochemical equivalence. The high velocity of photosynthesis, and the two quantum nature of the basic photochemical process, become understandable if we assume that each chlorophyll molecule does not act as a separate unit but that a large number of chlorophyll molecules in the organized structure of the chloroplast function as a unit, and that energy absorbed at several points migrates rapidly through the structure to act at a single point.

How the chemical processes occurring in organized structures result in duplication is far from clear. The results of such processes are readily followed, and we observe the results in every mitosis.

It is clear that genetic continuity depends upon the complementarity

of structure and process. In fact, one might consider that genetic continuity subsumes this generalization.

The nineteenth century gave us a broad descriptive basis concerning the cell, its parts, their reproduction, and its role as the dominant unit in development and in activity. The first thirty-five years of the twentieth century established the basic knowledge of genetics and cytogenetics. Further, the biochemists had largely established the major chemical compounds of the cell, and the individual proteins were being isolated and characterized. Much knowledge had accumulated concerning cellular permeability, osmotic properties, and active transport of ions. The major steps of fermentation and glycolysis had become clear, and the Embden-Meyerhof-Parnas scheme had gained acceptance. Much progress had occurred in our knowledge of cellular respiration.

If we arbitrarily take 1935 as the end of the classical period of cytogenetics and cell physiology, and consider the current period as one of experimental cell biology, we will not overly distort the truth.

The modern period can be thought of as one in which the study of the cell is no longer the province of a few hundred cytologists but is an area of exploration and experiment for many thousands of experimental biologists. These biologists have come from the sub-disciplines of biochemistry, biophysics, microbiology, pathology, genetics, in fact from the whole range of biology and medicine.

The great changes in our knowledge of the cell, and in the methods used to study it, can be thought of as largely determined by new instruments. As these volumes should make clear, instrumental advances have been great. However, the intellectual advance has been equally great, and in part is father of the new instruments.

The older biologists were limited in their outlook by their education and the conventions of biology. They were content to describe with precision the diversity of form and structures of cells, and the changes that cells underwent in differentiation. It was more or less assumed that if one piled up enough valid observations the great underlying principles would become apparent. The observational methods used by classical cytologists built a great and sound body of knowledge and were successful, combined with experimental attack in genetics, in establishing the chromosome theory of heredity. Even though cell physiology was becoming a respectable science, and microdissection had become a successful art, the biologists "knew" in advance that the disruption of the cell would not lead to advances in our knowledge. Although the localization of structures and occasionally compounds by staining methods had become a highly developed technique, these methods only extended the range of qualitative description.

In the 1930's newer methods were introduced. Bensley and Hoerr disrupted the cell and by differential centrifugation isolated cell particles. Behrens isolated cell nuclei, Granick and Robin Hill isolated chloroplasts. These isolated particles could be counted, analyzed by chemical means, and their enzyme activities assayed. Warburg had already determined the photochemical action spectrum of the reversal of carbon monoxide inhibition of respiration, and even earlier (1925) Keilin had, with a visual spectroscope, observed the correlation between cellular activity and the oxidation-reduction of the cytochromes. Caspersson's introduction of the spectrophotometric measurement of nucleic acids in single cells and nuclei had a profound effect, for it opened up a completely new level of quantitative analysis.

The discovery of bacterial transformation by Griffith in 1928, and the identification of deoxyribonucleic acid as the active agent by Avery, McLeod, and McCarthy in 1944, meant that the tools were at hand for the experimental study of the chemistry of heredity. The range of possible experiments was greatly extended by the recognition that some viruses were ribonucleoproteins while others were deoxyribonucleoproteins. Then, during the opening period of the war, came the Beadle and Tatum experiments in biochemical genetics. Great as has been the impact of biochemical genetics on genetics, the change in biochemistry has been even greater, for the biochemists gave up being taxonomists of enzymes and proteins and began to attack problems of biosynthetic pathways and the localization of enzymes; in other words, biochemical genetics was the stimulus that introduced biology into biochemistry.

Since 1945 the advance in our knowledge of the cell has been almost explosive. The problem of enzyme localization has been attacked with vigor, as has the study of the chemical composition of cell organelles. When one recognizes that an "average" wet cell may have a mass of 10^{-9} gm. and a dry weight of 2×10^{-10} gm., the analytical problems of cell chemistry become more clear. Individual substances may occur in cells in amounts of 10^{-17} to 10^{-19} moles.

The recognition of the problems to be solved has resulted in a marked refinement of the old instruments and the development of the newer ones. The optical microscope had reached its limit of resolution by 1890; improvements have been in the correction of spherical and chromatic aberration, better illumination, and ease of use. The ultraviolet microscope extended the resolution by a factor less than two; its greatest value has only come with the recent reflecting optics that allow spectrophotometric studies to be readily made in the ultraviolet. The phase contrast and interference microscopes have allowed the visualization of structures such as chromosomes and mitochondria in unstained living cells.

Although the electron microscope has not yet been successful in distinguishing the chemical components of the cell or in measuring the amounts of the substances, it has extended the resolution by nearly three orders of magnitude, so that a great advance has been made in our knowledge of fine structure. A whole generation may be involved in redescribing the structure of cells, at a new level of organization.

Fortunately, the interference microscope and X-ray microscope promise us tools for quantitative determination of cellular components. Old instruments, such as the polarization microscope and the fluorescent microscope, when combined with photography and densitometry (or photoelectric scanning) now offer the possibility for quantitative determination, where until recently they have been used only for qualitative observations.

The differential spectrophotometer, particularly as developed by Chance, has made possible beautiful studies on cell suspensions, and the correlation of cellular activity with the rates of oxidation or reduction of particular enzymes in the cell system.

The advances in microbiology have had a marked influence on our understanding of cellular phenomena. The study of adaptative enzymes has furnished another tool to investigate biosynthetic pathways. The methodology of microbiology when coupled with the new genetic techniques has been fruitful not only in microbiological problems but now also in the study of mammalian cell cultures. Problems in population genetics that might take decades to work out with multicellular plants and animals may be readily undertaken with microorganisms or with cell cultures.

Although Schleiden in 1838 went rather far when he asserted that every cell had a dual life, a life of its own and a life as part of the organism, his statement has some merit. When tissues or organs are analyzed, or their metabolism is studied, the results are most meaningful when they can be expressed in cellular terms. The recognition that the cell of the multicellular plant or animal is a metabolic unit becomes clear when the experimental results from various tissues or organs are compared on a cellular basis. Then it is apparent not only that the DNA per haploid chromosome set is a constant for the species but that the chemical composition and metabolic rates of cells of dissimilar origin are far more uniform than would be indicated from bulk tissue analysis. If a developing cell is followed through its history, changes in metabolic rates and chemical composition may be charted, and, although differences will be noted in the light of its history, the cell is found to be a self-regulating unit. Its minimum and maximum activities are found to lie within rather closely prescribed limits. The consideration of chemical

and metabolic changes in cellular terms makes obvious relationships among composition, rate of growth, and metabolism that would be missed otherwise. The lowly plant cell is found, during its periods of rapid growth, to be as active as its animal counterpart. Something of the dynamic aspects of a rapidly growing corn root cell becomes apparent when one can calculate that it may form 10^7 peptide bonds and 10^8 glucosidic bonds per second. We need such concrete data if we are to have a base line against which to compare the rates of protein synthesis in isolated nuclei or microsomes.

The new techniques, and particularly the change in intellectual climate, has meant that the experimental attack on the cell has greatly broadened. Biologists no longer hesitate to undertake nuclear transplantation, and we may expect chloroplast transplantation to follow. Thus cytoplasmic-nuclear interactions are possible targets for experimental attack. The culture of cell organelles, such as mitochondria and chloroplasts, still lies in the future.

Some progress has been made in theoretical biology, through our recognition of the role of nucleic acid in the transfer of genetic information, and the efforts to interpret this information in terms of the molecular arrangement of these macromolecules. However, no progress has been made in any general theoretical treatment of the cell. The cell theory is not such a treatment; it does not permit predictions, nor is it particularly useful in relating distinct observations. The new experimental biology has largely replaced cytology as an experimental science, but has not yet found a logical structure for its results. In this sense, it is still far short of the success of genetics. Perhaps the next twenty-five years may see an advance in theoretical approaches to the cell that will equal the advances in instrumentation and experiment of the past generation. The biologist now has the opportunity to construct a logical system of the cell. Perhaps he will come to real understanding of the cell by the use of all available approaches, experimental and theoretical. When we truly understand the cell, we will understand life itself.

DAVID R. GODDARD

University of Pennsylvania

CHAPTER 1

Optical Methods in Cytology

By RALPH W. G. WYCKOFF

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I. INTRODUCTION

Without microscopes there could be no cytology. Few cells can be seen with the unaided eye, and we became aware of the cellular basis of living organisms only after the invention of the microscope that made them visible. What is known about the internal structure of these universal units of life is for the most part what the compound optical microscope has allowed us to see. The limits of cytology have been largely those set by this instrument, and in a very real sense they can be transcended only through further developments in our ability to visualize and thus deal adequately with the very small. In recent years these developments have been rapid, and it is the purpose of this chapter to point out those that are helpful to further studies of the cell.

Cytology is not unique in its dependence on what we can see. All natural science, as an attempt to describe and understand the material world, is necessarily based on the impact this world makes on our organs of sensation. Most of the information arising directly from this contact is qualitative and limited in scope; our modern flourishing science is rooted in a steady invention of new instruments that extend this sensory contact and give it precision.

RALPH W. G. WYCKOFF

Originally science was necessarily concerned with the description of matter in bulk, with the appreciation of the orderliness of its behavior and with the attempt to discover the rules that govern this behavior. The invention of optical instruments in the seventeenth century drew attention to worlds beyond those directly accessible to our unaided senses; and the revelation by the first microscopes of a world of objects too small otherwise to be seen was the beginning of that investigation into the fine structure of matter which is so overwhelmingly the preoccupation of modern scientists. Many things, including the gradual improvement of microscopes, have fostered this preoccupation; early in the nineteenth century it began to dominate scientific effort. Once chemical experimentation had demonstrated the atomic and molecular basis of all matter it was evident that the description of any material object, inanimate or living, would not be considered adequate until it covered all degrees of organization down to and including these atoms and molecules. Most objects have more than one chemical constituent, and examination with even primitive microscopes made it obvious that their admixture is an exceedingly intimate one. The sizes and interrelationships of the minute constituents define the texture of the object as a whole and determine many of its physical characteristics. There could be little knowledge of this essential texture without microscopes.

In the biological sphere the textural unit of all but the simplest forms of living matter is of course the cell. It has already been said that cytology is dependent on microscopic vision and is mainly the record of what has been seen with the compound microscope as perfected during the last century. To some extent new cytological knowledge can come through further studies with this microscope; modern histochemistry with its mounting list of specific color reactions for compounds to be found within cells is an example. But the cytology of the future must largely grow through information the classical microscope cannot furnish. Before the end of the last century, the immediate possibilities of the compound microscope using visible light had been rather fully realized; its theory had taken on a completed form and the instruments themselves were already giving all the resolution consistent with this theory. For many years around the turn of the century microscopy was comparatively dormant except for the introduction of the ultraviolet microscope; but recently there has been a renewed effort to deal visually with the very small, and microscopy is currently in a state of healthy and rapid growth. Its new developments are directed toward an understanding of the microstructure of inanimate as well as of living matter; many of its new instruments, especially those that give quantitative data on pure

and crystalline substances, are not directly applicable to cellular structure. They will be omitted from the present chapter, which seeks only to survey, but not describe, those new methods that hold special promise for cytology.

A complete description of any object will include knowledge of (1) its form and that of its parts, (2) the chemical composition of its components, and (3) its texture, i.e., the distribution of these components with respect to one another. Microscopy can give some information bearing on these points for all rigid objects. For those that are living, including cells, we also want to know (4) what they do and what happens to their components during life, and sometimes at death. The microscope can supply information of this sort which in biology has a special significance. Microscopic methods, new and old, are important to us here in the measure that they can advance our knowledge of the cell in one or another of these four directions. It is thus helpful to evaluate new instruments and techniques in terms of the contributions they can reasonably be expected to make to morphology, to composition, and to a knowledge of the dynamics of the living state.

Starting out from the optical microscope as the instrument that has given to histology and cytology their present form and content, we can note three directions in the further development of microscopy which are useful in extending our knowledge of the cell. These are: (1) extensions in the use of light for microscopy, (2) the use of electrons instead of light as the agent for microscopic vision, and (3) the use of X-rays to examine microscopic detail. It will be convenient to consider these developments separately.

II. EXTENSIONS OF OPTICAL MICROSCOPY

There are a few principles laid down by optical theory which must be borne in mind if we are properly to appreciate the possibilities for, and the limitations to, a direct visualization of microscopic objects. One of these is the relationship that exists between the wavelength of the light used for seeing and the smallest dimensions of an object that can be imaged by this light. It applies equally to the infrared and ultraviolet, and to electrons. Customarily this smallness is defined in terms of a resolving power which expresses the ability of the optical system to show as separate two objects very close together. As usually stated, resolution for a microscopic system is:

$$r \text{ (this smallest separation)} = 0.61\lambda/n.a.$$

where λ is the wavelength of the illumination and n.a., the light-gathering power, is the numerical aperture of the system. Thus for the objective

lens of a microscope it is determined by half the angle α formed by the specimen and the maximum opening of the lens and by the refractive index of the medium through which the light is passing: it is μ (the refractive index) $\times \sin \alpha/2$. The attainable value of n.a. somewhat exceeds unity for optical lenses, and, therefore, it is approximately true that the smallest detail one can hope to image will have linear dimensions about half the wavelength of the light with which it is seen. Objects with diameters less than r can be detected, but what is then seen is only the light they diffract, and this does not indicate either their shape or true size. The ultramicroscopes used years ago by colloid chemists gave such imageless indications of the presence of submicroscopic particles. The attainment of this limiting resolution, which for blue light is about $4000 \text{ A}/2$ or 0.2 micron, requires an adequately corrected objective lens and an illuminating system of a numerical aperture high enough to fill the entire objective with light. Before the end of the last century microscopes capable of reaching this maximum resolution with light were in use. Thereafter still smaller objects could be seen only through the use of some form of illumination other than visible light.

We see anything that is nonluminous by reason of its interaction with the light that strikes it. What the image in a microscope reveals is determined partly by the resolution and partly by the contrast resulting from this interaction. Absorption of light in details of the specimen is the chief cause of contrast in the image produced by the conventional optical microscope, but contrast may also be contributed by the action of particulate matter in scattering light away from the objective. Some of the most valuable recent advances have come by creating contrast from differences in refractive index. Cells in the living state have always been difficult objects to study microscopically because they and their contents are for the most part transparent to visible light. Vital staining can make them somewhat absorbing; but this procedure has no more than a limited value, and nearly all we know about cellular structures has been gained from tissues that have been fixed and then rendered absorbing with stains. Because of Brownian motion there is no possibility of seeing the finest cellular structures in unfixed material, but we must always be troubled by the realization that fixed details are coagulation artifacts of the living structures. This is a consideration which must always give added weight to any procedures that will augment what can be seen in cells that are still alive.

The oldest technique for using differences in refractive index to introduce contrast into a microscope image uses polarization, and much of petrology is built on it. The polarizing microscope is helpful in the study of natural fibers; but the birefringence with which it deals is rare in cells and for cytology it is an instrument of secondary importance.

This is not true of the phase contrast microscope (see, for instance, Bennett *et al.*, 1951, which also includes an extensive bibliography) that has more recently been developed for creating contrast by taking advantage of local differences in optical path within a specimen. How it acts and what it accomplishes are directly apparent in terms of fundamental theory. Parallel light striking an optically inhomogeneous specimen is diffracted, with the deflected part taking a path different from that which is directly transmitted. The directly transmitted component is united in the back focal point of the objective, while the deflected portion, seeming to originate in the specimen, is focused farther away in the ocular plane. The image is considered to arise from interference between these beams. The light is everywhere retarded in the sample by amounts that depend on both thickness and refractive index, but the deflected beam from a detail will always be a quarter of a wavelength out of phase with the light directly transmitted through its surroundings. If the deflected part can be further retarded by a quarter wavelength there will then be either destructive or constructive interference to yield strong contrasts. To make them apparent there must be some absorption of the more intense directly transmitted part. These considerations indicate that phase contrast can be obtained by placing an annular quarter-wave plate in the back focal plane of the objective to give the requisite additional retardation to the deflected component; covering the hole at the center of this plate with a suitable absorbing layer brings the direct beam down to a comparable intensity. In this simple and most useful phase contrast the light must be monochromatic, but quarter-wave plates can be provided for different wavelengths which are not limited to the visible spectrum. Observation under phase contrast instead of by the classical absorption contrast does not reduce the resolving power of an optical system, and therefore it is bound to have great possibilities for the examination of inhomogeneous transparent objects. For cytology its most obvious, but not its only, application is to living tissues; it also offers a unique way to compare stained and unstained fixed tissues, and, since it enhances the contrast in weakly colored (absorbing) systems, it can add much to the value of vital staining.

The greatest contribution of phase contrast to our knowledge of cells probably will prove to be its superior way of following the changes in structure that occur during their life and multiplication. These can be followed visually, or through a series of photographs, or best by cinematographic procedures in the form of automatic time-lapse photographs viewed as a motion picture. Such micro motion pictures of growing cells were made a generation ago, but this necessarily was done under condi-

tions of specimen illumination showing much less cellular structure than is now visible under phase contrast. Our present more sensitive photographic emulsions compensate for the less bright phase contrast images, and with our improved and simplified tissue culture techniques a wide variety of tissues can be photographed. By cinematography, a tissue can automatically be kept under continuous observation for days or weeks, and the accelerated viewing of the film draws attention to many phenomena that are missed in single photographs. Enough cinematographic records have now been made under phase contrast to leave no doubt as to their usefulness. More of them undoubtedly will be made when suitable equipment is commercially available. To be genuinely useful for the photography of living cells it must be built around an inverted microscope, which, having the objective below the specimen, allows a culture to grow uninterruptedly in its nutrient fluids throughout the period of photography.

Phase contrast is not the only practical way to use differences in refractive index and optical path to create contrast within transparent objects. In microscopes this can be done by causing interference between different parts of the beam passing through a sample or between this beam and some of the illumination which has bypassed the sample. Several types of interference microscope now exist. They bring about interference in different ways and have different fields of usefulness; those that best measure the surface characteristics of an object have little value in cytology. The possibilities of interference for the examination of transparent objects can be appreciated through an experiment (Merton, 1947) easily carried out with an ordinary microscope and monochromatic light. When a thin inhomogeneous transparent object like an unstained tissue section is placed between a pair of half-silvered glass slides, its details appear in strong contrast; similarly a small particle lying between such plates is seen surrounded by a system of interference fringes whose number is a direct measure of its thickness in terms of the wavelength of the light used. The contrast is, of course, due to interference between the immediately transmitted and the reflected components of the beam as affected by the locally changing path differences in the specimen. The Dyson (1950) and the Linnik (1933) microscopes operate on this principle of multiple reflection.* The Baker microscope,† also applicable to the examination of cells, uses polarized light which is split before passing through the object, recombined in the objective, and then again split into opposed circularly polarized beams by a quarter-

* The Dyson microscope is manufactured by Cooke, Troughton and Simms of York, England.

† Manufactured by C. Baker, Ltd., London.

wave plate. The plane polarized result of their interference is passed through an analyzing plate to give an image whose appearance depends on the phase shifts introduced by the specimen. There has not yet been sufficient experience with these microscopes to support a sound preference for one or another in cytological investigations.

The experiment referred to above illustrates the two most obvious applications of interference microscopes in cytology. One is the visualization of detail in cells and tissues through the contrast resulting from the differing refractive indices within them. This field of application is very much the same as that of phase contrast, but more work must be done before their relative merits can be judged. The second application is quantitative. It consists in using interference fringes to measure the optical path differences in a tissue and to gain in this way a knowledge of the distribution of mass within and between cells (Walker and Richards, this volume, Chapter 4). Such measurements on cells as they grow have obvious value in studies of their metabolism. They are made possible by the fact that the nonaqueous component of cells is predominantly protein and that the increment of refractive index per unit weight is practically the same for all proteins.

A determination of the distribution of "dry weight" within a tissue can also be made by X-rays (see page 17), though this is possible only on dried preparations. It may be that such X-ray determinations are capable of greater accuracy, but they are more difficult to carry out and to evaluate as well as being more restricted in the kinds of specimen to which they can be applied. Both techniques have as yet been too little used to permit an adequate comparative evaluation.

Unlike earlier and cruder arrangements for using refractile differences to visualize details in a transparent specimen, phase contrast and interferometric microscopy can make use of the full resolving power of the optical microscope. They do not, however, portray smaller details of structure than can be seen with it. This requires light of a wavelength shorter than the visible. The first ultraviolet microscopes (Köhler, 1904) were devised and made at the beginning of this century. Fundamentally they differ from conventional microscopes only in having lenses of some material other than the optical glass which is opaque to radiations shorter than about 3500 Å. Fused quartz was chosen for ultraviolet lenses inasmuch as it has the necessary optical properties, including freedom from birefringence, and is transparent down to wavelengths somewhat below 2400 Å. Simple lenses of quartz can have no color correction and therefore they give acceptable images only with strictly monochromatic light. Such monochromats for the original Köhler microscope were calculated for 2700 Å. Nevertheless, though their focal length shifts, the

image they form remains of good quality for monochromatic light of other wavelengths, with the result that the microscope can be employed with considerable success throughout the region from approximately 2400 Å. to the lower limit of visible light at approximately 4000 Å.

Quartz is not the only material available for making ultraviolet lenses. The mineral fluorite, CaF_2 , is transparent to still shorter light and can be combined with quartz to build lenses achromatized to a pair of wavelengths. It has, however, not been widely employed, partly because crystals of natural fluorite pure enough to be transparent far into the ultraviolet and sufficiently perfect to have no birefringence are hard to find. Now that the crystals can be grown in the laboratory, more satisfactory material probably could be obtained. Lithium fluoride is perhaps even better. It is transparent far below 2000 Å., is isotropic, and is currently being produced commercially as large single crystals of optical quality. With lenses of such pure substances transmitting the farther ultraviolet, microscopes could be made to employ radiations down to about 1500 Å. Little has been done because their probable value appears to be so limited. Quartz ultraviolet microscopes have, in fact as well as in theory, given resolutions somewhat higher than microscopes using visible light, but the incentive to push this resolution still higher by employing shorter ultraviolet radiations has been removed by the electron microscope.

Thus the quartz ultraviolet microscope has become an important tool in cytology, not on account of its success in fulfilling its original function, but because certain constituents of cells show a characteristic absorption in the spectral region with which it deals. All nucleoproteins have a strong absorption maximum in the neighborhood of 2750 Å. and this makes it possible to photograph the chromosomes and other chromatin-containing components of unstained, living cells. Studies of many pure compounds have shown that all those containing conjugated double bonds have, in this region, maxima which become stronger, the greater the number of these bonds per molecule. The nucleic acids therefore are particularly opaque, but there is some absorption from proteins containing cyclic groups.

This highly characteristic absorption of the nucleoproteins is mainly responsible for our continuing interest in ultraviolet microscopy. It is useful in two directions: to observe the formation and fate of chromosomes within living and dividing cells and to measure quantitatively by spectrophotometric techniques the distribution of chromatin throughout cells which have been fixed. The apparatus for microscopy with ultraviolet light need not be much more complicated than for visual examination, but that required for quantitative microspectrophotometry is exceedingly complex.

It is unfortunately true that along with the increased absorption of ultraviolet light in the chromatin structures of the living cell goes a correspondingly increased sensitivity to damage from these radiations. The cells in a living preparation can easily be killed by the ultraviolet light used to photograph them. Short of this enough damage may be done so that what is subsequently observed will be as much a response to this injury as an expression of normal cellular behavior. It is therefore essential in all ultraviolet examinations of living organisms that close and continued attention be given to the total dosage the organisms are receiving. The situation is more or less saved by the fact that more illumination is required for focusing than for the actual taking of pictures. The ultimate usefulness of ultraviolet in the study of living cells, therefore, depends largely on finding out how to focus without injuring the preparation. Several procedures for focusing have been employed. The simplest involves examining a fluorescent screen placed in the plane of the photographic plate. Such screens have, however, been so inefficient that the resulting damage to living matter was early recognized. Exact focusing for an ultraviolet wavelength can be achieved by first finding focus visually with monochromatic visible light and then moving the objective and eyepiece a predetermined distance to reach the invisible focus. This has been done (Davies and Wilkins, 1950), but it is inconvenient and, since the setting must be very accurately made, the microscope must have a very precise and expensive mounting. A better arrangement (for instance, Johnson, 1934) would employ an achromatic quartz-fluorite or quartz-lithium fluoride objective to allow direct visual focusing, the required change of illumination for photography being made by shifting filters. Such achromats have been constructed, although not of high numerical aperture. The arrangement that would be most convenient for focusing, however, would use reflecting lenses.

In recent years there has been a revival of interest in reflection instead of refraction optics for microscopy. A reflecting lens consisting of properly shaped mirror surfaces can be employed over a great range of wavelengths and has a common focus for all (i.e., it is fully achromatic); thus it permits a direct focusing with visible light valid without change for all invisible wavelengths that may be of interest. The image quality of available reflecting objectives is still inferior to that of the quartz monochromats, but when adequate reflectors are made they will simplify in a revolutionary fashion the practice of microscopy outside the visible region.

Reflecting microscopes are as old as microscopy itself. Newton had such an instrument, and they continued to be made until early in the last century when they were rendered obsolete by the general avail-

ability of good optical glass from which the more durable refracting lenses could be made. There is little likelihood that reflectors will again be widely used for visible microscopy, but an increasing concern with invisible parts of the spectrum has renewed interest in them; since 1940 a number have been computed and built (see Barer, 1951). Pure reflecting objectives are limited by the fact that their numerical aperture cannot exceed about 0.65, with a correspondingly restricted resolving power. Combinations of reflecting and refracting elements (for instance, Grey, 1950) can, however, be constructed that are achromatic over a large spectral region and have higher numerical apertures and resolutions. If of sufficient quality, they will have many advantages over refracting lenses not only in the ultraviolet microscope but in many other new instruments that can be devised for use with invisible light.

One of these is the so-called color-translating microscope (Brumberg, 1939), which is arranged to present a composite ultraviolet image taken at more than one wavelength. With it the object is photographed three times with three separate ultraviolet wavelengths chosen according to the experimenter's wishes. When these photographs are projected simultaneously through three separate colored filters, the resulting composite image will have color in its various parts determined by the relative opacities in the three ultraviolet negatives. An elaborate instrument (Land *et al.*, 1949) has been built for doing this automatically and quickly (including the rapid processing of the photographs for projection). The few published photographs of cells and tissues taken with it do not establish the cytological value of this technique. The data it supplies could always be obtained by simpler arrangements, and its value must accordingly depend on whether or not this novel method of presenting data brings out details and relationships that would not otherwise have been apparent.

The possibility of minimizing the exposure of living cells by focusing the ultraviolet microscope with harmless visible light makes it especially important to know how the amount of ultraviolet needed to photograph a cell compares with the lethal dose of radiation. Such comparisons have been made (Mellors *et al.*, 1950) and they indicate that a number of successive photographs are permissible but that the kind of indefinitely repeated photography that is needed for a motion picture strip would probably be out of the question. The fact that the damaging dose is so near to that required for photography, however, gives point to a search for registering devices more sensitive than photographic emulsions. One possible substitute for the photographic plate is the television pick-up tube which for ultraviolet reception can have a fused quartz window. Television microscopes (Zworykin *et al.*, 1952) have been made for

both the visible and the ultraviolet, and it has been pointed out (Zworykin and Hatke, 1957) that color translation can also be achieved by coordinating successive illumination by three ultraviolet wavelengths with a pick-up that uses presentation on a color television screen. Ultraviolet television microscopes simplify the problem of focusing if quartz lenses are used, and they make it possible to show brilliant microscope images to many persons at once. It is not yet clear, however, if they will be valuable in research as well as in teaching.

The photomultiplier tube is the one type of receptor which could replace the photographic plate in ultraviolet microscopy and is known to have a greater sensitivity. Under ordinary conditions it is about a hundred times more sensitive than the best ultraviolet emulsions and by using special precautions this sensitivity can be further increased (Caspersson, 1950; Wyckoff, 1952). For the quantitative measurements involved in spectrophotometry it very definitely replaces the photographic plate, and in combination with television techniques it offers the possibility of ultraviolet microscopy with reduced exposure of the specimen.

Perhaps the most developed technique for microscopy using photoelectric registration is the flying-spot microscope (Roberts and Young, 1952; Causley *et al.*, 1953). It reverses the direction of the light through the ordinary microscope. Thus the source, placed in front of the eyepiece, is a television tube having a brilliant phosphor of very short persistence. The objective lens will focus on the specimen a greatly reduced image of the phosphorescing spot; as the electron beam of the tube scans its screen, the specimen will thus be scanned by this demagnified spot. When the output of a phototube placed beyond the condenser to pick up the unabsorbed part of the microbeam is fed to a television tube synchronized with the source, the microscopic image is thus displayed. This novel arrangement has a number of possible applications, qualitative and quantitative, in both the visible and the ultraviolet spectral regions. The instrument, like all scanning devices, is complicated, and it will probably be some time before its fields of genuine usefulness become apparent.

Fluorescent microscopy (see, for instance, discussion in Glick, 1949) is another procedure now used for the study of cells which requires their irradiation with ultraviolet light. It consists, as its name implies, in strongly illuminating an object with light of short wavelength and observing the image produced by visible light excited in any fluorescing components. Excitation is most conveniently brought about by an ultraviolet source from which the visible has been filtered. The shorter ultraviolet wavelengths so strongly absorbed by cells are not needed,

and therefore a condenser of quartz is not required. Many cells give a general fluorescence under this illumination and a few substances associated with tissues fluoresce in a way sufficiently characteristic for their identification. Such fluorescence has, for instance, been used to examine the vitamin and fatty content of tissues. Many problems of the circulation can be studied after the introduction of a fluorescent dye into an organism or tissue. Protein, and especially antibody complexes with such dyes can now be made and their uses in immunology are only beginning to be exploited. Nevertheless, the application of fluorescent microscopy to cytology itself is limited by the weakness of most fluorescence and the consequent need for intense illumination of the specimen.

The techniques for extending microscopy into the infrared do not differ in principle from those described for the ultraviolet. Reflection optics are well adapted to the long-wavelength region. Refraction lenses can also be employed: glass itself remains transparent for some distance beyond the red, and lenses of alkali halides or of silver chloride have been made. Cells absorb in this region, but not in the sharply characteristic fashion of the ultraviolet. Their examination in the infrared cannot in any case be very interesting because the low resolving power of this illumination prevents us from ever seeing appreciable detail within them. The infrared is chiefly valuable in the examination of pure compounds; and here microscopy (see, for instance, discussion by Blout, 1954) can be helpful in dealing with minute crystals and those that show a dichroism related to molecular structure.

The very strong absorption around 2750 Å. has stimulated the development of methods to measure it accurately and in this way to determine quantitatively the absolute amounts and distribution of the nucleoproteins throughout the cell. Such information has obviously great value for an understanding of chromatin fine structure and metabolism. Though simple enough in principle, the quantitative spectrophotometry which measures the absorption in microscopic regions throughout a sample requires elaborate and precise equipment (Caspersson, 1950; Wyckoff, 1952). A number of factors must be closely controlled. Thus light of the desired wavelength obtained by means of a monochromatizing spectroscope must strike only a minute region of the specimen passing in a precisely controlled fashion across this microbeam. The intensity of the source may not remain constant during the time necessary to map the specimen, and therefore a simultaneous and continuous record must be made during this traverse of the intensities of the light transmitted and of the incident beam. In order to measure absorption within cellular details, the illuminated area cannot exceed a micron on a side and the reproducibility of the mechanical movement of the

specimen must be considerably better than this. Obviously measurements of this sort, at several wavelengths, are so time-consuming and tedious that automatic registration both of specimen position and of the intensities is essential. The electronic circuits for this and for the photomultiplier tubes are complicated, but all these conditions must be met if significant results are to be obtained; there is little of value to be accomplished in this field with simplified and less costly experimental arrangements.

Even when the results are as precise as possible, many problems and uncertainties arise in evaluating measurements of ultraviolet absorption in cellular structures. Information from living cells can be only approximate because of the motility of their contents and the complex intracellular changes that begin in response to irradiation. The most accurate measurements have been made on frozen-dried tissues with ultraviolet micrographs controlling the photometric data. Even then the accuracy of nucleoprotein determinations is restricted both by the weaker absorption of other proteins and by light scattering. The latter is particularly troublesome since it increases rapidly at shorter wavelengths and is further enhanced by the coagulating effects of fixation. In spite of these difficulties, results obtained in this way concerning the distribution of nucleic acids bear in the most direct fashion upon their role in cellular life. The controversy that has arisen around theories involving these results is the inevitable consequence of any effort to construct a picture of a dynamic process from a few static views of this process. It can be resolved only by more measurements and should not blind us to the unique quality and importance of such experiments.

III. THE ELECTRON MICROSCOPE IN CYTOLOGY

Electron microscopes have now replaced all others as the means for discovering and studying the finer details of cellular structure. [For a general description and early literature, see Wyckoff (1949); for a thorough treatment, see Hall (1953); for a recent bibliographic orientation, see Wyckoff (1957).] Their resolving power is more than a hundred times greater than that attainable with light, and preparations are now good enough so that some at least of this high resolution is useful. It is important to realize the fundamental way in which this microscope differs from all those using light. Electrons are not part of the electromagnetic spectrum but are corpuscles having associated wavelengths as short as those of hard gamma rays. Because they are electrically charged they can be deflected and focused by passage through suitably shaped electrical and magnetic fields, and their very short wavelengths do not in practice limit the size of what they can image. This limit is set

instead by defects in the focusing lenses. The first electron microscopes superior to the optical microscope were built about twenty years ago; since then they have been improved until they can now form images of particles which, having diameters of less than 10 Å., are only five or six atoms across. This resolution is of course not the only factor determining the smallest details which can profitably be examined. Those in cells and tissues can be worth looking at only to the degree that they represent without excessive alteration the contents of the living cell.

At first the problem of utilizing for cytology the higher resolution possible with electrons was the essentially physical one of building microscopes that would be good enough and convenient enough for routine use. This stage has now passed, and current instruments can be successfully operated by any competent cytologist who seriously wants to do so. There will undoubtedly be further developments, but they are not likely to provide still greater resolution. Probably the present instruments capable of high resolution will be made more convenient to use and will give more photographs showing this maximum resolution; but increasing attention will also go to simplified and less expensive microscopes that will have a large output showing high, but not the highest, resolution. It is perhaps inevitable in this early stage of electron microscopy that everybody should want instruments of maximum resolution; but these must always be relatively cumbersome to use, and it will be a long time before most problems require them. The simpler microscopes are so much more economical in the time and effort spent in using them that they should be chosen for the majority of present-day applications to cytology. The need to survey large areas of tissue taken from very diverse sources is at least as urgent as the examination at highest resolution of a few cells fixed by the best procedures yet available; and it will always be impractical to reproduce such large areas at the very high magnifications required to demonstrate maximum resolution.

Every scientific instrument has its own clear-cut and characteristic limitations. For the electron microscope these are twofold: the sample must withstand evacuation and must be unusually thin. The need to operate in a vacuum prevents the study of living material. The first photographs of cells made it obvious that the greater resolution of the electron microscope could become of real value only after better methods of specimen preservation had been found. Tissues prepared by conventional methods were seen to be masses of artifacts so coarse in texture that nothing was to be gained by looking at them at these higher magnifications. Fortunately an improved fixation was not too difficult to achieve. The buffered, nearly neutral, osmic acid now generally employed gives a preservation of cellular detail startlingly better than that seen after acid fixation. It has no competitors, however, and there is

urgent need for other equally good fixatives with which its results can be compared. It is excessively superficial in its action; the adequately fixed layer is often no more than a few cells thick. Perhaps this is the inevitable price we shall have to pay for a fixation that does not produce a coarse and easily penetrated coagulation of cellular contents, but we should not lightly accept so hampering a conclusion.

Before adequate thin sections could be cut, isolated cells and even single layers of cells growing in tissue culture were subjected to electron microscopy, but they were too thick unless grossly flattened; and with flattening there inevitably went a disturbance of their contents that limited the value of the observations. Stimulated by what appeared then to be a need to examine thicker specimens, there was enthusiasm for building electron microscopes to operate at a high voltage. Some work was done, but experience soon showed that these would not facilitate the seeing of fine cellular details. In a thicker preparation they would simply show more and more detail confusedly superimposed. Some separation of this detail should in theory result from stereoscopic examination, but this has not helped in the study of cells.

From the outset it was apparent that the best cytological preparations would be the thin tissue sections which have now largely replaced all others. The methods for preparing them are superior to any that cytologists have heretofore employed; nevertheless, they are still far from ideal. Their most unsatisfactory features are neither fixation nor the actual cutting and handling of sections nearly a hundred times thinner than those routinely employed by optical microscopy: they are the shrinkage that accompanies chemical dehydration of the tissue and the damage produced when this dried material is embedded prior to the cutting. Freeze-drying naturally suggests itself as the most logical way to prevent shrinkage and the changes in structure that are its inevitable consequence; but thus far the electron microscopy of tissues desiccated after freezing has always revealed gross and obvious damage. No chemical desiccant has yet been shown to be outstandingly superior to others, and there is thus no clear indication of how shrinkage can be eliminated.

Tissues embedded in paraffin cannot be cut thin enough for electron microscopy. Successful sections have been obtained only from plastics polymerized after their monomers have infiltrated the dehydrated tissues. Methacrylates were the first to be employed and are now in general use. Those which are most convenient to use and which give blocks having the best physical properties for cutting, however, contract considerably as they polymerize. We are now coming to realize more and more clearly how serious and universal is the resulting cellular damage. The amount immediately recognizable under the microscope varies markedly with the tissue, but all cells are probably affected and some, like bacteria, are

almost always ruptured during the embedding. This artifact must be an especial source of worry to all who use the electron microscope to investigate diseased tissues; one is often uncertain whether or not many of the abnormalities seen can properly be ascribed to genuine pathological changes. To some degree this uncertainty can be relieved through experience, but it is essential to discover an embedding medium that does not shrink and produce this damage. It can be reduced by embedding in partly polymerized methacrylate, but this is too viscous to impregnate tissues well. Less tissue damage has sometimes been obtained with an epoxy resin such as that which goes under the trade name Araldite. Though these resins do not show the same contraction as the methacrylates, they are inconvenient to handle and difficult to cut; the monomers for impregnation are as viscous as partly polymerized methacrylates. For these reasons they may not find wide acceptance, but the superior quality of tissue preservation when they have been successful suggests that we may be able in the future to avoid this serious form of specimen damage.

The actual cutting of ultrathin sections is no longer a major difficulty, though it remains an annoying and time-consuming procedure. Several successful microtomes have been devised and built especially to give ultrathin sections, and some standard microtomes can be modified with equally good results. Most ultrathin sectioning is now done with knife edges of broken glass but these are very fragile and the need for better knives remains. Diamonds have been ground to give sharp and durable edges; they or some especially hard synthetic material will probably become standard.

Though there evidently are various ways in which current methods can be improved, it is important to realize that with enough care and attention preparations worth photographing at very high resolutions can now be obtained. For the future of cytology, it is also important to realize that where something less than the highest resolution suffices, the far more instructive electron micrograph of a tissue section at approximately 5000 times is easier to prepare than is a first-class photomicrograph of such a section at 1500 times.

The familiar electron microscope which so closely resembles the optical microscope in both the arrangement of its components and the general field of its application is only one of a number of new microscopic devices being developed from our growing knowledge of electron optics. It is, however, by far the most valuable for the study of cells. In early days an emission-type electron microscope was constructed to examine the residue left when cells were ashed; there has, however, been no recent attempt to extend this investigation, and it would appear that the results sought can better be attained in other ways. Most of the

new electron optical techniques now attracting attention either require specimens less readily damaged than cells or are devoted to the ultimate chemical analysis of areas of sample still too big to give the cytologist much useful information. It is too early to say what their future evolution will be.

IV. THE X-RAY MICROSCOPY OF CELLS

Attempts to use X-rays for microscopy began very shortly after their discovery and have been renewed from time to time ever since. Unlike the rest of the electromagnetic spectrum interesting to the microscopist, they cannot be manipulated by any of the extensions of optical microscopy already discussed; they cannot be focused by lenses, and no metal shows for them the spectral reflection that underlies reflection optics. Nevertheless, practical ways of making X-ray photographs of microscopic objects are gradually being developed, and some of these are applicable to the study of cells. In one procedure X-rays are focused by total reflection from appropriately shaped surfaces, and the instrument for doing this is thus a true microscope; it is not, however, sufficiently developed to be of present cytological interest. The useful techniques are all recent developments of radiography.

The simplest is a form of contact radiography improved through the use of fine-grained film and X-rays that are unusually soft (see, for instance, Engström, 1950). A contact radiograph is made by causing X-rays to pass through an object before impinging on a photographic plate or film with which it is in close contact. Its applications in medicine and industry are familiar to all. If the object is thin and the contact with the photographic emulsion very good, the resulting shadow picture can be magnified to give a somewhat enlarged view. Limits to what can be seen in this way are set by the thickness of the specimen and this intimacy of contact, as well as by the photographic grain of the developed image. X-rays of the hardness usual in radiography are not appreciably absorbed in biological material as thin as a cell, and the grain of emulsions sensitive to such X-rays is so coarse that the photographs cannot profitably be enlarged more than a few times.

A different situation exists if very soft X-rays are employed. Those excited at a few kilovolts are strongly absorbed in the conventional paraffin sections of soft tissues; when between 500 and 1000 volts are used, best results are obtained with tissue no more than one micron thick. Sections as thin as this make excellent contact with a sensitive photographic surface to yield remarkably sharp images and do not give rise to a superposition of detail that will confuse the elements of structure. Fine-grained emulsions (such as Eastman 649) are highly sensitive to these very soft X-rays (though not to harder X-rays), and their

developed grain remains so small that the microradiographs can profitably be examined at the highest magnifications of the optical microscope. Soft X-rays are strongly absorbed in air; for those having wavelengths up to approximately 10 Å, it is sufficient to fill the space between the tube and the plate with helium, but photography with the still longer X-rays produced below approximately 2 kilovolts must be carried out in a vacuum. Though much work with living cells is thus precluded, this is not in itself a serious limitation, since desiccation is needed to produce appreciable contrast. This technique is so recent that enough micrographs have not yet been made to indicate those problems in cytology to which it can make its greatest contribution. Nevertheless, it will be a way to recognize concentrations of any elements heavier than carbon, oxygen, and nitrogen and to measure quantitatively the distribution of dry weight in a tissue. In this latter respect it competes with interference microscopy and may be more accurate.

The resolution attainable by contact microradiography cannot of course exceed that of the optical microscope with which the photographs are examined, and it is poorer unless the rays are very soft and the preparations very thin. An escape from some of these limitations may come through a new microradiographic technique made possible by the development of electron lenses. This (Cosslett and Nixon, 1952; Nixon, 1955) makes use of the fact that detail in the shadow cast by any object is sharper, the smaller the source of illumination. Electron lenses, like other lenses, are able to demagnify as well as to enlarge, and a minute electron beam thus produced is at the heart of the projection X-ray microscope. In it a demagnified electron beam strikes a thin metal foil to furnish a correspondingly small source of X-rays. An object interposed between this foil and a fluorescent screen or photographic plate will then cast a shadow which will be larger, the closer the object is to the source and will show a resolution approximately equal to its diameter. This can routinely be made of the order of 1–2 μ and sometimes as small as about 0.2 μ , the limit of optical resolution. Further developments may give even smaller sources. Though substantially greater resolution cannot now be obtained than by contact radiography, this arrangement has other advantages. Thus initial magnifications with it are great enough to permit the use of coarser-grained emulsions more sensitive to harder X-rays. With this instrument, too, there is no loss of sharpness no matter how thick the specimen. Accordingly, it is now most valuable in the study and stereoscopy of thick samples and whenever any but the softest X-rays are required. Its advantages for microanalysis are probably greater with inorganic materials than with tissues.

An analysis for chemical elements present in a sample using their characteristic X-ray absorptions can be based on either of these forms

of microradiography. Under favorable conditions this can give a knowledge of the amounts and distribution on a microscopic scale of any but the lightest elements. In principle it depends on the fact that an element absorbs least those X-rays which it would itself emit and very strongly rays a little harder than this. By photographing with rays of lengths lying on either side of this absorption limit characteristic for an element, it is possible to identify it and tell where it is concentrated. Where a specific element is in question, it may be sufficient to work with a pair of wavelengths on only one side of the absorption limit. The method can be made quantitative though this requires careful microphotometry. Its most obvious applications are to inorganic materials, but it has been applied in an interesting fashion to measurements of calcium and phosphorus in bone. There are other evident histological problems in which it would be useful, and these will become more numerous as we gain experience with the very long X-rays characteristic of the lightest elements.

V. CONCLUSION

The newer techniques for microscopy that have been discussed in this chapter differ greatly in the complexity of their experimental arrangements and in the range of their applications to cytology. Both these factors are important in determining whether or not a new instrument will find widespread use. One reason why phase contrast microscopy for instance has found such general acceptance is that the necessary equipment is neither especially expensive nor more difficult to use than the ordinary microscope. Interference, in some respects its competitor, is being accepted much more slowly, in spite of its added value as a device for weighing cells, largely because interference microscopes are still rather complicated. Nevertheless, both microscopes will have a great future value in the expanded study of living cells made possible by our simplified tissue culture techniques. The electron microscope is the other new instrument which is universally applicable. Its direct and unanticipated visualization of cellular structures down to molecular dimensions will have an even greater ultimate impact on cytology. It is now within the range of the average laboratory to participate in that reinvestigation of all types of cell which this microscope has made inevitable.

Spectrophotometry, and most other techniques devoted to quantitative measurements on cells and their contents, will presumably continue to be the specialized concern of a few laboratories. This will also be true of the quantitative applications that can be made of X-ray microscopy. As instruments, X-ray microscopes are simple enough to be widely used; if further exploratory work shows them to be as valuable in the

study of cells and tissues as they are in some other fields they, too, may be found everywhere. The future of the new quantitative aspects of ultraviolet microscopy is less certain. Its expanding use seems to depend mainly on a broader acceptance of reflection optics and perhaps of television-like image receptors. Whatever may be the developments in these and the other directions that have been described, it is obvious that there is at present no dearth of studies that can be made of cellular structure using the novel techniques of a rejuvenated microscopy. (For discussion of many of the problems mentioned see Oster and Pollister, 1955.)

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CHAPTER 2

Fixation and Staining

By ISIDORE GERSH

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I. DEFINITION OF FIXATION AND STAINING

Fixation may be defined as the selective preservation of cell or tissue structures and components for subsequent study. Fixation is selective in the sense that those methods are selected which are most suitable for the particular purpose of the research study. For example, for the study of cellular interrelations and tissue patterns over large areas in sections, one would select Bouin's fluid, formalin, or formalin-Zenker; because of its poor penetration and its interference with subsequent staining, osmium solutions would be unsuitable. Again, for the study of fat droplets, one would select solutions of formaldehyde, potassium bichromate, or osmium; because it dissolves lipids, alcohol would be unsuitable. Similarly, one would select methods using solutions of osmium, silver, or chromium for the Golgi apparatus or mitochondria, and avoid Bouin's fluid. For studies on chloride, glycogen, cytoplasmic basophilia or certain enzymes, one would select freezing and drying over ethyl alcohol or osmium solutions. The same selectivity extends to include any method which may give useful information on structure or structural components whether this be at the atomic level (as with X-ray diffraction, automicroradiography, X-ray absorption, or inorganic histochemistry), at the molecular level (as with birefringence or fluorescence microscopy), at the macromolecular level (as with electron microscopy or X-ray diffraction), or at the level of the various types of light microscopes.

It need only be pointed out that over this great range of dimensions, various cell structures and components can be studied after adequate preservation without further staining. The possibilities for study are, however, enormously increased by the use of stains, particularly at the range of the light and electron microscopes.

It is particularly difficult to define the term "stain" without injuring

this very proper word. Correctly used, the term stain refers to a colored organic compound which "has a special aptitude for being retained" by tissue elements (Baker, 1950). These properties are referred to the chromophoric group and the auxochrome group. This usage excludes many situations which are customarily thought of as staining. For example, the Sudan and other stains for fat droplets are not really stains, as their staining effects depend on their differential solubility in fats (they lack an auxochrome group). The various silver, osmium, and chromium mixtures for the study of mitochondria, fat droplets, and the Golgi apparatus should not be called stains in the strict sense. The use of silver salts in the identification of various inorganic elements and organic substances similarly does not qualify the silver compounds as stains. Certainly the numerous inorganic and organic compounds, colored or uncolored, used to identify sites of enzyme activities, do not qualify as stains. Finally, when dyes or inorganic substances are used in electron microscopy, the element of color is inconsequential. If the term "stain" is to remain in technical use, it will have to be considered as any substance which when added to living cells or to fixed structures or structural components makes them more clearly visible or detectable with any instrument. As in the consideration of fixatives, the dimensions of the structures or components which are made visible range from the interatomic and atomic to the microscopic. If stretching the technical meaning of the term stain is too offensive, some other expression such as visualizing agent or detector shall have to be used.

These general remarks serve to indicate the scope of this chapter. More extensive treatment of many of the topics should be sought in some of the more general references (Fischer, 1899; Mann, 1902; Lison, 1953; Gomori, 1952a; Pearse, 1954a; Danielli, 1953; Lillie, 1954; Conn, 1953; Eränkö, 1955; Baker, 1950; and Oster and Pollister, 1956), in one of the numerous good handbooks on histological and cytological technique, and in various chapters in this book.

II. FIXATION

A. Fixation by Immersion in Fluid Fixatives

1. Separation of the Aqueous from the Solid Phase

The essential effect of fixation is the separation of the solid phase of protoplasm from the aqueous phase. This was shown most graphically by Hardy, who tried unsuccessfully to separate water from gelatin by the application of 400 pounds' pressure per square inch. However, after fixation, water could be separated from the gelatin by simple hand pressure. The solid phase may separate out as fibrils (Flemming, 1882),

as granules (Altmann, 1890), or as nets (Hardy, 1899) or vacuoles (Bütschli, 1892, 1894), depending on the conditions of fixation. The single significant feature, regardless of the form which the solids assume on the separation from the aqueous phase, is that the process involves a shift or movement of the solids. This may be visible microscopically in cells, as after alcohol fixation, or may be minimal and not detectable with the light microscope, as after formalin or osmium fixation of certain cells (Strangeways and Canti, 1927; Porter *et al.*, 1945; and others). Even though no microscopically visible changes could be observed after fixation by osmium solution, it is questionable that one could infer from this that no changes take place also at submicroscopic dimensions (Sjöstrand, 1956). In fact, the submicroscopic dimensions of some of the nets produced by a variety of fixatives have been recorded by Bretschneider (1950). In any case, recent work with high-resolution optics by Frédéric (1956) casts doubt on the assertion that no microscopically visible changes occur during fixation by buffered osmium solution.

It is possible to describe in only the most general terms other changes in the properties of protoplasm which accompany, precede, or follow the essential effect of fixation. One may assume that, when a fixative diffuses toward and into a cell and reaches a sufficient concentration, the fixative would have a "toxic" effect. The cell components would be expected to change at least at the submicroscopic level, owing to the chemical combination of the fixative with some amine, amide, carboxyl, sulphydryl, or other active groups of protoplasmic proteins, or to molecular distortions, aggregations or disaggregations. With continued or increasing concentration of the fixative one would expect changes in the concentration and localization of the small molecules and atoms of the affected regions and changes in the state of hydration of the proteins. At some point in the process, one might expect that the solid phase separates from the liquid phase, with concurrent displacement of cell proteins and some loss of enzymatic activity. As the process of fixation continues, the physical displacements progress. In addition, one would expect extraction and adsorption of some components, solution and reprecipitation of other components, and what can be described only as a physical rending apart of still other components. Some of these effects are gross enough to be detected directly with the microscope or by chemical means and have led to disuse of fixatives with such effects. Sometimes, as with osmium, these effects have been too subtle for direct observation. There seems to be no reason why the general sequence of events described in this paragraph should cease at the level of resolution of the light microscope. In fact, Bretschneider (1950) showed that it does not.

2. *In Vitro Experiments*

The separation of the solid from the liquid phase has been studied in model experiments with a variety of fixatives acting on protein preparations which currently would not be regarded as pure. It was found that the form and dimensions of the separated solid phase depend on the concentration and nature of the protein, the concentration, temperature, and nature of the fixative, the occurrence of "strains" in the protein preparation, the nature of the protein mixture, and the presence of nonprotein particles or droplets (Hardy, 1899; Fischer, 1899; Mann, 1902; Baker, 1950).

B. Fixation by Freezing and Drying

1. Separation of Water from Solid Phase as Ice

The separation of the liquid from the solid phase of protoplasm is achieved rather more rapidly and uniformly throughout the specimen as compared with immersion fixation, and the water is removed by sublimation in a vacuum system. Specimens are immersed in a cooling bath and chilled as rapidly as possible. Usually, the time interval is great enough for the water molecules to rearrange themselves to form ice crystals. In this process, the solid components are displaced to the outside of the growing ice crystals. However, if the specimens are frozen more rapidly still, so that they pass through a critical range with very great rapidity, water molecules may be frozen before they can be rearranged in the form of a crystal lattice. In such a situation, the water would be solidified in an amorphous, vitreous, or glassy state. The following are some of the factors which determine whether the specimen is frozen sufficiently rapidly to avoid the formation of ice crystals: the size and shape of the specimen, the initial temperature, the temperature of the cooling bath, the heat conductance of the tissue and of the cooling bath, and the water content or state of hydration of the specimen. In practice, the specimen is about 0.1 mm. thick (though it may be larger in the other dimensions) and the cooling bath is about -175° C., obtained by condensing a large volume of propane in liquid nitrogen, which serves to keep it at the low temperature. Under such conditions, specimens are cooled at a rate exceeding 5000° C. per second, and specimens of about 0.1 mm. in thickness are frozen in about $\frac{1}{50}$ second. It seems fairly certain that there would be minimal shift or displacement of the solid components of protoplasm in specimens whose water has been frozen in an amorphous state (Stephenson, 1956; Gersh *et al.*, 1957a).

2. Removal of the Aqueous Phase

The frozen specimens are dried in a vacuum system at a low temperature. In practice this temperature ranges from about -30° C . to about -60° C . Below this range, the vapor pressure of water is so low that drying times are unduly prolonged. The rate of drying depends in addition on the actual temperature of the interface between the ice and the vacuum, the resistance of the dry shell to the passage of water molecules, the resistance of the drying apparatus to the passage of water molecules, the partial pressure of water in the vacuum, and the efficiency of heat transfer to the interface (Gersh and Stephenson, 1954). Specimens may be dried also by having the water molecules swept away by a stream of cool, dry gas, a technique described by the Treffenburg (1953) and by Jensen and Kavaljian (1957). It cannot be proved that water does not rearrange itself in the frozen tissue in crystalline form while the tissue is at the elevated temperature used during the drying period (Pryde and Jones, 1952; De Nordwall and Staveley, 1956). But there is no indication from work with the electron microscope on suitably frozen tissues that crystals larger than 80 Å. may occur. Whether these spaces represent crystalline ice has not yet been determined. The evidence thus far obtained indicates that the maximum displacement of solid components of protoplasm during freezing and drying may be approximately 40 Å.

The dried specimens may be cut directly; or they may be infiltrated with paraffin or some other medium and sectioned; or they may be post-fixed and then infiltrated with some medium to facilitate sectioning. As in fixation by immersion, the specific treatment of the specimen during postfixation depends strongly on the purpose of the investigation. For this reason, the technical details are very varied and are summarized in several books and articles (Hoerr, 1936, 1943; Gersh and Stephenson, 1954; Gersh, 1956; Gersh *et al.*, 1957a, b; Bell, 1951, 1956; Neuman, 1952). Recent examples on the application of the method to electron microscopy are to be found in Afzelius (1956), Sjöstrand (1951, 1953c, 1956), Bell (1956), and Gersh (1956).

3. Advantages and Disadvantages

From observations to be cited later, it is clear that fixation by freezing and drying is superior to immersion fixation in certain respects: (1) The displacement of the solid components during the process is limited, small, and to a certain extent controllable. (2) Post-mortem changes are minimal. (3) There is no extraction of diffusible components, and, if the

suitable infiltration medium is selected, there is minimal extraction of proteins and lipids. (4) Most enzymes are not markedly affected, and the solubilities of proteins are in general probably not markedly affected.

Many of these useful features are compromised by subsequent manipulation (embedding, sectioning, deparaffinizing, and rehydration). To this qualification should be added the following additional disadvantages of the freezing-drying method: (1) The specimens are usually small, and it is difficult to prepare large patterns without interference by the size of the ice crystals. Reduction of specimen size as for electron microscope studies tends to prolong the post-mortem time and results in some greater manipulative distortion. (2) Some structures are quite disturbed or even disintegrated between growing ice crystals when these occur. (3) The apparatus and processing are costly and have not yet been standardized.

C. Fixation by Freezing-Substitution

Some of these disadvantages of fixation by freezing and drying are overcome by a simplified procedure known as freezing-substitution. The specimen is frozen rapidly, and the water is removed at a low temperature (-78° C. to -20° C.) by one of a series of reagents (Methyl Cellosolve, ethanol, propylene glycol, *n*-propanol, *n*-butanol, tertiary butanol, or methanol). When the water is removed, the specimen is allowed slowly to reach room temperature. While diffusible substances and lipids are extracted, proteins are mostly precipitated, and enzymes are inactivated. In favorable specimens, the morphology is excellent. Some substances are also made insoluble, notably glycogen. The distortions arising from ice-crystal formation during the freezing are as in similar material prepared by freezing and drying (Simpson, 1941a, b; Lison, 1953; Blank *et al.*, 1951; Bennett, 1951; Davies, 1954; Gouréwitch, 1953; Woods and Pollister, 1955).

D. Fixation of Viruses by Freezing and Drying

For the preparation of bacterial or viral cell suspensions, where air-drying results in the exposure of the organisms to enormous forces of surface tension, two different methods of preparation have been exploited. The critical-point method of Anderson (1956) and the freezing-drying procedures of Williams (1954) yield dried specimens for study with the electron microscope which retain to a remarkable degree the external shape of the organisms. Some details of internal structure can also be made out by an ingenious method described by Steere (1957).

*E. Some Practical Aspects of Fixation**1. Speed of Fixation*

The findings of the *in vitro* experiments on fixation summarized above are reflected practically in some precautions and interpretations of fixation of tissue specimens. It need hardly be emphasized that cells deteriorate rapidly after death and in the process may show marked structural changes. For this reason, the time between the withdrawal of the cells from their normal environment and fixation must be as short as possible. Fixation is probably most rapid in thin outstretched regions exposed directly to the fixative, as in certain parts of tissue culture outgrowth. In thicker specimens, penetration of the fixative depends on (1) its rate of penetration, (2) the kind of fixative used, (3) the size of the specimen, and (4) the density of the specimen. In the fixation of proteins in solution, of fibrin clots, and of slices of certain organs, the rate of penetration depends on the rate of diffusion of the chemical involved (Baker, 1950; Medawar, 1941). It is a matter of common experience that some fixatives penetrate very rapidly (ethyl alcohol, formaldehyde), while others do so very slowly (osmium solutions) (Dalton, 1953). With all fixative solutions, the advancing zone of the fixative is diluted by the water of the specimen. At any given spot, the fixative becomes more concentrated in time. The situation with mixed fixatives, whose different chemical components diffuse at different rates, must be extremely complicated. There must be a series of advancing zones corresponding with their rates of penetration, each chemical exerting its effect on an already altered region of protoplasm. Because of the post-mortem changes, including the time elapsing between the withdrawal of the tissues from their normal environment and the penetration of the fixative in an effective concentration, the outer portion of the block has most often been regarded as showing minimal post-mortem change. This problem is more acute in fixation for electron microscopy, and it has been estimated that only the cells in the outer 40 μ of an immersed specimen show minimal changes (Sjöstrand, 1956).

Another practice which reduces post-mortem change is the perfusion of the animal or organ as rapidly as possible, and completion of fixation by immersion in excess fixative. This procedure has also been adopted for electron microscopy. Endothelial cells lining the blood vessels are in an especially favorable position for quick fixation.

Post-mortem changes are greatly reduced by fixation, by freezing and drying, or by freezing substitution.

2. Extraction and Solution

The extraction of proteins, lipids, and basophilic substances (chiefly

nucleotides) during fixation has been shown to take place in a variety of ways. Berenbom *et al.* (1952) found that acetone extracted acid-soluble substances. Fixation by freezing and drying was rather complete, the specimen yielding some protein and acid-soluble substances when it was embedded in paraffin, deparaffinized, and rehydrated. Lipids behaved in a similar way. Hack (1952) found that extraction of lipids from frozen-dried material could be prevented by use of low viscosity polyethylene glycol (Carbowax) as embedding medium. Sylvén (1951) found that protein could be more readily removed from frozen-dried material than from the same material postfixed in formaldehyde, Carnoy's fluid, or absolute alcohol. Postfixation in formaldehyde after freezing and drying caused more lipid to be retained. Sylvén also found that basophilic substances were retained in frozen-dried material better than by other procedures. Similarly, Harbers and Neumann (1955) found that more ribonucleic acid was lost after fixation in Carnoy's fluid than after freezing and drying. Hartleib *et al.* (1956), on the other hand, found little evidence that protein or basophilic substances were removed by a variety of fixing procedures including freezing and drying; and Edström (1953) found that ribonucleic acid was not extracted from sections of nerve cells prepared by freezing and drying and postfixed in Carnoy's fluid. Stowell and Zorzoli (1947) showed that the amount of basophilic material extracted from sections depended on the nature of the fixative. Engström and Glick (1950) found that postfixation of frozen-dried material resulted in a marked loss of total mass of certain cell types only. Additional references on this subject are given by Boyd (1955).

Davies (1956), by direct measurement of nuclei in tissue cultures, found that some labile material absorbing at 265 m μ was lost after fixation in 10% neutral formalin and in Carnoy's fluid, while no loss was detected after fixation by freezing-substitution.

It is clear that buffered osmium solutions extract protein during fixation (Dallam, 1957), even when the fixation period is short (Bahr, 1955), and renders the residue more readily extractable by water. Rhodin (1954) presented evidence that certain granules are extractable by osmium solutions, and Low (1954) found that certain cells were disintegrated on prolonged fixation. Dettmer (1956) describes the gradual disappearance of elastic fibers during immersion in buffered osmium solution for 90 hours. It is almost uniformly agreed that no pulmonary alveolar membrane exists between the cells of the alveolar wall and the air space. This may also be attributed to the effect of buffered osmium solution (plus subsequent treatment?), as after freezing and drying and subsequent postfixation, a homogeneous, continuous alveolar membrane can be observed clearly with the electron microscope (Chase,

unpublished data). It is not yet clear how much of the loss of morphological entities is caused by extraction by the fixative and how much by subsequent treatment.

3. Reprecipitation and Displacement

It seems highly probable that if substances are soluble in the fixative and extractable, incomplete extraction would result in the reprecipitation of the material in some sites other than their original ones. This is clearly visible in edematous tissues, where the intercellular material which survives fixation seems to be aggregated on fibers and other surfaces. Another form of gross displacement occurs in certain mucigen cells. The mucigen droplets seem to swell in all fixatives; they may then fuse and become secondarily reprecipitated as granules or nets. This difficulty seems to be entirely prevented by fixation by freezing and drying.

An equally gross displacement of cell components may take place as a result of uneven exposure of cells to the fixative. For example, after fixation of even small specimens of liver, the cell components, including glycogen, are displaced toward the more slowly penetrated portion of the cell, where they are finally precipitated. This difficulty seems also to be avoided by fixation by freezing and drying.

4. Diffusion

The impressive rapidity of diffusion of small molecules or ions during fixation by immersion has been calculated by Bell (1956). Even relatively insoluble calcium compounds and PAS-reactive material in bones in certain experimental states may diffuse in fluid fixatives (Heller-Steinberg, 1951). The diffusion of the ferrocyanide ions in muscle from their extracellular site into the muscle fiber can be readily observed after improper fixation. In these instances, as in others (chloride, phosphate-carbonate) (Gersh, 1938), diffusion may be reduced or prevented by fixation by freezing and drying.

5. Inactivation of Enzymes

Suitable preservation of the activity of enzymes for microscopic study has proved to be difficult. After fixation in cold acetone, alcohol, or formaldehyde for short times, the activity of certain enzymes has been found to be greatly reduced or destroyed (alkaline phosphatase, acid phosphatase, cytochrome oxidase, esterase, cholinesterase, and peptidase) (Berenbom *et al.*, 1952; Chang and Berenbom, 1956; Doyle, 1950, 1953; Doyle and Liebelt, 1954; Gomori, 1952a; Pearse, 1954a; Chen *et al.*, 1952). All are better preserved by freezing and drying, although the

enzymatic activities are frequently reduced or destroyed by subsequent treatment. The maintenance of a high level of enzymatic activity during the postfixation following freezing and drying has not been possible, with few exceptions. An outstanding one is the preservation of dopa oxidases in human skin (Rappaport, 1955).

F. Freezing and Section-Drying

The difficulties attributable to paraffin infiltration, deparaffinization, postfixation, and rehydration were avoided altogether by Lowry and his students (1953, 1956, 1957), who extended a procedure used earlier by Linderstrøm-Lang and Morgensen (1938). Rapidly frozen specimens are cut at a continuously maintained low temperature and the sections are dried *in vacuo* at a low temperature. Cells or morphological regions are removed for analysis in the dry state and analyzed chemically by elegantly sensitive methods. Regions as small as $100 \times 100 \times 20\mu$ may be analyzed, and the chemical analysis may be based on either their protein content, their dry weight, or their volume. It is not known how much diffusion takes place at the advancing cutting edge of the knife or during the drying.

A similar technique was used for the quantitative analysis of sections of perikaryon of single nerve cells for total mass, by X-ray absorption and direct weighing, and for ribonucleic acid (Brattgård and Hydén, 1954).

The preservation of the antigenic properties of certain compounds for subsequent staining with specific fluorescent antibodies has been achieved by freezing and drying, with and without subsequent embedding (see fluorescent staining, page 46).

G. Artifacts—General Discussion

It is axiomatic that all forms of fixation result in artifacts. Some of these are "good," or useful, while others are "bad," or misleading. An example of a good artifact is the formation of peripheral vacuoles in the colloid of thyroid gland follicles. These occur rarely in the living animal, are more numerous and give a scalloped appearance to the colloid in fixed material when the colloid is more dilute. They occur infrequently in fixed material when the colloid is dense. The occurrence of the vacuoles in fixed material indicates, then, that in the living animal, the colloid was probably dilute and possibly of low viscosity. An example of a bad artifact is the fixation of glycogen in liver cells by alcohol. The distorted distribution of glycogen in one portion of the cell, its aggregation into granules, plates, and other figures can give no indication of the distribution of the glycogen in the living cell.

There are at least three useful criteria for deciding whether a given artifact of fixation may be good or bad. The first is a comparison of the appearance in the living cell with that after fixation. For shape, size, and relations of nucleus, mitochondria, and other cell organoids this is often possible, though it is impossible in many situations, as for example with nerve cells. It is also impossible to apply this criterion with any substance or structure not visible directly in the living cell *in situ*: as, for example, the distribution of glycogen, synapses, cell infoldings, various protoplasmic membranes or granules. The second criterion is a comparison of the appearance in the fixed material with pertinent information derived from some other discipline. For example, the distribution of chloride largely in the extracellular connective tissue was in line with the same distribution which physiologists and biochemists had deduced from their analyses of muscle. Similarly the distribution of cholinesterase in nerve endings of skeletal muscle is in close agreement with what we have learned from pure morphologic studies and pharmacologic studies on nerve endings. However, in many instances, there is not sufficient dovetailing of the findings on the subject in neighboring disciplines. A third criterion is of special interest in studies of sub-microscopic structures or components. In these subjects morphologic structures can be regarded as probably more sound (or not artifactual) when they can be confirmed by other information obtained by other methods based on different assumptions. Such instances may be regarded as examples of high reality probability. These include (1) spacings of collagen fibrils, myelin sheaths, retinal rods, and lamellae of the grana of chloroplasts, and apatite crystals of bone, where the observations made with the electron microscope agree very closely with the findings made in studies based on the use of X-ray diffraction and birefringence (Sjöstrand, 1949, 1950, 1953a, b; Finean *et al.*, 1953; Steinmann and Sjöstrand, 1955; Carlström and Finean, 1954; Schmitt *et al.*, 1942; Fernández-Morán, 1952); (2) cell processes of brush borders in such epithelial cells as those of the gall bladder and intestine, where they may be seen directly by phase contrast and compared with images in the electron microscope. In this group are also probably the cell processes of cells where they are too small to be seen with the light microscope though they are readily visible with the electron microscope, as in thyroid gland and parietal cells; (3) glycogen particulates, retinal rod, and myelin sheath layers as seen in the fixed preparation as well as after cell fragmentation or differential centrifugation; (4) shape, size, and relations of mitochondria and nuclei, where they can be seen with the light microscope and the electron microscope.

In contrast with the above examples are the structures described in fixed cells by electron microscopists, which have not yet been confirmed

by other methods involving different technological assumptions. These may be referred to as examples of low reality probability: (1) single or double membranes of the cell or nucleus, and internal and external membranes of mitochondria; (2) ergastoplasmic lamellae or the B-cytomembranes; (3) various kinds of granules, vacuoles, and fibrils. The preparations of mitochondrial membranes in separated mitochondria, of the ergastoplasmic lamellae, and of the "basophile" granules by differential centrifugation are not yet convincing that the structures pre-exist in the living cells as such (Palade and Siekevitz, 1956a, b; Siekevitz and Watson, 1956; Watson and Siekevitz, 1956).

H. Fixation for Electron Microscopy

1. History

During the last century shortly after fixatives were first introduced, their use was expanded rapidly through application of a multiplicity of chemical substances in a variety of concentrations and mixtures. Only near the peak of inventiveness did some workers become concerned with the mechanism of action of the fixatives used. The impact of such studies led to a very pessimistic view of the artifacts of fixation and stimulated the development of techniques of studying fresh or living cells. About this time, when knowledge of the structure of proteins was well advanced, studies appeared on the kinds of possible chemical linkages between fixatives and proteins. This literature is summarized ably by Wolman (1955). It is an impressive commentary on our ignorance of the mechanism of fixation even now, that we do not know why osmium solutions should result in a homogeneous-appearing nucleus, while other fixatives like formol-Zenker result in nuclei with crisp chromatin clumps, or why Bouin's fixative preserves mitochondria only rarely, while potassium dichromate seldom fails to preserve them. The grand array of fixatives of thirty years ago has been greatly reduced, and new ones are very scarce indeed. It is good, in this period of retrenchment, to see the careful report on the action of formaldehyde (Crawford and Barer, 1951) and the very extensive analysis of dichromate as a fixative (Casselman, 1955a, b).

In the brief development of electron microscopy, some of the century-long history of fixation is being repeated, though in abbreviated form. At first, many fixatives were tried. Most of them have been discarded, and emphasis has shifted to the use of buffered osmium solution. Freezing and drying, which arrived late in practical histology, has finally made an appearance in electron microscopy. New fixatives are continuing to appear, though at a reduced rate.

It was thought worthwhile to document this changed emphasis on

TABLE I
PARTIAL LISTING OF THE NUMBER OF REPORTED USES IN ELECTRON MICROSCOPY OF VARIOUS
FIXATIVES FOR PROTOPLASM OVER THE PERIOD 1945 TO MID-1957^a

Fixative	1945	1946	1947	1948	1949	1950	1951	1952	1953	1954	1955	1956	1957
Acetic acid	1	—	1	—	1	—	2	1	1	—	—	—	—
Acetone	—	—	—	—	—	—	—	—	1	—	1	—	—
Alcohol	2	—	—	2	3	1	1	3	1	1	—	—	1
Alcohol-ether	—	—	—	1	—	—	—	—	—	—	—	—	—
Alcohol-ether acetone	—	—	—	—	—	—	—	1	—	—	—	—	—
Altmann*	—	—	—	—	—	—	—	2	—	—	—	—	—
Ammonium molybdate	—	—	—	—	—	—	—	1	—	—	—	—	—
Apathy*	—	—	—	—	—	1	1	—	—	—	—	—	—
Aoyama	—	—	—	—	—	—	—	—	—	—	—	1	—
Baker	—	—	—	—	—	—	—	—	1	—	—	—	—
Benda*	—	—	—	—	—	—	—	—	1	—	—	—	—
Bensley*	—	—	—	—	—	—	—	1	1	—	—	1	—
Bouin	—	—	—	—	—	2	2	—	3	—	2	—	—
Cajal	—	—	—	—	—	—	—	—	1	—	—	—	—
Calcium-formalin-acetic	—	—	—	—	—	—	—	—	1	—	—	—	—
Carnoy	—	—	—	1	—	1	2	1	3	—	1	—	—
Champy*	—	—	—	—	—	1	3	1	2	—	—	—	—
Chromic acid	1	—	1	—	2	2	1	2	1	—	—	—	1
Chromic acid + formalin	—	—	—	—	—	—	—	—	—	—	—	1	1
Copper salt	—	—	—	—	1	—	—	—	—	—	1	1	8
DNA stains	—	—	—	—	—	1	—	—	—	2	—	—	—
Dubosq-Brasil	—	—	—	—	—	—	—	—	1	—	—	—	—
Erlke	—	—	—	—	—	1	—	—	—	—	—	—	—
Flemming*	1	—	—	—	—	2	3	3	2	9	4	—	4
Formalin	—	—	—	—	5	4	10	2	9	9	—	3	—
Formalin-alcohol	—	—	—	—	1	—	1	—	—	1	—	—	—

TABLE I (*Continued*)

Fixative	1945	1946	1947	1948	1949	1950	1951	1952	1953	1954	1955	1956	1957
PMA ^b	1	—	—	—	—	—	1	1	1	—	—	—	—
PTA ^c	1	—	1	2	—	2	1	—	4	—	—	1	—
Rabl	—	—	—	—	—	—	—	—	—	—	1	—	—
Rawitz	—	—	—	—	1	1	—	—	—	—	—	—	—
Regaud	—	—	—	—	1	4	2	5	2	1	1	—	—
Romeis	—	—	—	—	—	1	—	—	—	—	—	—	—
Schaudinn	—	—	—	—	1	1	—	1	—	—	—	—	—
Serra	—	—	—	—	—	—	—	—	—	1	1	—	—
Silicotungstate	1	—	—	—	—	—	—	—	—	—	—	—	—
Silver salts	—	—	—	—	—	—	—	—	1	1	—	—	—
Sulfosalicylic acid	—	—	—	—	1	1	—	—	—	—	—	—	—
Tellyesnicsky	—	—	—	—	—	—	—	1	—	—	—	—	—
Uranyl salt	—	—	—	—	—	—	—	—	1	—	—	—	—
Zenker	—	—	—	—	3	3	1	3	—	1	1	1	1

^a Osmium solutions, unbuffered or containing mixtures of other regents are marked with an asterisk.

^b Phosphomolybdic acid.

^c Phosphotungstic acid.

the fixation of protoplasm with Table I. The tabulation is based on some 525 references where sufficiently accurate notes were made of the method of fixation. These references were culled from some thirty-four journals, which were examined systematically over the period covered (1945 to about the middle of 1957). Papers dealing primarily with extracellular components were not included. The papers comprised under Palade's fixative (introduced in 1952) include the use of buffered osmium solutions, warm or cold, isosmotic or not. Other osmium solutions, unbuffered or containing mixtures of other reagents, are marked with an asterisk. The list is incomplete. The table serves to point out that we are approaching a dangerous period, when too much reliance may be based on the empirical use of a single fixative. It emphasizes that the need for the development of other less empirical fixatives is very acute.

2. Use of Buffered Osmium Solution

a. Advantages. The predominant place of buffered osmium solution in current studies in electron microscopy justifies a thorough knowledge of the use of this fixative. There is general agreement that it is at present the most satisfactory fluid fixative. The best objective evidence for this is the very close agreement between the spacings in certain structures observed in electron micrograms and values for the same spacings obtained by studies in birefringence and X-ray diffraction (see p. 32). Some of the structures could also be fragmented into the component layers. The intrinsic organization of these layered structures must be firm enough to withstand the fragmentation process (Sjöstrand, 1956). It is possible that the layered structures are so rigid that they may be able to withstand the disruptive and solvent action which osmium solution seems to have on other, less rigid structures. In addition, it is important that contrast and sharpness of detail are very satisfactory. How much of this favorable contrast should be attributed to staining (Maaløe and Birch-Andersen, 1956), how much to solution of material intervening between regions of high contrast, and how much to adsorption of finely dispersed or colloidal osmium (Owens and Bensley, 1929; Bensley, 1953) is not known. While osmium solution causes undoubtedly some physical change in the appearance of internal structure in certain cells, these changes are not as large as with most other fixatives. The cutting properties of material fixed in osmium solutions is very favorable. Finally, because the method is reproducible, the processing of specimens does not suffer from becoming routine.

On the other hand, it is generally admitted that osmium is not favorable for the study of the submicroscopic structure of the nucleus,

although the elegant observations of Gibbons and Bradfield (1956) show that the distribution of chromatin is at least partly preserved by this fixative. They suggest that the situation is confused by the enhanced contrast imparted by the osmium to the non-DNA components in the nucleus, so that it is difficult to distinguish the reactive from the non-reactive portions of the nucleus, the latter having an innately higher contrast because of their density before staining.

b. Rate of penetration. Osmium is the most slowly penetrating fixative. Because this slowness may permit significant post-mortem changes to take place between the withdrawal of cells from their normal environment and the time of cell fixation, Sjöstrand (1956) regards only the outer 40μ of a specimen to be well fixed. The criteria for limiting the region of good fixation to 40μ , rather than some other larger or smaller value, are not clear. Some workers have applied osmium solutions to specimens *in situ*, with the blood circulation continuing, in order to reduce the interval between interruption of the circulation and fixation. It seems, however, that the actual effect would be to prolong the "toxic" phase of fixation and delay the accumulation of a sufficiently effective concentration for fixation.

The chemical action of osmium was studied comprehensively more than a generation ago in connection with the staining of lipid and other morphologic structures and has been restudied by Porter and Kallman (1953) and Bahr (1954) and reviewed by Wolman (1955). Osmium in solution oxidizes aliphatic and aromatic double bonds and sulfhydryl groups, alcoholic hydroxy groups, and some amines. It also has an affinity for certain nitrogenous groups. Sites of oxidation of adjacent hydroxyl or ethylenic groups of adjacent molecules are thought to be bridged by reduced osmium. Bahr (1954) states particularly that carbohydrates and nucleic acids do not react with osmium.

c. pH, tonicity, temperature. The pH of the osmium solution is said to be critical (Palade, 1952), though Sjöstrand (1956) finds a wide range to be equally effective. The tonicity of the osmium solution is also claimed to be important (Sjöstrand, 1956), though Sager and Palade (1957) find no difference attributable to marked variations in tonicity. It is possible that, as for light microscopy (Baker, 1950; Crawford and Barer, 1951), tonicity is important with some cell types and not with others. If so, the reasons for the greater effectiveness of isotonicity must lie in some other direction, as membrane permeabilities are probably nonexistent. For plant cells where vacuoles show osmotic effects in the living state, isosmotic fixation was recommended by Hairston (1956). For satisfactory fixation of bacteria, the ionic environment was believed to be important (Maaløe and Birch-Andersen, 1956). Reducing the temperature during fixation is thought to favor good fixation (Sjöstrand,

1956), though Bahr *et al.* (1957) could find no evidence that low temperature was more effective than room temperature in controlling volume changes.

d. Swelling and shrinkage. The major detailed study on volume changes occurring during fixation of specimens for electron microscopy is that of Fernández-Morán and Finean (1957) on various nerve fibers. Study of low-angle X-ray diffraction patterns of the same specimens studied with the electron microscope showed that fixation by osmium solutions causes a slight shrinkage, which becomes more marked during dehydration. The swelling in methacrylate and during polymerization compensated for the effects of dehydration. It is of interest that fixation with potassium permanganate causes little change in the low-angle X-ray diffraction pattern. The breakdown of organized structure in methacrylate does not take place after embedding in glycerinated gelatin. Whether such changes are duplicated in other submicroscopic structures not so highly ordered is not known.

It is interesting that similar shrinkage and swelling effects were observed also in some studies on fixation of larger specimens prepared as for light microscopy. All recent workers who have concerned themselves with the question have been unable to control completely the initial volume changes which specimens undergo during fixation by variations in tonicity of the fixative. There seems to be a diminution of the initial swelling when large molecules are incorporated in the fixative. The detailed studies by Bloom and Friberg (1957) and by Bahr *et al.* (1957) were successful in estimating progressive volume changes during fixation, dehydration, and embedding. While methyl alcohol causes swelling, the other alcohols tested resulted in shrinkage. However, the subsequent clearing in xylene and paraffin infiltration resulted in a more marked shrinkage in the lower alcohols than in the higher ones. All specimens tested swelled in formaldehyde, but the swelling was least marked in the 4–10% solutions and more marked in more dilute and more concentrated solutions. During dehydration and infiltration with paraffin, the specimens shrank; the shrinkage accompanying dehydration was overcome to a certain extent by a swelling coincident with polymerization of methacrylate. Similar changes were found to occur during fixation with osmium, and the subsequent dehydration and embedding in paraffin and methacrylate. Polymerization in methacrylate resulted in swelling, also, but the net volume change was such that the specimen was only slightly larger than in the fresh state. Certainly the implication is that bald measurements of vacuoles, granules, etc., must be softened to permit the possibilities in the living cell that the structures may be larger or smaller than the measured values.

Davies (1956) followed the effects on nuclei of fixation of cells in

tissue culture. He found that rather marked shrinkage took place after fixation in 10% formaldehyde and in Carnoy's fluid.

e. Hardening of specimens. An important property of fixatives which is so commonplace as to be largely ignored is the favorable hardening action on the specimens which makes them more suitable for sectioning. The brittleness and increased difficulty of sectioning of tissues mordanted in bichromate and osmium solutions is well known. Because of the thinness of sections to be studied by electron microscopy, the distortions at the knife edge are probably greater than in the thicker sections prepared for light microscopy, and the correct hardness of the specimen and embedding material are more difficult to achieve. Overfixation results in shattering of the sections, while underfixation results in swelling of the specimen either during the polymerization process or on floating in the water bath. Underfixation is overcome in part by double embedding in nitrocellulose and methacrylate. Moreover, the oxidizing action of the catalyst used to accelerate the polymerization of the methacrylate may be disadvantageous, especially when it causes oxidation to a more soluble state of a dye used in the staining process. These difficulties are lessened by the use of epoxy resin, though its high viscosity introduce still other troubles (Maaløe and Birch-Andersen, 1956; Glauert *et al.*, 1956).

I. Fixation of Lipids

Microscopically, the lipids are preserved by three major fixatives—formaldehyde, bichromate, and osmium solutions. Formaldehyde seems to dissolve some lipids, but this effect is reduced by the addition of calcium chloride. It seems to affect the staining properties of the lipids also, but the chemistry of this process is unknown. Bichromate oxidizes certain unsaturated lipids and renders them insoluble. The reduced insoluble chromium may in turn act as a mordant and thus makes possible staining even after paraffin sectioning. Osmium acts in a similar way, but is generally ineffective as a mordant, except perhaps with gallic acid derivatives. However, when the osmium is reduced directly by the unsaturated bonds, or secondarily through the action of the dehydrating alcohols on the osmium dissolved in the lipid droplets, black lower oxides of osmium are deposited (Lison, 1953). Lipids may also be preserved by freezing and drying. Subsequent infiltration with low-viscosity polyethylene glycols at about 0° C. makes frozen sections possible. With aqueous fixatives, the protoplasmic partitions between lipid droplets in lipid-rich cells are not always preserved, especially when at a distance from the surface region. The lipid droplets may then fuse and result

in artifactual appearances. These physical shifts are prevented by freezing and drying and replacement of the aqueous phase by polyethylene glycol (Hack, 1952).

J. Fixation of Inorganic Substances

Little can be added on the subject of the fixation of inorganic substances to the information presented in Gomori (1952b), Lison (1953), and Pearse (1954b). It is here particularly that the advantages of the method of freezing and drying are clearest—it is the only general method of preparation, for which with some ions (chloride, potassium) there is no substitute. It seems that the full advantages of the method have not been reaped in autoradiography. Diffusion of readily diffusible inorganic components cannot yet be avoided in the methods aiming at the finest possible resolution. Only by sacrificing resolution by effecting separation of sensitive emulsion and the frozen-dried section can one prevent diffusion (Russell *et al.*, 1949; Holt *et al.*, 1949; Harris *et al.*, 1950; Taylor, 1956).

III. STAINING FOR LIGHT MICROSCOPY

A. Staining with Acid and Basic Dyes

This account is based mainly on the excellent analyses of Baker (1950), Conn (1953), and Singer (1952, 1954).

Dyes are colored organic compounds which have a special aptitude for attaching themselves to tissue components and contain chromophoric and auxochromic groups. The property of color is imparted to the dye by its chromophoric groups, which are aromatic compounds of varying degrees of complexity. The color is determined in large part by certain unsaturated groups, chiefly the following: carboxyl, azo, nitroso, nitro, quinoid, and ethylene. The property of attaching themselves to tissue structures is attributed to the auxochrome group, which confers on the dye its solubility and dissociability in water. The water-solubilizing groups of acid dyes are usually sulfates, carboxyls, or hydroxyls, and the cation is usually sodium. The water-solubilizing groups of basic dyes are usually amines, and the anion is usually chloride. Both acid and basic groups may be present in one dye, in which case their relative strengths determine the over-all net charge. Some of the more commonly used acid dyes are: acid fuchsin, alizarin, Bismarck brown, Congo red, eosin Y, erythrosin, fluorescein, Janus green B, orange G, phloxin, picric acid, rose bengal. Some of the more commonly used basic dyes are: acridine red, aniline blue, the azures, basic fuchsin, crystal violet,

light green, malachite green, methylene blue, methyl green, pyronine, safranine, and thionine. To this list should be added the lakes (see p. 43).

Staining with dyes for light microscopy consists primarily in staining proteins. In the fresh undenatured state, proteins seem to show little tendency to take up dye. After denaturation caused by the fixative, the tendency is more marked. This increase is presumably attributable to some physical change in the protein resulting in exposure of a larger number of reactive groups.

Most of the staining properties of most proteins are referable to their amphoteric nature and to their conjugated groups. These include free acidic or basic side groups, terminal acidic and basic groups, and the acidic phosphoric, uronic, sulfuric, and carboxyl groups of the conjugated components. The net charge of the protein depends on the number of dissociable groups and on their degree of ionization. The latter depends primarily on the pH of the staining solution. When the aqueous solution is on the acid side of the isoelectric point of the protein, the dissociation of the acid group is depressed and that of the basic groups raised, with a net fall in the negative charge. In this condition, less basic dye will combine with proteins and the reverse takes place with acid dyes. There are, however, a number of exceptions to this rule, suggesting that forces other than salt linkage play important roles in the combination of proteins with dyes. Other forces suggested are resonance bonds, van der Waals' bonds, and covalent bonds. The latter may indeed be the primary factor in special situations, as for example in the staining of elastic fibers (Dempsey and Lansing, 1954).

One would expect that if salt linkages were the dominant force, proteins would show little tendency to bind a particular acid or basic dye exclusively. On the other hand, some dyes are bound more readily than others, depending on the dissociation constants of the dye, the size and composition of the dye, and the shape of the dye molecule.

The dependence of the staining of proteins in solution and in sections on the pH of the solution has been used to estimate their isoelectric range. For this purpose, the isoelectric range is defined as that pH range at which there is equal (minimal) retention of acid and basic dyes. The isoelectric range is affected by the dye pairs used and the kind of fixative used. The important effect of the fixative has been explained by the introduction of ionizing (hence dye-binding) sites, by the interference with certain reactive groups (as formaldehyde with amines, mercuric chloride with carboxyls), and by the mordanting action of the polyvalent metal (see p. 43).

The temperature of the staining solution influences the rate of stain-

ing chiefly by its effect on rate of diffusion and perhaps swelling of the protein. The latter may be especially important when the dye exists wholly or very largely as colloidal aggregates.

The ionic strength of the staining solution has a marked effect on staining with acid and basic dyes: increase of ionic strength reduces the amount of dye taken up. Increase in the concentration of the dye increases the rate of staining.

As staining with acid and basic dyes is largely interpretable in terms of ionic reactions, destaining may be understood in the same way as the result of a shift in an equilibrium reaction. The strongest influence in destaining is the pH of the destaining solution: increase of pH is effective in removing acid dyes; decrease of pH in removing basic dyes.

It would seem obvious that the term basophilia is only a relative one, for by controlling the various factors discussed it is possible to reduce basophilia almost to extinction or to increase it up to a certain maximum. Moreover, as when the staining of nucleic acids is suppressed and the avidity of the associated proteins for acid dyes is heightened, it becomes possible to stain nucleoproteins with acid dyes. The degree of basophilia varies, depending on the fixative, the pH of the staining solution, the nature of the dyes used in staining, the concentration of the dyes, etc. This is especially important in analyzing the significance of staining with methyl green-pyronine.

1. Mordants

Many dyes are used in conjunction with mordants. These are incorporated in certain fixatives, especially those containing osmium, calcium, iron, copper, or chromium. These are bi- or multivalent metals which have the property of combining on the one hand with reactive groups of proteins or lipids, and on the other, with dyes. Destaining with the appropriate reagent results in selective staining of some neurological components, intracellular granules, Golgi apparatus, and certain cellular lipids, especially those containing unsaturated bonds. Mordants are also used as lakes, which are combinations between certain dyes and certain di- or multivalent metals that serve to attach the dye to the tissue component. All lakes are strongly basic. The most common mordants used are double salts of potassium or ammonium with aluminum or ferric sulfate. The most common lakes are the various hematoxylin, carmine, and alizarin stains. Lakes may stain progressively or regressively. The progressive stains are self-terminating, as for example gallo-cyanine-chrome alum. The regressive stains all overstain and must be destained subsequently, as, for example, iron hematoxylin. In this instance, selective destaining is achieved by decrease of the pH of the

destaining fluid, by using excess mordant to shift the direction of the equilibrium so that dye leaves the tissue, or by bleaching of the excess dye.

2. Staining for Substances Containing Strongly Acid Groups

It was pointed out (p. 42) that staining of proteins by basic dyes is depressed to a minimum by increase in the acidity of the staining solution. This range of minimum staining varies in different structures and components, depending on the degree of dissociation of the anion. For example, staining with methylene blue of cornea, Wharton's jelly, synovial fluid, and vitreous humor was minimal at pH 4.0, while hyaline cartilage and nucleus pulposus continued to stain strongly even at pH 1.5. This difference in stainability probably reflects the occurrence of the weaker carboxyl groups in the first group of structures, and the stronger sulfate groups of the second group. Combined with the use of enzymes with specific activity, this method is particularly useful for indicating the probable presence of conjugated groups containing carboxylic, phosphoric, or sulfuric acid radicals (Dempsey and Singer, 1946; Dempsey *et al.*, 1946; Wislocki and Dempsey, 1946; Dempsey *et al.*, 1947; Pearse, 1949). A successful search for specific blocking agents such as that described by Friedenwald (1947) would increase the probability.

3. Metachromatic Staining

This is the staining of certain tissue components in a color different from that of the dye solution used. The phenomenon is called metachromasia. The substrates which are stained metachromatically are called chromotropes. The most common metachromatic dyes, which are all basic, are Toluidine Blue O, thionine, methylene blue, the azures, crystal violet, and pinacyanol. For example, the normal or orthochromatic blue color of Toluidine Blue in stained sections is replaced by red, pink, or violet where it selectively stains structures metachromatically. Dyes in their metachromatic state have an absorption curve different from that of the normal or orthochromatic state, in that a new and/or heightened absorption band appears at shorter wavelengths in the visible spectrum. The most common explanation of the appearance of a heightened so-called μ band is that the dye in solution has changed from a monomer to aggregate as dimers or higher polymers. The first reaction of the dye seems to be that of a simple salt linkage with the chromotype, with secondarily an association of adjacent dye molecules on the chromotrope to form the dye dimer or polymer.

In general, all chromotropes are anionic or negatively charged in aqueous solution, and of high molecular weight (or with the capacity

of associating to form micelles of high molecular weight under the conditions of staining). The degree of metachromasia seems to be greatest with sulfate polymers (heparin), least with carboxyl polymers (hyaluronic acid), and intermediate with phosphate polymers (nucleic acids). The degree of metachromasia is also influenced by the temperature of the staining solution, the concentration of the dye, the concentration of the chromotrope, the ratio of the concentration of the chromotrope to the concentration of the dye, and the salt concentration and protein content of the dye-substrate environment. Metachromasia is reduced or abolished by dehydration with alcohol. That which persists has been called "true" metachromasia, while the rest has been called "false." Largely because metachromatic staining of certain structures (mast-cell granules presumably containing heparin, or cartilage matrix containing chondroitin sulfate) resists treatment with alcohol, it has been claimed that true metachromasia suffices to identify the presence of a polysaccharide sulfate in a structure. "There can be little doubt that polysaccharides containing ester sulfate groups show the most marked alcohol resistant metachromasia. But the idea that alcohol resistant metachromasia of tissues of unknown composition indicates exclusive presence of polysaccharide ester sulfate would still not seem justified" (Schubert and Hamerman, 1956). Essentially the same view was expressed by Hale (1957) after a very judicious survey of the literature.

B. Staining of Lipids and Steroids

Lipids may be stained directly in frozen sections or in sections of frozen-dried specimens infiltrated with Carbowax (Hack, 1952). Staining is done by any one of a series of dyes through their differential solubility in the lipid. These include the Sudan dyes¹ (dissolved in various lipid solvents and in hydrotropic solutions), the fluorescent dye 3,4-benzopyrene (dissolved in hydrotropic solutions), and aqueous Nile blue, rhodamine B, and phosphine 3R (Pearse, 1954b).

Lipids may be made insoluble and then stained. Bichromate, osmium, calcium, and cadmium solutions are used for this purpose. Bichromate oxidizes the unsaturated bonds of lipids, which become insoluble and may remain so even after embedding in paraffin. The insoluble lipids are visualized by use of one of the colored fat-soluble stains mentioned above, or by forming a lake with a dye like hematoxylin. Osmium also oxidizes unsaturated lipids, which may be recognized because of the deposit of the lower oxides of osmium. The latter may also remain attached to the insoluble lipid and serve as a mordant in the formation of gallic acid lakes (Wigglesworth, 1957).

The apparent inability to distinguish between aldehyde and ketone

groups in tissues, despite the development of several colored reagents which combine with them, has thrown doubt on claims for specific staining (or identification) of certain steroids (Gomori, 1952b; Lison, 1953).

The only lipid test about whose reliability there has been some measure of agreement is that of Feulgen and Voit (1924) for plasmal—an acetal phosphatide in which the saturated long-chain fatty aldehyde is joined to glycerol by an acetal linkage. The specificity of this reaction is assured by the use of certain blocking agents as emphasized by Danielli (1953), Pearse (1954a), Hayes (1949), and Cain (1949).

The identification of specific lipids by means of stains is not very reliable. Not all lipids can be stained. In addition, the miscibility of many lipids seems to affect their extractability by solvents, which limits the use of differential solubilities based on those of purified lipids as a means of identifying them. There are reservations even for the rough characterization of lipids by stains, the end result of a progressive analysis being suggestive only (Cain, 1950; Lison, 1953; Pearse, 1954a). Now, with the increased resolution of the electron microscope available, the need for the development of methods for the staining and characterization of lipids in protoplasm (especially the masked lipids) is more pressing than ever.

C. Staining with Fluorescent Dyes

The principles of the use of fluorescent dyes for staining are in general the same as those governing the use of nonfluorescent dyes. In general, because of their sensitivity, they are used in very low concentrations and the staining times are short. The most useful basic dyes appear to be berberine, acridine orange and yellow, and coriphosphine; the most useful acid dyes appear to be primulin and thioflavine S. Several are useful as fat stains (see p. 45).

The most important property which underlies other uses of fluorescent dyes is the fact that fluorescence can be detected in extreme dilutions with a suitable microscope and light source. This made possible the identification of riboflavin, vitamin A, and thiamin in the low concentrations in which they occur normally (Sjöstrand, 1944; Popper, 1944). The administration of dye permits the study of supravitally stained cell nuclei (De Bruyn *et al.*, 1950), the circulatory and biliary system of liver (Ellinger and Hirt, 1929), renal excretion (Grafflin, 1947), blood vessels, and pinocytosis (Holter and Marshall, 1954). The extreme sensitivity also makes possible the localization of certain kinds of naturally fluorescent dust particles after inhalation (Yagoda and Donahue, 1945). When specific antibodies of high titer are concentrated and coupled with a fluorescent dye, the complex may be used to identify

the antigen in suitably prepared tissues; as, for example, adrenocorticotrophic hormone identified in certain cells of the adenohypophysis (Marshall, 1951), chymotrypsinogen, procarboxypeptidase, deoxyribonuclease and ribonuclease in the exocrine portion of the pancreas (Marshall, 1954), perhaps insulin in the endocrine portion of the pancreas (Lacy and Davies, 1957), and myosin in the muscle fiber (Finck *et al.*, 1956; Holzer *et al.*, 1957). Similarly the method has been successful in the identification of infectious agents and tissue-specific antigens. A very comprehensive discussion of these subjects is to be found in an article by Coons (1956) and in another by Price and Schwartz (1956). Fixation methods are dictated by the need to preserve antigenicity; freezing and drying, and freezing and section-drying have been the methods of choice.

D. Specific Staining after Extraction

The differential solubility of proteins is one of their most delicate properties. It has been used to analyze the contents of thyroid gland follicles, which stain with the Hotchkiss method (Gersh, 1949), and the ovarian-stimulating hormones in certain basophilic cells of the adenohypophysis (Catchpole, 1949). These substances were stainable with the Hotchkiss method after denaturation, but were absent and hence not stainable after extraction of the undenatured frozen-dried sections with certain reagents. It was possible also by staining sections of frozen-dried material before and after extraction by a series of buffers to approximate the isoelectric point of some specific components. For this purpose, the pH of minimum solubility (and maximum stainability) was regarded as the isoelectric point. (See p. 42 for a consideration of isoelectric range with acid and basic dyes.) The method has also been used to characterize the state of aggregation of ground substance of connective tissue. Readily soluble, Hotchkiss-positive material was interpreted as less highly aggregated than similarly staining material which resisted extraction (Gersh and Catchpole, 1949).

A similar method was proposed by Barrnett *et al.* (1956). They extracted the adenohypophysis with a variety of reagents and correlated the presence or absence of certain granules in the basophile cells with an analysis of the extract for hormonal activity. While in general they were able to confirm the findings of Catchpole, there are certain reservations about a general extension of the method. The reservations arise from the fact that when a wet specimen is penetrated by the reagent, its concentration varies from zero to full strength over a finite period of time. Extraction of a substance or activity from an original structure could take place at any concentration up to that of the extractive.

This method is inapplicable to the identification of lipids (see p. 40).

E. Staining of Nucleic Acids

The methods for the identification of nucleic acids have been critically reviewed recently by Kurnick (1955), Swift (1955), and Walker (1956). The chief staining methods in use are the Feulgen method for deoxyribonucleic acid, and staining of both kinds of nucleic acid by basic dyes, supplemented by the use of specific enzymes and extractives. Present evidence is largely consistent in showing that the Feulgen method is specific for deoxyribonucleic acid. A negative test, however, may indicate that some of the reactive material which combines with the leucofuchsin has been dissolved during fixation or in the various steps of the test, or that the reactive material is present in small amounts in submicroscopic structures and the color is too weak to see.

Walker and Yates (1952), for example, find a consistently lower content of deoxyribonucleic acid by the Feulgen method than is estimated from the same nuclei in the living state. Woods (1957) found that a certain labile fraction, which may be quite appreciable, is lost during the staining procedure. The theory of staining and the numerous possibilities for error during spectrophotometric measurements are analyzed in great detail by the first three authors cited above and by Swift and Rasch (1956).

The staining of nucleoproteins by basic dyes may be interpretable largely in terms of ionic forces (page 42). When so considered, staining of both types of nucleoproteins should take place indiscriminately with all basic dyes. The discrimination of ribo- from deoxyribonucleic acid may then be made by the use prior to staining of selective enzymes (ribonuclease or deoxyribonuclease) or of certain extractives, such as solutions of sodium chloride, perchloric acid, or tricholoroacetic acid under strictly controlled conditions. The degree of separation achieved by the solvents is not yet agreed on, variables being the nature of the fixative and of the specimen, and the time, temperature, and concentration of the reagent. Loring *et al.* (1947) and Lagerstedt (1957) present evidence that ribonuclease may not remove all ribonucleic acid, and Zamenhof and Chargaff (1949), that deoxyribonuclease may not remove all deoxyribonucleic acid.

There seemed to be, and still is, no reason to expect to find basic dyes with a selective affinity for one or the other nucleoprotein, such as is claimed for the use of the methyl green-pyronine method of Pappenheim and Unna reintroduced into cytology by Brachet (1940). When this staining procedure is followed precisely with normal specimens,

deoxyribonucleic acid is stained green and ribonucleic acid is stained red. Under certain conditions, despite the fact that both dyes are basic, methyl green alone stains only the former, while pyronine alone stains both. Kurnick (1955) especially favors the view that the apparent specificity of methyl green is for the highly polymerized state of nucleic acids alone and that it does not stain ribonucleic acid or "depolymerized" deoxyribonucleic acid because they are not sufficiently highly aggregated. When nuclei are stained red with dye mixture, this is accordingly interpreted as the consequence of depolymerization of the deoxyribonucleic acid of the cell nuclei. Swift (1955) gives reasons for questioning this interpretation and suggests alternatively that the altered staining may be caused by changes in the amount or nature of the associated proteins.

F. Histochemical Staining of Proteins

Various types of proteins have been identified by staining methods. The examples in this section were selected only to illustrate different principles.

1. Specific Staining of a Minor Component

The best example of this kind of staining is the brilliant red color of mucigen granules and mucin with the periodic acid Schiff method independently described by McManus (1946), Lillie (1947), and Hotchkiss (1948). The method consists in the controlled oxidation of vicinal hydroxyl or amino-hydroxyl groups to aldehydes by periodic acid, and their combination with leucofuchsin to form the red dye. The use prior to staining of enzymes to remove polysaccharides and of lipid extractives to remove most or all of the reactive lipids, together with the use of blocking agents on other control specimens, results in a red staining of mucoprotein which is probably specific. The protein is thus identified by the staining of a small component, which characterizes the protein.

This identification may be confirmed by some additional properties, such as the determination of the isoelectric range by the use of acid and basic dyes (page 41), the extinction of staining with a basic dye in solutions of differing pH (page 42), the isoelectric point as determined by solubility (page 47), the solubility in different reagents (page 47), or by metachromatic staining (page 44). The use of certain radioactive exchange compounds may also be helpful.

2. Staining with Immunochemical Specificity

Fluorescent dyes are firmly linked to antibodies without impairing their reactivity with intra- or extracellular antigen which stimulated their

synthesis. The procedure is designed (ideally) so that only the site of combination of antigen and fluorescent antibody should be visible. The specificity is theoretically that of immune reactions, and the identification of a protein depends on certain specific immunochemically reactive parts, which may be of limited extent. Denaturation if severe enough, or chemical blocking if extensive or specific enough, would reduce or abolish the sensitivity of the antigen-antibody reaction. This most promising method of staining has already been applied fruitfully (page 46).

3. Specific Staining by Means of Autoradiography

A very good example is the identification of iodinated proteins by their emission of beta particles as studied in the thyroid gland (see Boyd, 1955, for references). The method in this instance is specific and sensitive, though it has a maximum resolution of about 1μ and is only roughly quantitative. The slight to large losses which take place in aqueous and alcoholic fixatives (Kaminski, 1955; Hagmüller and Hellauer, 1955) make freezing and drying or freeze-substitution almost mandatory. The broad application to other simple and conjugated specific proteins is limited to those which can be characterized by the method of solubilities or specific enzymatic action.

4. Staining of Specific Enzymatic Reaction Products *

The ultimate object is the identification of the locus of a particular enzymatic protein through description of sites where the products of enzymatic activity are located. For light microscopy this calls for accurate, specific, sensitive detection of generally a colored or light-absorbing product of enzymatic activity. Ideally, a successful test should be confirmed by quantitative studies on the kinetics of the reaction. This has been achieved only in part in analyses of the Gomori method for alkaline phosphates (Doyle, 1953), and may be approached by use of the interference microscope as a means of detecting increase in mass at a specific site during the development of the histochemical test (Barter *et al.*, 1955). Quantitation is important also for the analysis of change in functional state. Quantitation is especially important for a proper interpretation of the observed distribution of an enzyme activity. If, for example, only a portion of the normally occurring enzymatic complement of a cell structure is active, and if this particular portion of the total enzymatic complement is lost during fixation, dehydration, embedding, section-mounting, and solution or inactivation in the testing reagents, and if the

* After this section had been written, a more extensive analysis by M. M. Nachlas, A. C. Young, and A. M. Seligman appeared in *J. Histochem. and Cytochem.* 5, 565-583.

remaining enzyme complement is visualized entirely satisfactorily, then the latter may be misleading in terms of normal cell physiology. On the other hand, the pattern of distribution of the fraction of the enzyme complement which survives the technical manipulations may successfully identify which cells of a group of tissues, or which tissues, are responsible for this particular enzymatic function. In this connection, it would be useful to ascertain whether the enzymatic activity observed was present in a more or less readily soluble or more or less fixed state in the living cell. This cannot be determined with certainty by the very nature of the testing procedure, for as soon as a cell is injured or altered (as by fixing or sectioning) a more or less soluble enzyme may become more or less immovable by entrapment in a close-meshed net of surrounding proteins. Similarly a relatively insoluble enzyme could become soluble through swelling or solution of the protein matrix to which it was attached before fixation. The terms lyo- and desmoenzyme in histochemistry are operational, and it is important to realize that the conditions may be quite different from the operational usage of these terms with biochemical extracts.

When one considers whether a histochemical method is accurate or precise, one wonders at what level this is so. There is little question that despite the difficulties, certain histochemical tests for enzymatic activity are probably satisfactory at the tissue level (for example, alkaline and acid phosphatase, lipase). Others are probably accurate at the cellular level (for example, esterase, cholinesterase, sometimes alkaline phosphatase). Some methods are probably applicable at the level of cell organoids (for example, succinic dehydrogenase). None have yet been proved to be accurate at submicroscopic levels, though the probability is good that this is so with certain enzymes in sperm (see page 58).

All histochemical staining methods for enzymatic activity involve the exposure of sections to specific substrates. The reaction product in nearly all instances is precipitated eventually in a form visible with the light microscope. In this process, two possibilities of error arise—diffusion of the enzyme and diffusion of the reaction product. The reduction and control of these two factors has called for the exercise of admirable ingenuity, especially in the tailoring of organic molecules used as substrates. The contribution of organic chemists in recent years has been most notable.

In the remaining part of this section, a few types of methods developed for the localization of some enzymes will be outlined briefly.

Gomori method for alkaline phosphatase by staining phosphate (Gomori, 1952a): Sections are incubated in a buffered solution (pH 9.0) of β -glycerophosphate containing calcium and magnesium ions. After a

rinsing with water, sections are treated with cobalt acetate, rinsed again, and treated briefly with a solution of ammonium sulfide. Phosphate is liberated from the ester by enzymatic activity and precipitated as calcium phosphate which is converted to cobalt phosphate, and this in turn to the black cobalt sulfide. Apart from the losses which take place during fixation and embedding, there is some question about diffusion of phosphate at sites of very low and very high activity. For this reason, short periods of incubation are preferred. The conversion of the calcium phosphate to the cobalt sulfide may not be quantitative (Doyle *et al.*, 1951). The occurrence of precipitated phosphate in nuclei and the spreading to neighboring tissues are regarded as indications of diffusion.

Method for alkaline phosphatase by staining for aromatic part of ester: The simultaneous coupling type reaction was devised by Menten *et al.* (1944). They incubated sections in a buffered solution (pH 9.4) of β -naphthol and diazotized α -naphthylamine. The β -naphthol liberated as a result of enzymatic action coupled with the diazotized compound to produce an insoluble red precipitate. To reduce diffusion and nonspecific staining, the substrates have been modified successively by Danielli (1946), Manheimer and Seligman (1949), and Gomori (1951). A variety of other naphthyl phosphate derivatives and diazonium salts may be used. The following have been recommended by Pearse (1954b): for β -naphthyl phosphate, 6-bromo-2-naphthyl phosphate; 2,4-dichloro-1-naphthyl phosphate; 2,4-dibromo-1-naphthyl phosphate, and for the diazotate of α -naphthylamine, the diazotates of 5-chloro-*o*-toluidine and of *o*-dianisidine.

Method for acid phosphatase by staining for phosphate (Gomori, 1952a): Sections are incubated in a buffered solution (pH 5.0) of sodium β -glycerophosphate containing lead nitrate. After a brief rinsing with water, sections are immersed in dilute ammonium sulfide and washed. Phosphate is liberated from the ester by enzymatic activity and precipitated as lead phosphate, which is then converted to the black lead sulfide. To the possibilities of diffusion of the enzyme and the phosphate should be added the erratic nature of the reaction in practice. This method has been compared critically by Gomori (1956) with the one which follows.

Method for acid phosphatase by staining for the aromatic part of ester: Seligman and Manheimer (1949) proposed a modification of the simultaneous coupling reaction like that used for alkaline phosphatase. Other substrate reagents were proposed to reduce diffusion, and greater accuracy is claimed with the use of α -naphthyl phosphate or 2,4-dichloro-(or 2,4-dibromo-)1-naphthyl phosphate and the diazotate of *o*-dianisidine (Pearse, 1954a). These are dissolved in a buffer (pH 5.0), and the section is incubated in the solution and washed.

Simultaneous coupling azo dye method for nonspecific esterases: Nachlas and Seligman (1949) introduced this method, which was subsequently improved by Gomori (1950). A later modification (Gomori, 1952b) resulted in even less diffusion. Sections are incubated in a neutral solution consisting of Naphthol AS acetate (2-hydroxy-3-napthoic acid anilide) and a stable diazotate, and washed.

Indigo method for nonspecific esterases: Introduced by Barrnett and Seligman (1951), the method has been exploited particularly by Holt (1952, 1956) in a series of very thoughtful articles. A soluble ester of indoxyl is used as substrate under controlled conditions. The free indoxyl is rapidly oxidized to the very insoluble blue indigo dye. After synthesizing a series of derivatives, Holt found the best to be 4,4'-dichloro-5,5'-dibromoindoxyl acetate. Resolution may be as small 1μ . His analysis of requirements for a sensitive, precise, specific test includes the following: (1) The substrate must be hydrolyzed by the enzyme by a zero-order reaction. (2) The hydrolysis product must be converted to an insoluble, colored final reaction product which should be completely nondiffusible. (3) The precision of localization depends on: (a) the diffusion coefficient of the primary product of enzymatic action, (b) the size of the reactive site, (c) the velocity constant of the reaction resulting in the final product, and (d) the tendency to stain or combine with proteins adjacent to or at the site of enzymatic action. Pearse (1954a, b) expressed the opinion that this method would supplant all azo-dye methods, and could be extended to demonstrate phosphatases, sulfatases, glucosidases, and glucuronidases, in addition to esterases.

Method for lipase (Gomori, 1952a): Sections are incubated in a buffered solution (pH 7.0-7.4) of water-soluble esters of long-chain fatty acids with polyglycols or polymannitols (Tweens) in the presence of calcium ions. After being washed, sections are immersed in a solution of lead nitrate, washed again, and treated with a solution of ammonium sulfide. The liberated fatty acids form calcium and then lead soaps and become visible as black crystals of lead sulfide. The results are somewhat irregular, but suitable at the tissue level in certain organs.

Acetylthiocholine method for cholinesterase: Developed first by Koelle and Friedenwald (1949), the method has been perfected by the senior author (1951). Sections are incubated in a buffered solution (pH 6.0) of high ionic strength containing acetylthiocholine, magnesium and copper ions, glycine, and a trace of copper thiocholine. Sections are washed in solutions of thiocholine containing decreasing concentrations of sodium sulfate, treated with a solution of ammonium sulfide saturated with copper sulfide, rinsed, fixed, and dehydrated, cleared, and mounted in fluids saturated with copper sulfide. The dark-brown copper thiocholine precipitate is sharply localized in nerve endings and in certain nerve cells.

Nerve endings were studied with an indoxyl method for esterase first introduced by Holt (1954). Using 5,5'-diindoxyl diacetate, he and Couteaux (1955) described very precise localization of the reduced indigo dye.

Tetrazolium methods for succinic dehydrogenase. The type reaction is represented by reduction of the nearly colorless tetrazolium chloride to a very insoluble red formazan. To improve the color intensity and specificity and reduce diffusion of the end product, several derivatives were synthesized: neotetrazolium-2,2'-(*p*-diphenylene)-bis-(3,5-diphenyltetrazolium chloride) (Antopol *et al.*, 1948), blue tetrazolium—a dimethoxy derivative of neotetrazolium (Rutenberg *et al.*, 1950), and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-(phenyltetrazolium chloride) (Atkinson *et al.*, 1950). Nachlas *et al.* (1957) synthesized and applied a superior reagent, Nitro BT (2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-3,3'-(3,3' dimethoxy-4,4'-biphenylene ditetrazolium chloride), which is precise at the cellular organoid level.

Farber *et al.* (1956) extended the use of tetrazolium stains to the identification of certain specific oxidative enzymes by changes in the substrate; i.e. di- and triphosphopyridine nucleotide diaphorase. The Nitro BT compound could be used for the same purpose (Nachlas *et al.*, 1957).

IV. STAINING FOR ELECTRON MICROSCOPY

There is no general agreement on the principles of staining for electron microscopy. The field is still too new, and possible theoretical and practical approaches are fluid. Cytochemical stains are even more exploratory. For this reason this section, by comparison with others, consists in part of critical compilations, with trends appearing in some aspects.

A. Morphological Stains

1. During Fixation by Immersion

The end product of all electron microscopic work is a photographic image in which parts are recognizable because they are darker or lighter than other parts, that is, because they differ in contrast. Such differences in contrast have been recognized for many years in unstained collagen fibers (Schmitt *et al.*, 1942; Burge and Randall, 1955) and are also apparent in unstained frozen-dried material. The contrast becomes greater when stains are used to increase the mass of certain sites selectively through their combination with inorganic substances containing certain elements, chiefly those of high atomic weight. The following is a partial

list of such elements: osmium, molybdenum, copper, lanthanum, silver, chromium, gold, iodine, lead, mercury, platinum, tungsten, cadmium, uranium. Some organic compounds, with or without heavy elements, have also been used: methylene blue, hematoxylin, mercurochrome, chloramine-T, orcein. Several others containing iron, iodine, or bromine have recently been proposed by Locquin (1955). The use of many of these stains is limited by the quality of the fixation coincidentally effected by the stain, or of the fixation prior to staining. It is doubtful, for example, that any stain will show mitochondrial membranes after picric acid fixation. Lamb *et al.* (1953) proposed a method of staining by introducing silver into an organic molecule and made some preliminary estimations of increased contrast caused by the stain.

There are at least fifty examples in the literature where fixation by a small molecule such as formaldehyde was sufficient to introduce or retain enough contrast for clear recognition of certain structures. (See also Bradfield, 1953; Afzelius, 1956; Maaløe and Birch-Andersen, 1956). There are also instances in which an atom of moderate atomic weight could produce more contrast in the same structure than another with more than three times that atomic weight (Luft, 1956). Ornstein (1957) cites other evidence in an analysis that questions the primacy of osmium in the production of high contrast in sections of material fixed in osmium solutions. Following is a partial list of other factors involved in contrast in addition to staining: (1) the occurrence of unequal intrinsic local densities, as, for example, that caused by deoxyribonucleic acid in the chromatin of the nucleus; (2) swelling and the consequent local decrease in mass; (3) extraction of substances between two regions; (4) swelling or solution of some components, displacement, and deposition on some other structure; (5) the pattern of separation of the solid from the liquid phase of protoplasm (see page 23).

2. After Fixation by Freezing and Drying

Freezing and drying of specimens without gross displacement of the solid components of protoplasm permits use of a greater variety of stains. Some of the metals used in stains made up in 95% alcohol include: copper, gallium, gold, indium, iron, lead, mercury, molybdenum, platinum, tin, tungsten, uranium, vanadium, zinc (Gersh *et al.*, 1957a). In addition, it is possible to use organic dyes: Azure II (Finck, 1955), leuco-fuchsin (Bondareff, 1957), orcein (Chase, unpublished data). Other mordant dyes like gallocyanine-chrome alum (Finck, 1958) and iron hematoxylin (Vergara, unpublished data) are also effective. Isenberg (1956, 1957) developed a theoretical analysis and proposed the following mechanism of staining: the stain must combine in high concentration

with certain tissue elements, and the molecular density of the dye must be higher than methacrylate, the embedding medium. Heavy atoms need not necessarily be present. In general, his analysis shows that common histological organic dyes may be used profitably as electron microscope stains. This interpretation of actual findings has been questioned by Ornstein (1956), who states: "The differential increase in specimen contrast which can be produced after treating with reagents such as OsO₄, depends on a stabilizing effect of the reagent on the cell structures such that material that was easily sublimed by the electron beam before treatment is stable afterwards." This statement is based on the work of Morgan *et al.* (1956), but the derivation of the inference is unclear. In either way, the site of deposition of the stain, inorganic and organic, remains the same whether the increased contrast is caused by the increased mass at the site due to the deposition of dye or to the "stabilization" by the stain of cell structures or components in the electron beam. Incidentally, it is difficult to see how the electron microscope could distinguish between organic material originally present and organic material added in the form of a dye.

B. Cytochemical Stains

1. After or During Immersion Fixation

Just as the development of histology paced histochemistry in the last century, cytochemistry remains not far behind the development of knowledge of the fine structure of cells. At even this early date, a number of cytochemical studies have appeared. Morgan and Mowry (1951) attempted to describe the distribution of glycogen in liver cells of humans many hours after their death. Fawcett (1955), Luft (1956), and Wislocki *et al.* (1957) inferred the locus of glycogen (in liver cells and in metrial gland cells of the gravid rat) by comparison of material fixed in buffered osmium solution or in potassium permanganate solution and studied with the electron microscope, with material fixed, stained selectively for the glycogen and viewed with the light microscope. While this method is effective when interpreting relatively large, homogeneous bodies such as starch granules (Strugger and Perner, 1956; and others), it is subject to doubt when the bodies are small and inhomogeneous, where many inhomogeneities could occupy the space of a structure observed with the light microscope. For this reason, the method of thick and thin sections used by Moses (1956) to translate the Feulgen-positive material observed with the light microscope to submicroscopic structures fixed with buffered osmium solution and observed in the electron microscope seems to be of dubious value. Bretschneider (1949) developed a

complicated method designed to deposit silver specifically where deoxyribonucleic acid occurred in the heads of sperm. The method was modified later by van Winkle *et al.* (1953). However, both procedures seem to be too drastic to preserve fine structure. Gay (1956) was successful in using deoxyribonuclease on salivary gland nuclei fixed in a variety of ways. The result was a marked reduction in electron scattering of the chromosomes, as also of certain granules in nuclear blebs. Bernstein (1956) presented convincing evidence for the specific staining of the deoxyribonucleic acid core of the T₂ phage of *Escherichia coli* by the use of ferric chloride.

A series of enzymes has been localized by the extension of the cytochemical technique suitable for study with the light microscope to electron microscopy. The procedure of Sheldon *et al.* (1955) was to immerse the intestine briefly in buffered osmium solution, wash, and expose the specimen to the Gomori reagents for a long time. Increased contrast over and above that attributable to the specimen fixed only in osmium solution indicated to the authors the sites of acid phosphatase activity. The later finding of Novikoff *et al.* (1956) that appreciable amounts of enzyme activity resisted brief fixation (in subsequently homogenized specimens) is not sufficient to prove that any enzyme activity survived in the regions fixed by the osmium solution. The same general comment may apply to the attempt to demonstrate the sites of alkaline phosphatase activity in intestinal epithelium by a similar procedure (Brandes *et al.*, 1956). Brandes and Elston (1956) described the end products of the method for alkaline phosphatase in the cell wall of *Chlorella vulgaris*. A more convincing experiment showing that some esterase activity survives fixation in buffered osmium solution was described by Novikoff and Holt (1957), who followed up the earlier studies of homogenized organ pieces fixed in osmium solution (Novikoff *et al.*, 1956). The site of enzymatic activity was not studied with the electron microscope; it seems certain that contrast caused by the colored indicator of the enzymatic activity would be reduced or wiped out by the background density caused by the prior use of the osmium solution. An even more convincing experiment would consist in a quantitative analysis of sections for enzyme activity and osmium after brief fixation. Barrnett and Palade (1957) described the use of potassium tellurite as an indicator of succinoxidase activity in mitochondria of cardiac muscle. The prolonged incubation time and the appearance of the end product as crystals suggest there may have been an opportunity for diffusion of both the enzyme and the end product.

Buffered osmium solution has been used for the visualization of catechol amines (among which are adrenaline (epinephrine) and nor-adren-

aline (norepinephrine)) by Sjöstrand and Wetzstein (1956), and by De Robertis and Vaz Ferreira (1957a, b, c). The numerous other cellular structures showing high contrast after the use of this fixative raise the question of specificity of the method. Dense particles have been described in polychromatophile erythroblasts, which are thought to be iron particles 40-100 Å. in diameter (Bessis and Breton-Garius, 1957), and in hemosiderotic hepatic and splenic cells, where the dense particles were about 55 Å. in diameter (Richter, 1957). Kuff and Dalton (1957) also describe similar particles in hepatic cells. In all these instances the dense particles were believed to resemble the ferritin particles described by Farrant (1954). Bradfield (1956) has summarized evidence on the occurrence of chemical substances in bacteria as observed with the electron microscope.

It should be pointed out that, just as fixation by osmium solutions prohibits or interferes with the later use of many stains in light microscopy (Romeis, 1948; Baker, 1950), it seems also likely to interfere with cytochemical stains for electron microscopy, though for different reasons. Through combination with, or reduction by, a vast number of chemical substances, it must impart a background density which tends to obscure added density from some specific chemical reaction (Bahr, 1954). In addition, even brief contact with osmium seems to reduce or abolish the activity of enzymes. It may be of some interest to list some of the substances which have been identified in protoplasm because osmium is thought to be reduced or not reduced by the substance in question: deoxyribonucleic acid, ribonucleic acid, lipid or lipoprotein, protein, glycogen, catechol derivatives (adrenaline or nor-adrenaline), starch.

2. After Freezing and Drying

A number of stains have been used on material prepared by freezing and drying. Bahr (1957) published an electromicrograph showing the increased contrast caused by methyl mercury chloride in nucleoli of salivary gland cells of *Chironomus* larvae. This compound was described three years earlier with Moberger (1954) as a means of identifying protein-bound sulfhydryl groups. Afzelius (1956) also used the same reagent as well as *p*-chloromercuribenzoate to identify sulfhydryl groups in certain cortical granules of sea urchin eggs. In both instances, the mercurial compounds increased contrast in the electron microscope. Nelson (1957a) used *p*-chloromercuribenzoate and *N*-ethylmaleimide on frozen-dried sperm. Enhanced contrast occurred in the matrix, the helical coil, and the peripheral fibers of the sperm tail.

Glycogen has been identified in liver cells by the use of the periodic acid-Schiff method. The increased contrast caused by the added dye was

lacking with prior digestion of the specimen in saliva (Bondareff, 1957). Gallocyanine-chrome alum has been used to stain ribo- and deoxyribonucleic acid in liver cells (Finck, 1958) and in the nuclei of salivary gland chromosomes of *Drosophila* larvae (Isenberg, unpublished data). The increased contrast caused by the mordant-dye complex was not observed in the small particles of the chromatin and chromosomes when deoxyribonuclease was used prior to staining. In the same way, the small concentrates in the nucleolus and cytoplasm which appear after gallocyanine-chrome alum were barely detectable when the preparations were digested with ribonuclease prior to staining.

Nelson (1957b) studied also the distribution of certain enzymes in sperm tail after freezing and drying. The site of adenosinetriphosphatase activity was visualized in the peripheral fibers, which developed increased contrast due to the deposit of calcium phosphate. Alkaline phosphatase was more prominent in the helical coil than in the fibrils, the increased contrast in this instance having been caused also by the deposited calcium phosphate. Succinic dehydrogenase was visualized by the increased density of the fibrils, attributable to the deposition in these sites of formazan (from polymeric tetrazolium in one series of preparations) and to the formation of an enzyme-inhibitor complex with malonate in a second series of preparations (Nelson, 1958). It seems clear that cytochemical stains of different sorts may be used with material fixed by freezing and drying, all of them serving as indicators of sites of enzymatic activity through increasing mass locally and thus increasing contrast in the electron microscope: (1) insoluble inorganic reaction products, which need not be colored; (2) insoluble organic reaction products, which need not be colored; (3) combination with specific reagents which act as specific enzyme competitors, specific enzyme inhibitors, or form specific complexes.

A very interesting example of histochemical staining studied with the electron microscope is the detailed study of the distribution of ferrocyanide in the ground substance of connective tissue between muscle fibers. Chase (unpublished data) found that with the light microscope ferrocyanide appears in the form of discrete droplets of about $1-2\mu$, confined to the connective tissue. These are further resolvable as clusters of droplets of about $1/4\mu$. With the electron microscope, these are still further resolvable into clusters of smaller vacuoles of about 600 Å. or more, with sharp edges. There was no indication of diffusion of the ferric ferrocyanide, which appeared in marked contrast because of its high molecular weight and high concentration. The possibility of detecting with the electron microscope other diffusible substances through suitable staining is very clear.

3. Calcium Deposits

The chief insoluble inorganic component of the body consists of calcium crystals. The difference in mass between them and the organic matrix of connective tissue is so great as to make it unnecessary to stain them to increase their contrast (Scott and Pease, 1956; Speckman and Norris, 1957).

C. General Comments

In general, cytochemistry with the electron microscope suffers from all the defects of cytochemistry with the light microscope, and in addition, some others. With the light microscope, specificity, sensitivity, diffusion, and fixation are the major problems. With the electron microscope, in addition to these difficulties are others which enter from the lack of color and the more stringent technical requirements associated with the increased resolution of this instrument. The greatest of these is the retention of fine structure of protoplasm despite the vigorous chemical and physical treatment incurred by the specimen during the cytochemical manipulations. Ideally, every cytochemical procedure requires that the substance be treated as if it were in solution, yet it must remain insoluble and not shift more than a few Ångström units.

There are, however, certain cytochemical procedures which are simpler in electron microscopy than in light microscopy. These arise from the fact that it is only increased mass in circumscribed or limited sites which becomes important, thus obviating the element of color intensity or opacity. For example, in the Gomori technique, it is unnecessary to "convert" calcium phosphate to cobalt sulfide, thus avoiding successive treatments with aqueous reagents. Again, by relying on complex-formation and inhibitor or competitor combinations to increase mass locally, numerous specific, sensitive reactions become possible in electron microscopy. Especially advantageous is the fact that the same reagents in the same concentrations used biochemically for the identification of certain groups can be used in cytochemical work. Thus it seems that the same increase in density exploited in the study of the distribution of alkaline phosphatase with the interference microscope by Barter *et al.* (1955) may become more generally extended to electron microscopy.

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CHAPTER 3

Autoradiography

By A. FICQ

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I. INTRODUCTION

Autoradiography (or radioautography) dates from the discovery of radioactive elements by Becquerel (1896), for this first detection of radioactivity was by means of a photographic plate.

The present chapter will be concerned with the application of the technique in determining the site and the amount of radioactive substance in biological material.

At the time, Becquerel's discovery could not have a great repercussion in biology because, potassium excepted, natural radioactive elements are neither very frequent nor very abundant in living matter. Therefore, autoradiography acquired its present significance only after the discovery and large-scale preparation of artificial radioisotopes.

As late as 1953, Pearse was writing, "The routine application of autoradiography will necessarily for many years lag behind its application to research."

This view was very pessimistic: four years indeed were sufficient to allow many biologists to obtain quantitative and reproducible results at the intracellular level by this method, which is often simpler than most of the cytochemical procedures.

The reasons for this success are in particular: (1) the production on

an industrial scale of photographic emulsions sensitive to electrons at minimum ionization, and considerable progress in the manufacturing process, (2) the use of radiotracers in the study of the most varied problems of biology, (3) the synthesis of a great number of labeled molecules acting as specific precursors, coupled with progress in our knowledge of their metabolic pathways. (4) the possibility of obtaining quantitative data in addition to information concerning the localization of radioisotopes.

Owing to this progress and to the number of valid results obtained, many reviews have been devoted to autoradiography. Therefore, we shall refer the readers to those general papers which are especially concerned with the theoretical aspects of autoradiography and where mention is made of the work undertaken with the blackening or grain techniques. Among the most recent pertinent works, the book of Boyd (1955) and the reviews of Doniach, Howard and Pelc (1953), Pelc (1957, 1958), Norris and Woodruff (1955), Fitzgerald (1955), and Taylor (1956) should be consulted.

In the present chapter, we shall be especially concerned with the method of "nuclear emulsions," or "track method," because it has not found so far the place it deserves in the literature. We will discuss its limitations and advantages as compared to the more classical methods; the presentation of results which have been obtained by various investigators will show the utility of this method.

II. OUTLINE OF THE VARIOUS AUTORADIOGRAPHY TECHNIQUES

The techniques of detection and measurement of radioactive radiations are based upon the property of these radiations to ionize the medium they cross. The ionizing power varies directly with the square of the charge of the particle and inversely roughly with the square of its velocity (or energy) down to a certain minimum value after which, for the velocities considered here, it can be assumed to be constant.

The recording of the ionizing particles is performed by special photographic emulsions. First, we will briefly review the radioautographic techniques used prior to the appearance of nuclear emulsions; let us recall that they are based on the observation that total blackening occurs, and not on the study of the individual tracks emitted by radioelements.

The contact method is the simplest and oldest; it consists (Lacassagne, 1924) in pressing the biological sample on the sensitive pellicle, usually an X-ray film. This method, on account of the imperfect contact between the radioactive object and the film, allows only a poor resolution; therefore there are difficulties in localization (Hamilton *et al.*, 1940).

A more refined technique described by Evans (1947), Endicott and Yagoda (1947), and Bourne (1949), is that of mounting histological preparations on the photographic plate used as a glass slip. The section is protected by a celloidin film 2μ thick; but this film often prevents the penetration of the photographic baths. Furthermore, pieces of tissue may be lost while dissolving the celloidin.

The method of Bélanger and Leblond (1946), consists in melting the sensitive gelatin, previously detached from a plate, and placing it onto the sample; the sensitivity of the emulsions they used is much lower than that of the nuclear emulsions. Moreover, this method is handicapped by the following difficulties: when the emulsion is dried, bubbles often appear which prevent good observation; melting of the emulsion increases the number of background grains; it is difficult to apply a homogeneous layer of uniform thickness. This attempt, however, represents great technical progress, since the superposition of the section and its autograph allows resolution at the cellular level by simultaneous observation.

The "stripping film" method, which has been described by Pelc (1951), Andresen *et al.* (1952), Taylor and McMaster (1954), consists in covering the cytological preparation with a sensitive emulsion, 5μ thick, which adheres to a support of inert gelatin, 10μ thick, thereby making the manipulations easier.

The blackening methods, cannot give a very high resolution. Resolution may be defined as the shortest distance between two punctual sources of radiation, resolvable into two distinct images. Resolution is affected by several factors. First, the nature of the particle: if radiation exerts a very localized effect in the emulsion, resolution will be increased. In the particular case of β -emitters, the low energy electrons of H^3 , C^{14} , S^{35} , give better resolution than the more energetic ones of P^{32} . Second, the histological section must be as thin as possible and the distance between it and the film should be reduced to a minimum. Overexposure will also result in imperfect resolution.

It is useful to know the characteristics of the photographic emulsions, especially the concentration and the uniformity of the grains, whose dimensions should not exceed a certain diameter.

Some of these problems were studied in their theoretical aspects by Doniach and Pelc (1950), Berriman *et al.* (1950), Gross *et al.* (1951), Herz (1951), Nadler (1951, 1953), and Dudley (1951).

The greatest criticism which can be presented against all these methods is the possible ambiguity between the grains actually affected by radiations and those due to other causes: (1) mechanical agents: pressure, scratches, shocks; (2) physical agents: high temperatures, cos-

mic rays, red light used during manipulations; (3) chemical agents: reducing substances favoring the production of pseudo images, a phenomenon also observed after the use of certain stains. This last property has been advantageously applied by Boyd and Board (1949) to localize reducing groups *in situ*, in some living tissues, by "histochemography." These effects which decrease the efficiency of the blackening methods, are not important in the "track method" because in this case, only the well-characterized tracks produced by the radiation are considered, and not isolated grains or general blackening over cellular structures.

In 1909, Mügge observed that it is possible to obtain an image of the track of α -particles in a photographic emulsion. A little later, Wilson obtained similar images with his famous "cloud chamber."

A thick photographic emulsion is similar to the cloud chamber by its capacity to distinguish the nature of the ionizing particles. In contrast to the cloud chamber, which has a transitory type of recording, the photographic emulsion registers events in a permanent manner, resulting in an integral representation of the radiations; this fact presents an advantage in the case of low radioactivities.

In the photographic emulsion α -particles produce straight and continuous tracks. Their energies being a line spectrum, they appear in groups of definite length. Neutral particles such as γ -rays and neutrons do not ionize directly. Neutrons may activate other elements (lithium, boron, nitrogen).

The majority of radioisotopes used in biology are β -emitters. They

TABLE I
VALUES CORRESPONDING TO MAXIMUM ENERGY OF SOME β -EMITTERS

β -Emitters	Half-life	Energy ^a (Mev.) (β)
H ³	12.26 Years	0.018
C ¹⁴	5600 Years	0.155
S ³⁵	87.1 Days	0.167
P ³²	14.3 Days	1.71
I ¹³¹	8.04 Days	0.25 (3%) 0.36 (9%) 0.61 (87%) 0.82 (1%)
Ca ⁴⁵	164 Days	0.254
Fe ⁵⁹	46 Days	0.27 (46%) 0.46 (54%) 1.56 (0.3%)
Zn ⁶⁵	245 Days	0.32

^a Figures in parentheses are percentages of components of radiation.

have variable energies which spread over a continuous spectrum, from zero to a maximum value, characteristic for each element. Table I gives the value corresponding to the maximum energy of the electrons of some β -emitters used in biology.

Commercial firms (e.g., Ilford or Kodak) now produce types of emulsion which allow the registration of electrons at all speeds. Their characteristics have been analyzed by Yagoda (1949), Waller (1953) and Harrison (1954).

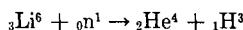
These emulsions are characterized by a very high concentration in AgBr: it is ten times higher than in ordinary photographic material. The grain is rather small, from $0.4\text{ }\mu$ to $0.7\text{ }\mu$ after development, which allows a good definition of the trajectory. Emulsion thickness can be chosen depending on the energies of the particles to be analyzed, e.g., $30\text{ }\mu$ for the dried emulsion in the case of C^{14} .

Even at this stage, electrons are difficult to analyze. At high energy they are weakly ionizing and so their grains are widely spaced. At low energies, they produce tortuous tracks which makes it difficult to follow and to count them, and to determine their orientation.

Electron-sensitive plates stored for some time acquire a high background due to radioactive contamination in the surrounding, in the glass and the gelatin and due to the action of cosmic rays. Liquid emulsion which can be poured fresh for each experiment minimizes this background. Types G₅ and K₅ (Ilford), in gel form, constitute the most sensitive emulsions for this kind of work.

The principle of the autoradiographic method by neutronic irradiation is different: stable atomic nuclei, when bombarded by nuclear particles (e.g., neutrons, protons, deuterons, tritons, α -particles) suffer disintegration. The products of this disintegration, if sufficiently energetic, will leave tracks in the photographic emulsion. The presence of a particular element can then be detected by its characteristic disintegration "star" after bombardment.

A typical reaction which has been used is the disintegration of lithium by neutrons:



When thermal neutrons, which can be obtained in great quantities from a nuclear reactor, are used, Li⁶ gives rise to the emission, in opposite directions, of the two particles: One α (helium nucleus) and one triton (tritium nucleus).

The histological preparation where such elements must be localized is covered by sensitive emulsion (types C₂ or E₁, Ilford, in gel form), dried, shielded from light, and exposed to a neutron beam; a bombard-

ment of 10^{10} neutrons/cm² is convenient for lithium (Faraggi, 1950). Photographic development must be performed as soon as possible after irradiation.

Possible errors and confusion lie in the existence of recoil protons and in the presence of other elements activated at the same time; a background measurement must be made in order to estimate the value of the contamination.

III. TECHNIQUES RELATED TO AUTORADIOGRAPHY

A. Histological Techniques

The purpose of the experiments being to follow the incorporation of a radioactive substance into a cell or tissue, all the chemical transformations eventually undergone by this precursor must be considered prior to the interpretation of the results. It is also important to evaluate eventual damage caused by radiation (Pelc, 1951; Plaut, 1954; McQuade *et al.*, 1956).

Finally one must take into account all the changes which may occur during the fixation of the sample, and it is necessary to modify the classical histological methods because of the presence of the emulsion and because of the photographic procedures.

The radioactive substance may be administered either *in vitro*, being added to the culture medium of the organism, or *in vivo*, by injection in the animal. During fixation and dehydration of the tissue, the solubility of the labeled molecule must be considered and any loss or migration avoided. This problem has been examined by Holt *et al.* (1949), Holt and Warren (1950), Mellgren (1952), Doniach and Pelc (1950), Winterringham *et al.* (1950), Harris *et al.* (1950), Canny (1955), Kaminsky (1955), and Gallimore *et al.* (1955). For these authors, fixation by freeze-drying (Altmann-Gersh method, Gersh, 1938) constitutes an ideal technique in the case of soluble substances.

Our own experiments have shown that the fixation by freeze-substitution, as described by Lison (1953), is more favorable and that it preserves perfectly well the soluble material *in situ* (lipids excepted). On the other hand, the activity of several intracellular enzymes is also well preserved (proteases, ribonuclease, for example).

In such cases we have modified the radioautographic technique, using thick stripping, which is particularly suitable for observing soluble material. To avoid hydrating the sections at any time, we proceed as explained on page 76.

Other methods of freeze-substitution have been described by Simpson (1941), Russell *et al.* (1949), Woods (1955), and Freed (1955).

But, in the case of precursors incorporated into complex molecules like proteins or nucleic acids, the best results are usually obtained by

fixing the cells in a mixture of 3 parts 94% ethanol and 1 part glacial acetic acid for 1 hour and washing them with 70% ethanol. This fixative eliminates many soluble substances whose presence often complicates the interpretation of the observations; one must not forget that this treatment may also remove some material which can be precipitated with 10% trichloroacetic acid.

One can also use the method of Wolman and Behar (1952), in which the fixation is slower and is performed at -8° C . for 4 hours; fixation is followed by washing in absolute ethanol for 12 hours at the same temperature.

All the fixatives containing sublimate, bichromate, and formalin must be avoided because they increase the number of background grains.

Embedding in paraffin is perfectly adequate. The sections are cut with an ordinary microtome at a convenient thickness of $5\text{--}7\mu$. The freeze-microtome may be useful in the case of short-life elements or when the tracer molecule is extracted by organic solvents, but the thickness of the sections decreases the resolution.

Sections are placed on slides, previously coated with a film of alum-gelatin (gelatin, 5%, plus chrome alum, 0.5 gr. per liter) instead of albumin, to which the emulsions stick imperfectly. The slides are deparaffinized and progressively rehydrated by the usual method.

It is recommended, at this point, to wash the labeled sections thoroughly in an identical, but unlabeled, solution; this washing favors the exchange of the isotope not incorporated into the organic constituents of the cell. The slides are then ready to receive the layer of sensitive gelatin.

Prior to this, some authors cover the sections with a protective film (e.g., Plexiglas, Formvar), but the resolving power is of course reduced if this is done.

B. Cytochemical Procedures Applied to Autoradiography

The first autoradiographic attempts had as purpose the localization of radioelements at the organ level. Later on, it soon became possible to determine, even at the cellular level, the structures responsible for the activity.

The cytochemical studies made so far show that it is possible to identify the nature of the radioactive molecule after treatments such as extraction, precipitation, specific staining, or enzymatic digestion. For example, the ribonuclease technique of Brachet (1942) can be used in autoradiography to determine whether the radioactive material is of a ribonucleic acid (RNA) nature. The same can be done with deoxyribonuclease, in the case of deoxyribonucleic acid (DNA).

Unfortunately, limitations to these cytochemical treatments arise from

the sensitivity of the photographic emulsion: reducing agents provoke the precipitation of silver grains; oxidizing agents, on the other hand, accelerate the fading of the latent image. Exposure of the sections to perchloric acid, according to the method of Ogur and Rosen (1950), sometimes leads to a total desensitization of the emulsion.

Hydrolysis of the sections in *N* HCl at 56° C. for 5 minutes, according to the technique of Feulgen (Feulgen and Rossenbeck, 1924) and of Vendrely (Vendrely and Lipardy, 1946), quantitatively eliminates the RNA and the purine bases of DNA: therefore, Feulgen staining can easily be utilized. But it will be important to establish, in the future, to what extent this acid hydrolysis also attacks the proteins. Taylor (1956) has very judiciously criticized the specificity of these different extraction procedures.

A way to identify with a high degree of certainty a radioactive molecule consists in using, as a tracer, a specific precursor of the molecule whose distribution among the cellular elements is to be studied. This is the case for orotic acid, which is the precursor of the pyrimidine groups of RNA (Hurlbert and Potter, 1952), for thymidine, which acts as a specific precursor of DNA (Friedkin *et al.*, 1956), or for amino acids, in the case of the proteins.

Using high enough doses of a precursor as specific as possible, during a treatment period sufficiently brief to reduce to a minimum the risk of degradation which would lead to unspecific labeling, one can hope to follow the metabolism of proteins or nucleic acid, for example, into the different cellular organelles.

Such a procedure will not solve, however, the most delicate problem of interpretation when radioactivity is observed: are we dealing with an actual synthesis or a mere turnover? The same question arises, of course, whenever one is using any type of radioactive measurement technique.

The quantitative estimation of specific radioactivity raises many questions and must be interpreted with caution; one must not forget that the number of labeled molecules depends on several factors: (1) the dilution of the radioisotope in the labeled medium; (2) the concentration of the labeled substance in the organisms and cells under study; (3) the content in different precursors of this substance; (4) the particular turnover of these precursors and the labeled substance; (5) the actual synthesis of the precursors and of the complex molecule under study.

For instance, experiments of Hultin (1952), designed to follow the incorporation of labeled amino acids into the proteins of developing sea urchin eggs, have shown that alanine enters much faster than glycine, owing to the high content, in a free state, of this latter amino acid in the egg.

Steinert (1955), in the case of amphibian oöcytes and eggs, made similar observations with hypoxanthine and glycine. Our own observations on the same material, but with adenine, have shown that soluble precursors of RNA (adenosinetriphosphoric acid (ATP) especially) are labeled fifty times more strongly than is RNA. Moreover, they show a chiefly cytoplasmic localization, while labeled RNA is localized in the germinal vesicle, mostly in the nucleoli. It may be that these precursors are retained, in an insoluble form, through the histological and extraction processes: it is therefore obvious that the specific radioactivity will have a significant value only if the autoradiography observations are corroborated by estimations made with other methods.

C. Photographic Techniques

As regards the "stripping film" method, we refer the reader to the above-mentioned (see page 69) papers describing in detail the process used for this technique.

Briefly, the procedure is as follows:

A pellicle of thin emulsion is cut to a convenient size with a razor blade and separated from the plate which supports it. The small fragment is turned over and allowed to float on the surface of clean, distilled water at room temperature in a petri dish. After a few minutes, the film has swelled and spread.

The slide with the histological preparations is immersed and brought in contact with the film, whose sensitive side is now upside down. Excess of water is kept off by gently pressing the photographic emulsion with filter paper.

Exposure is performed in a dry atmosphere. Development may be carried out with D.19 Kodak developer in 10 minutes; fixation in thiosulfate will take about 15 minutes. This is followed by washing.

We shall now describe the technique for spreading the nuclear emulsion onto the tissue. All manipulations are performed in a dark room, the only illumination being a red lamp (Wratten safelight lamp, series 1).

The photographic emulsion (G_5 or K_5 , Ilford, in gel form) is melted at $50^\circ C.$ in a water bath and diluted by one-third of its volume with distilled water; it is then filtered through cotton gauze in order to prevent air bubbles. The melted emulsion is then uniformly applied, to form a layer of about $30\ \mu$ thick (if C^{14} is used), after fixation and drying. Drying of the emulsion on the slide must be done rapidly (3–5 hours), with $CaCl_2$, on a horizontal support, in a light-tight container protected from radioactive dust. Only when the emulsion has dried will it reach full sensitivity.

In order to study soluble material and avoid any hydrating process, we have, in some cases, modified this technique as follows: a film of liquid emulsion spread on Plexiglass slides on which it is allowed to dry without adhering, is then softened in water vapor and very closely applied on the sections with the aid of a rubber roller; during the whole time of exposure, it is held tightly in contact with the section. A large proportion of the histological material will stick to the emulsion and can then be submitted to all developing processes.

Experience has shown that, at least during the first few days, the number of tracks observed is directly proportional to the time of exposure. Keeping the emulsion dry and cool, proportionality can be maintained during weeks.

The presence in the emulsion of one active center, negatively charged by the capture of one electron, is sufficient to render the grain sensitive to the developer. The larger the grain of the emulsion, the greater is the probability that such active centers will be formed by the passage of an ionizing particle. This explains why, all other conditions being equal, a coarse-grained emulsion is more sensitive than one of finer grain.

During photographic development, the radiation-sensitized grains are the first to be reduced, thanks to the latent image constituted by the metallic silver at their surface; if well chosen, the developer will reduce most of the sensitized and very few of the unsensitized grains.

A choice has to be made among the various organic reducers: di- or triphenols, aminophenols, hydroquinone, etc. Development is more pronounced when the pH is high, and it is blocked in an acid bath. Addition of KBr slows the reduction procedure and diminishes the number of unsensitized grains, whose development constitutes the "fog." Fixation eliminates the excess AgBr, complexing it with sodium thiosulfate. Nuclear emulsions having a high content of AgBr shrink as much as one-half after this manipulation; this must be kept in mind when precise measurements are desired.

Some particular problems arise during photographic development, owing to the thickness of the emulsion. For a homogeneous result, the time of diffusion of the solutions must be brief as compared to the duration of the development. In the case of plates less than 50μ thick, it is sufficient to dilute the developer. For thicker plates, developing at two temperatures (Dilworth *et al.*, 1950) is recommended: impregnation of the emulsion is done at $4^\circ C$, a temperature at which development is very slow: the reduction procedure begins only when the temperature is raised to about $20^\circ C$. or over.

The "amidol" developer (Dilworth *et al.*, 1950) which gave us the best results has the following formula:

Amidol (diaminophenol)	1.125 gm.
Anhydrous sodium sulfite	4.500 gm.
KBr, solution at 10%	2 ml.
Distilled water	250 ml.

The pH of this solution is near neutrality. Development is performed at 14° C. for 20 minutes. After a facultative stop bath, in a solution of 1% acetic acid, the slides are fixed in a cold solution of sodium thiosulfate, at one-third saturation. For plates 20–30 μ thick fixation is complete after 30 to 45 minutes. The slides are then thoroughly washed in tap water for at least 1 hour, thus progressively eliminating the thiosulfate in order to avoid distortion of the gelatin.

Before staining, a 70% ethanol bath constitutes a good pretreatment of the slides: it eases the diffusion of the stains through the gelatin. Two staining methods are suitable: the technique of May-Grünwald-Giemsa in the case of bone marrow cells, and that of Unna (methyl green-pyronine) in most other cases. Lajtha (1952) has also used the method of Giemsa. The stains formerly used in autoradiography were hematoxylin or hemalum. The methyl green-pyronine mixture has the advantage that it is especially simple to use and that it allows a cytochemical localization of nucleic acids as well.

The photographic emulsion takes up some of the stains. Differentiation in water or 70% alcohol must be followed under the microscope; gelatin becomes almost completely colorless in these baths.

Axelrod-Heller (1951) has observed that, in certain cases, staining removes a great deal of the radioactivity. This fact illustrates the necessity of staining only after the photographic process.

When dry, the slides may be mounted in balsam or a similar mounting medium; they may also be directly observed under the microscope. In certain cases (sections of yolk material, for instance), mounting in glycerin has some advantages; but, the refractive index of this medium is lower than that of the glass; furthermore, in time, glycerin may differentiate certain stains, pyronine for example.

Observations may also be performed in phase contrast or under the interference microscope.

IV. MICROSCOPICAL ANALYSIS

As far as localization of the radioactive source is concerned, various factors which affect resolution—thickness of the section, interspace, ex-

posure and size of the silver grains—have been mentioned. For several authors, the maximum resolution would theoretically be 2 or 3μ in the case of the blackening methods (Stevens, 1948). A theoretical approach to the problem has been achieved by Gross *et al.* (1951), who worked on models which allowed them to compare the scatter of the radiation in vertical and lateral directions.

In the case of nuclear emulsions, it is essential, for a precise localization of the origin of the tracks, to proceed to a complete and homogeneous development: the first grains of the trajectory must not be lost and the direction of the track must be determined. The range of the particles is 2,000 times longer in the air than in the emulsion, where they leave tracks of some tens of microns; observation should preferably be done at high magnification with immersion oil objectives in order to get a more exact idea of the spatial relations between trajectories and the histological section. Under the best conditions, resolution may approach the 2μ which are theoretically obtainable.

However, substances labeled with tritium are becoming easier to obtain and hence will be more commonly used in the future. Tritium allows a very precise localization because its β -particles, of very low energy (0.017 Mev.), affect only one silver grain. Moreover, tritium, which has a half-life of twelve years, is particularly convenient to use: its specific radioactivity is sufficiently high to produce a satisfactory autograph rapidly without having to take into account its decay during the experiments. Resolution of 0.3 to 0.4μ may eventually be obtained in the case of tritium-labeled precursors (Eidinoff *et al.*, 1951; Beisher, 1953).

Errors in localization may be due to the existence of a back-scattering phenomenon: electrons striking the glass plate may be reflected back into the emulsion, thereby giving a false point of origin. This implies the necessity of making a great number of observations before one can draw accurate conclusions about localization. In this case, again, tritium has great advantages because back-scattering is negligible for very low-energy β -particles.

We will now discuss the often-debated question of the quantitative significance of autoradiography. Whatever the method used, the conditions of development must be carefully established and standardized; if they are not, it is impossible to draw quantitative conclusions.

The measured intensity of radioactivity will depend: (1) on the concentration of the radioisotope, (2) on the length of its half life, (3) on the energy of the β emitted. On (1) and (2) will depend the choice of exposure time and on (3) the thickness of section and the thickness of the emulsion.

For the blackening method, if resolving power is low, radioactivity

may be estimated by a measure of optical density: Howard and Pelc (1951) and Herz (1951) have calculated that the radioactivity corresponding to a density of 0.6, after 15 days' exposure, is 3.6×10^{-4} microcuries per square centimeter; these data are valid for C^{14} and P^{32} . Marinelli and Hill (1948) for I^{131} , Lajtha (1952), Odeblad (1950); and Dudley (1951) for P^{32} have made similar calculations for standardized emulsions with sources of known radioactivity.

A method for the quantitative assessment of stripping radioautographs has been proposed by Mazia *et al.* (1955). It is based on the formation of a band pattern of the silver grains by means of a cylindrical-lens camera. The distribution of band intensities along the strip is recorded by a photoelectric system.

Dudley and Pelc (1953), on the other hand, have described an electronic counter using a flying-spot technique to count the grains.

If a more accurate measurement is needed, as is the case for low activities, it is necessary to count the grain under the microscope.

The numerous investigators who used blackening radioautography (Leblond, 1943a, b, 1944; Axelrod and Hamilton, 1948; Branson and Hansborough, 1948; Dalgaard, 1948; Dobyns *et al.*, 1949; Dudley and Dobyns, 1949; Berriman *et al.*, 1950; Pelc, 1951; Nadler, 1951; Fink, 1951; Chapman-Andresen, 1953; Pelc, 1957) have discussed the quantitative significance of their observations. Blundell and Rotblat (1951), who have compared the merits of emulsions by blackening and by tracks, have concluded that the latter method is superior. Campbell (1951), who has calculated the dosage of Fe^{59} and S^{35} by this method, has expressed the same opinion.

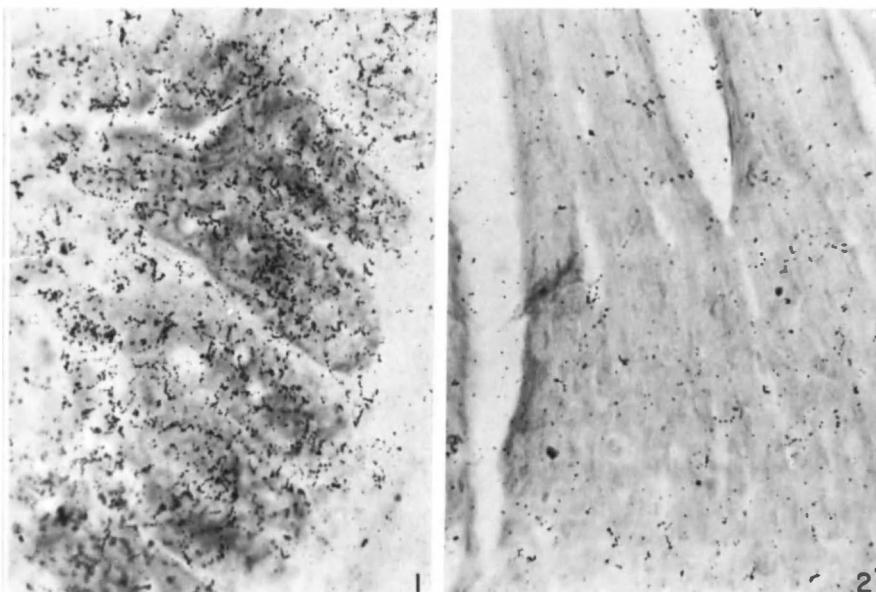
In the particular case of the nuclear emulsions, if all other experimental conditions are rigorously standardized, one can admit that radioactivity is directly proportional to the number of tracks. The number of these tracks per unit of surface compared to the quantity of active substance estimated by another method—by spectrophotometry or refractometry for instance must, in principle, give a measure of the specific radioactivity.

The use in each experiment of a stable standard, labeled in a constant manner, which could be incorporated into the photographic emulsion, would constitute an excellent control. Tubis and Jacobs (1952) have suggested the use of such standards. Recently, however, Levi (1957) has studied the distribution of radioactivity emanating from inorganic objects (crystals containing 300 atoms of S^{35}), which were supposed to be homogeneous and of the same size. She observed that the distribution of the tracks and, even more, of the grains present some deviation—but not a considerable one—from the Poisson distribution.

A critical study of the "track method" in its quantitative aspect

has been undertaken by Levi (1954, 1957, Levi and Hogben, 1955). Levi has obtained a reasonable agreement between track-counting and measurements with the ionization chamber. She has also studied systematic errors due to the observation and the variability of the photographic material.

She arrives at the conclusion that, for a trained observer, errors owing to track-counting vary from 4% to 10% (they increase with track density). Taking into account the corrections made for geometrical conditions—self-absorption, interspace—she estimates at 10% the number of disintegrations actually registered on the film. She observes that the sensitivity of the NTB Kodak emulsion used in her experiments is 2.5 times lower than Ilford G₅ emulsion.



Figs. 1 and 2. Autoradiographs of intestinal mucosa (Fig. 1) and cardiac muscle (Fig. 2) from a mouse injected with phenylalanine-2-C¹⁴.

V. CONCLUSION

In summarizing these few considerations about the autoradiographic method, it would seem desirable to present the advantages it offers as compared to other methods for radioactive measurements.

The principal one is to enable the localization of the radioactive

atoms in various cell structures. As we have seen, the resolution can be as low as 1μ .

The sensitivity of the method permits the use of very low doses



FIG. 3. Bone marrow cells labeled with glycine-1-C¹⁴. β -Tracks can be followed into the thickness of the emulsion.

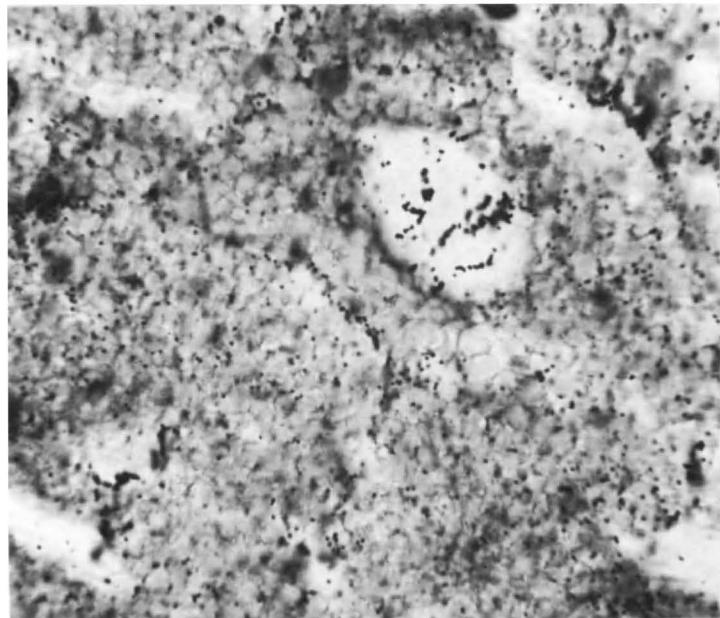


FIG. 4. Incorporation of C^{14}O_2 into nuclei of an amphibian morula.

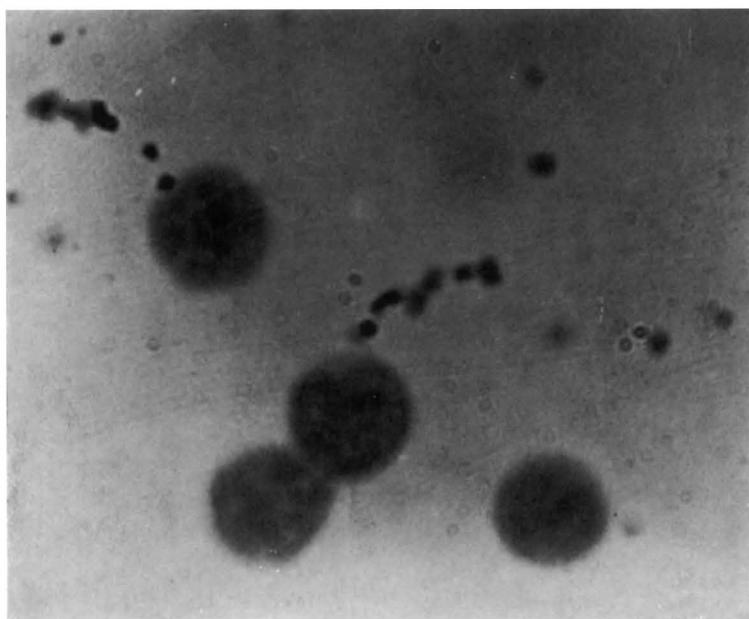


FIG. 5. Isolated thymocyte nuclei labeled *in vitro* with phenylalanine-2- C^{14} .

[detection of 10^4 atoms is possible with the track method (Webb, 1951)] and is not limited, as is the case for the Geiger counter or the ionization chamber, by the surrounding radioactivity or cosmic radiation. It has a cumulative character, if exposure is not too prolonged: in the latter case, fading of the latent image may occur.

As compared to the blackening technique, the sensitivity of the "track method" allows one to reduce the exposure time, and the danger of misleading background is considerably diminished by the mode of observation. Moreover, with emulsions in gel form, cosmic background interferes only after the application of the emulsion on the section.

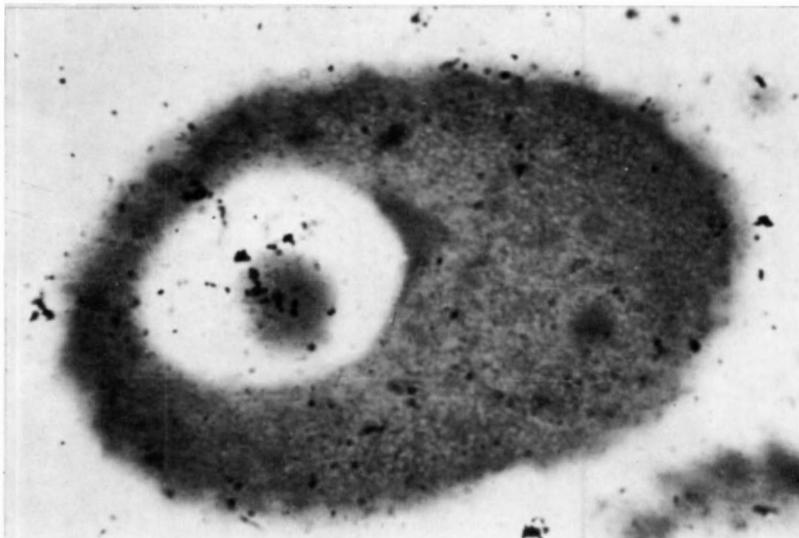


FIG. 6. Three β -tracks emerging from the nucleolus of a starfish oocyte, tagged with adenine-8-C¹⁴.

Further, let us point out the possibilities of combining autoradiography with other methods, e.g., ultraviolet absorption, chromatography, or electrophoresis. The work of Fink and Fink (1948) may be cited in this field, and especially the remarkable analysis of photosynthesis made by Calvin *et al.* (1950), who combined chromatography with autoradiography. Autoradiographic data might become much more valuable if they could be given a more specific biochemical meaning.

Although reasonably good incorporation curves of the number of radioactive atoms from a given precursor during increasing periods of

contact, either *in vitro* or *in vivo*, have been obtained, these data have not so far been related to the concentration of the cellular constituent concerned, giving adequate specific radioactivities.

It seems at present possible to relate the number of counts to the total dry weight of the cellular structures measured by electron micrography (Engström *et al.*, 1957) or by interference microscopy (Davies

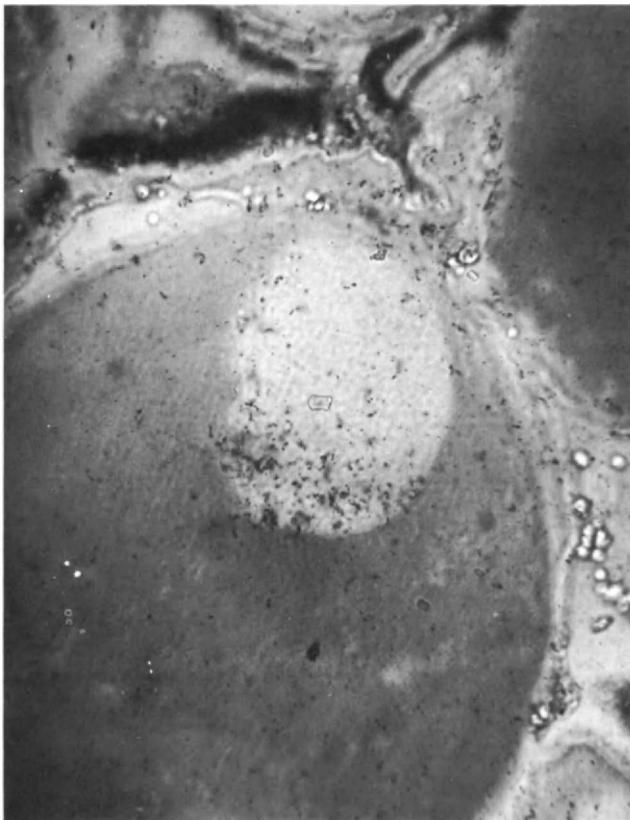


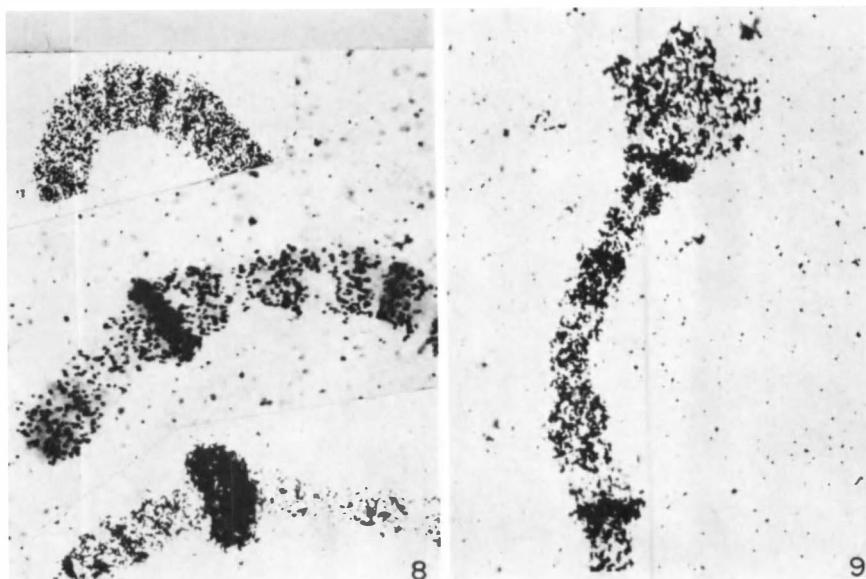
FIG. 7. Autoradiograph of a centrifuged *Triturus* oocyte; the loops of the lampbrush chromosomes which have sedimented inside the nucleus are specifically labeled with adenine-C¹⁴.

et al., 1957); to the nucleic acid concentration determined by ultraviolet or visible microspectrophotometry (Caspersson, 1956; Lison, 1950; Walker, 1957), or to the amount of proteins calculated by combining these methods (Davies *et al.*, 1957).

Further refinement of the biochemical localization of the radioactive compounds can probably be obtained if the specific extraction proce-

dures already mentioned are applied simultaneously to these microscopic procedures.

One can also hope that autoradiographic techniques will be combined with ultramicrobiochemical procedures like microchromatography or microelectrophoresis on silk threads. By these techniques, quantitative amounts of purines or pyrimidines extracted from single nerve cells have been determined (Edström, 1957). Such threads could be embedded in photographic emulsion and the radioactivity of individual constituents could be accurately measured. It is not impossible to imagine, in cases of very low radioactivities, that the photographic



Figs. 8 and 9. Autoradiograph of *Rhynchosciara* chromosomes showing differential incorporation of tritiated thymidine into the different bands during the larval development.

emulsion itself could be used as a support for chromatography or electrophoresis. Sensitive emulsion dropped in, or on, capillary tubes might permit one to record directly the concentration of migration of labeled molecules (Bonetti and Occhialini, 1951).

Finally, we wish to point out, as an illustration of its possibilities, that the "track method" has brought new contributions to our knowledge of cellular metabolism, especially in the case of the relations between nucleic acids and protein synthesis.

Figures 1 and 2 illustrate this problem, at the organ level, and show

the relative radioactivities of sections of muscle and intestinal mucosa from a mouse injected with phenylalanine-2-C¹⁴. The coincidence between the basophilia of the cells and rate of amino acid incorporation into the proteins is evident here (Ficq and Brachet, 1956).

Figure 3 shows bone marrow cells which have incorporated glycine-1-C¹⁴ into their proteins: the rate of labeling is directly related to the age of the cell and its degree of differentiation (Ficq *et al.*, 1954).

Figure 4 shows the high incorporation of labeled C¹⁴O₂ into the nuclei of an amphibian morula (Brachet, 1955).

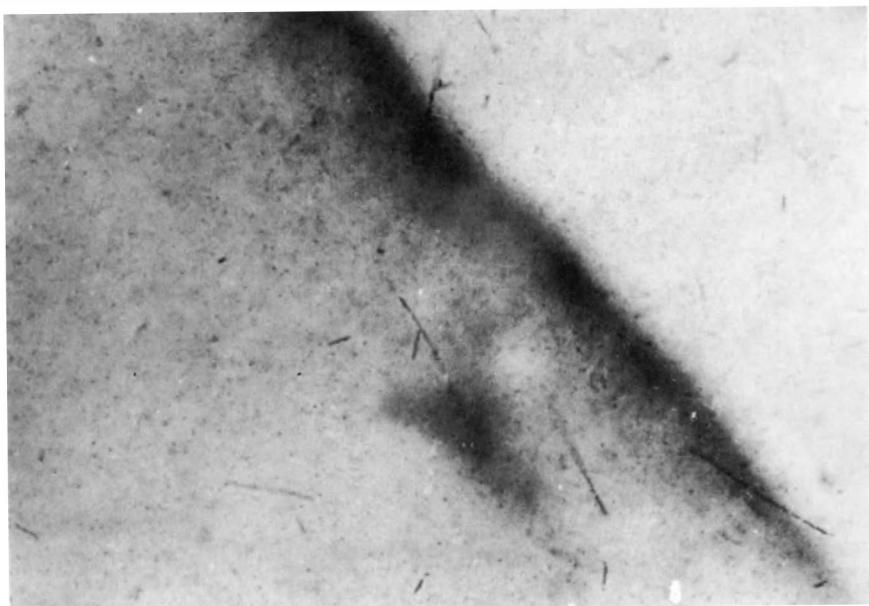


FIG. 10. Triton and α -tracks corresponding to lithium, after neutron irradiation, in a section of an amphibian embryo treated with lithium.

Figure 5 illustrates autoradiography experiments similar to those of Allfrey *et al.* (1955) on the metabolic activity of proteins in thymocyte nuclei free of cytoplasm (Ficq and Errera, 1958).

Figure 6 demonstrates the metabolic role of the nucleolus: labeling is fast and intensive, whatever precursor is used (amino acids or adenine) (Ficq, 1955).

Figure 7 shows the distribution of radioactivity in a centrifuged *Triturus* oocyte, after injection of adenine-C¹⁴. Labeled RNA is specifically localized in the loops of the lampbrush chromosomes, which have sedimented inside the nucleus (Brachet and Ficq, 1956).

Figures 8 and 9 represent the incorporation of thymidine labeled with tritium into *Rhynchosciara* chromosomes at different stages of larval development. These experiments seem to show that, at least at the isolated chromosome level, the constancy and metabolic stability of DNA are not as rigorous as was once thought (Ficq and Pavan, 1957).

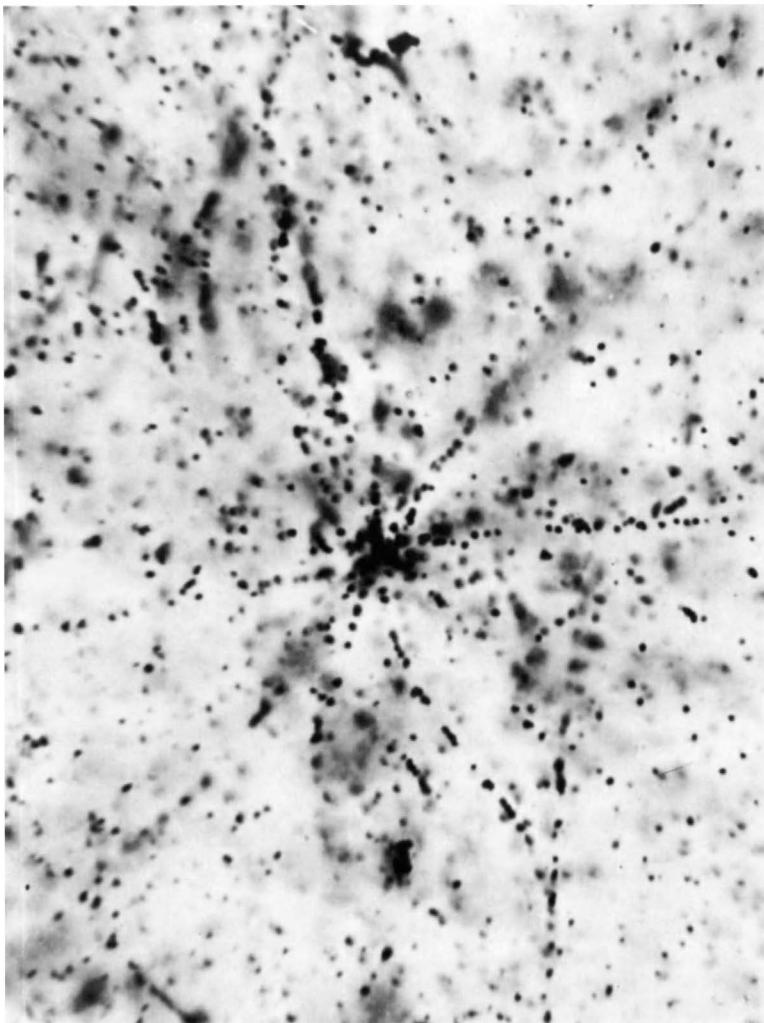


FIG. 11.

Figure 10 corresponds to a section of an amphibian embryo treated with lithium. Lithium was estimated and localized by the neutronic irradiation method (Ficq, 1954).

Finally, let us mention that this method has allowed Levinthal and Thomas (1957) to follow, in an elegant way, the problem of DNA duplication into single viral particles.

Figure 11 shows a "star" resulting from the disintegration of P^{32} atoms incorporated into the bacteriophage DNA.

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CHAPTER 4

Quantitative Microscopical Techniques for Single Cells

By P. M. B. WALKER and B. M. RICHARDS

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I. INTRODUCTION

The study of the chemistry of single cells provides great opportunities for the biologist. He need not be confined to what may be called the biochemical approach. This of necessity requires large populations of

cells for each experiment and thus smears out the differences due to function and to growth. It is these very differences in cells, either as they vary their function or as they proceed from stage to stage during interphase and mitosis, which is of increasing importance and which in principle may be investigated by the cytochemical techniques to be described here.

Properties of single cells such as their stainability have long been used to investigate the structure and qualitative chemistry of cells, but it is only recently that attempts have been made to correlate stainability and other natural properties of cells with the chemistry in a more precise and quantitative manner. The difficulties are great because the quantities of substances in cells are so small that checking the procedures is difficult and it is easy to suspect that the peculiar environment of the cell will influence the results.

Indeed it is possible that most of the stains which have been used by histologists during the last hundred years could be made the basis of a quantitative cytochemical technique, in the sense that under controlled conditions a suitable measuring instrument would give reproducible results. In fact, very few have been so used, and of these none commands universal confidence. In this chapter we will discuss the reasons for this paradox, and in so doing we will try to give some of the criteria which must be fulfilled before any technique which involves optical measurement on single cells can be considered quantitative. We will then consider in greater detail those few methods which have been or could be employed in this way.

We are concerned here primarily with techniques in which a microscope with ancillary equipment is used to measure the amount of color, refraction, or naturally occurring absorption within a cell. The quantities to be measured are therefore small, within the range 10^{-13} gm. to 10^{-10} gm., which is about 10^5 times smaller than the amount of material required by the biochemist for his well-tried and accepted methods. This scale difference makes it difficult to check cytochemical findings by other methods, and this is one of the uncertainties which the cytologist must accept if he wishes to study the growth and development of a single cell.

A. Specificity and Stoichiometry

Although it is essential to know which substances are measured, it does not matter how many of them are included within the range of the technique. For example, methods are available for measuring total dry mass or all substances containing purines and pyrimidines at one extreme and for a single amino acid at the other. In staining procedures

it is easy to see that specificity is concerned with defining the different chemical groups which will react and that the stoichiometry concerns the kinetics of the reaction between the "stain" and these groups, its sensitivity to the chemical environment and to the properties of the compounds that are finally formed. On the other hand, when the interference and ultraviolet microscopes are used to measure dry mass and naturally occurring absorption, the problems analogous to those of specificity and stoichiometry are not so evident. Difficulties concerning specificity arise when measurements are attempted on intracellular structures, or they may originate in the differential extraction procedures which are employed to extend the usefulness of these methods. Their stoichiometry, that is, the relation between the parameter measured (path difference or absorbance) and the mass of the substances investigated, depends on possible variations in the properties which we use in the two methods—specific refractive increment and absorptivity. In general, specific refractive increment is less sensitive to the cellular environment (Davies, 1958) than is absorptivity (Walker, 1958), but all these factors will be discussed in greater detail in Section III in relation to specific procedures.

Except for interference microscopy, the methods so far mentioned require the measurement of the absorption due to particular chromophores, such as heterocyclic nitrogen bases or the fuchsin-DNA (deoxyribonucleic acid) complex. Each of these chromophores has a specific absorption curve, whose height at a particular wavelength will determine the concentration of chromophores required if measurement is to be possible. On general grounds it would seem that in staining reactions the optimum condition will be that in which one molecule of stain is attached to each reactive group. The absorptivity of a stain or dye is, within limits, the sum of its constituent absorbing chemical bonds, and therefore the problem of increasing the sensitivity of any staining reaction is one of getting as large a molecule as possible containing these absorbing groups to the site. There are however two limitations to increasing the absorptivity of the stain, and therefore to the ultimate sensitivity of the method. First, steric difficulties will hamper the introduction of large molecules and, second, the more we increase the absorbance of those particles which are just below the resolution of the microscope, the more we increase errors due to inhomogeneity (see Section II, A, 2).

Information on the smallest measurable quantities has been collected by Walker (1956) for the naturally absorbing substances such as the nucleic and aromatic amino acids: it appears that 5×10^{-12} gm. is about the smallest amount of deoxyribo- or ribonucleic acid (DNA or RNA)

which may be measured to an accuracy of 10% in a cell. If the nitrogen bases are present as nucleotides, this figure may be reduced by one-half to one-third, but thirty times as much protein containing 5% tyrosine and 1% tryptophan will be required. These figures are calculated on the basis of a nucleus or cell having an area of $100\mu^2$; in smaller cells or if these substances are concentrated in small structures, they will be correspondingly reduced.

Comparable figures are not available for the measurement of stain, but our own experience with measuring Feulgen stain with an accurate scanning instrument (Deeley, 1955) suggests that under normal staining conditions the amount of DNA that is measurable is about the same as that for the naturally occurring absorption, after the greater instrumental accuracy in the stain measurement has been allowed for. It seems unlikely therefore that any staining technique will enable us to measure much less than 10^{-13} gm. of a cellular component. In interferometric measurements of dry mass different limitations apply, but Davies *et al.* (1957), using a specially designed instrument, were able to detect differences of dry mass of 10^{-11} gm. to an accuracy $\pm 1\%$.

The total dry mass of an average mammalian cell is about $2-400 \times 10^{-12}$ gm., of which one-fourth is contained within the nucleus. Cytochemical techniques can therefore in principle be used to investigate quite a small fraction of a cell's contents. Even so they will obviously exclude many important compounds or classes of substances.

B. Basis of Comparison

The purpose of cytochemical measurement is the investigation of the relations between different cells or between comparable parts of cells, and it is necessary to discuss the kinds of valid comparative measurements which may usefully be made. First, the concentration of substances may be determined in living cells by using the interferometer, phase contrast (Barer and Joseph, 1954, 1955a, b), or ultraviolet microscope. Second, the relative concentrations or proportion of two or more classes of substances may be measured in fixed cells and, third, total amounts of material may be found in comparable cell structures.

All these different approaches involve some definition of the part of the cell which is measured. It is of little use to compare the concentration of a substance in a part of the cytoplasm of one cell with any part of the cytoplasm of another cell. On the other hand, although local variations of great significance may thereby be concealed, the average relative concentration of material in the whole cytoplasm or nucleus will provide useful information when compared with another cytoplasm or nucleus. This is because nucleus and cytoplasm are structures we can

identify in different cells; nucleoli and chromosomes may also be compared; and, more doubtfully, the centrosphere region, which is often obvious in ultraviolet and interference photomicrographs, could be used as a subdivision of the cytoplasm.

These subdivisions depend on appearances in the light microscope and are subject to qualifications both of a structural and chemical nature. Thus electron microscopy has indicated structural continuities between nucleus and cytoplasm, and it is clear that the centrosphere region, containing the Golgi apparatus usually includes many constituents also present in the rest of the cytoplasm. It is also apparent that cytoplasmic constituents about which chemical information is urgently required are well below the resolution of the light microscope and therefore outside the scope of the methods to be considered here.

Chemically, the arbitrary nature of the cellular subdivisions is most clearly seen in connection with measurements of total amount, which must also be made on comparable cell structures. For example, it seems valid to compare the amount of DNA in different nuclei, and of proteins in nucleus or cytoplasm, because these substances are more or less confined to these particular organelles. Measurements of small molecular weight substances like nucleotides or amino acids, which can traverse cellular membranes, should not be confined to nucleus or cytoplasm. Random changes of the ratio between the volumes of cytoplasm and nucleus may cause changes in the amount of these mobile molecules, which are independent of the particular cellular process being investigated. Indeed, it may be that the cell is not the ultimate unit of comparison for some substances, but that the unit should be the tissue or even the whole organism.

An important method of studying the total amounts of substances in cells is to find out how they change during growth; one way of doing this is to study tissue cultures, which can be filmed prior to making the measurements (Walker and Yates, 1952), in order to provide a record of the previous history of the cells. In other tissues this approach may be impossible, and we will have to employ less direct methods such as that in which the time and rate of synthesis is related to the distribution of measurements from a large population of cells (Walker, 1954; Scherbaum and Rasch, 1957).

The two problems of the sensitivity of the techniques and of the spatial definition of the measurement within the cell are formidable limitations of the method, but they are only secondary to the general problem of the purpose and scope of cytochemistry. In the period when the cell was thought of as a more or less homogeneous colloidal system, it was reasonable to suppose that a precise knowledge of the quantities

and concentrations of all cellular components would eventually provide us with a clear picture of the mechanisms of cell growth and function. This view was well summarized by Danielli (1947) in one of the symposia which initiated in no small degree the great development of cytochemistry in the following decade. This attitude is now changing because of our more detailed knowledge both of the fine structure of cells and of the morphology of nucleic acids and proteins. We now recognize that cell functions cannot be explained by simply studying the concentrations of cell components, however attractive physical and chemical analogies make this type of quantitative approach.

It is necessary, therefore, to enquire whether the methods which we will discuss can provide information about the detailed chemistry of macromolecules and whether we can correlate this information with the morphology of the cell, which is now being revealed by the electron microscope. This more precise approach re-emphasizes a difficulty which has often been neglected with some justification in more general methods. While it may be considered that the processes of preparation like cytological fixation, could not affect the amount of substances present in gross amounts, it is certain that they will alter the kind of detailed chemical, and therefore structural, morphology which we must now consider. This is of course a very general and in some senses an inseparable problem common to all investigations, but it should not prevent new experiments. Indeed a very satisfying system has been built up concerning the microstructure of cells, although the living cell has a rather elusive role in much of this research. It remains to be seen whether a similarly detailed chemistry will fare as well.

II. METHODS

If we wish to measure the amount of an absorbing material, we must use the absorption law, which can be expressed as follows:

$$A_{\lambda} = \log_{10} \frac{I_o}{I} = kcl \quad (1)$$

A is the absorbance (extinction) which we measure at a wavelength λ and is derived from the energy transmitted (I) by an incident (I_o) on the object. k is the absorptivity (extinction coefficient) of the particular chromophore, while c is the concentration in grams per liter and l is the length or thickness of the material in centimeters.

Provided therefore that we know the value for k and the thickness of the object, we can easily calculate the concentration of substance. In many biological specimens, however, it may be difficult to determine the thickness to the required accuracy, and it will then be more convenient to use the second relation:

$$m = \frac{A \times a}{k} \quad (2)$$

where m is the mass in grams and a is the area in cm.². It is also evident from equation (2) that the smallest amount measurable is directly proportional to the area measured and indeed if this area is in μ^2 , then m is in 10^{-11} gm., which is within the range of the quantities of substances in cells.

It follows from these equations that we will require a suitable source of monochromatic radiation of the specified wavelength λ , a microscope both to define the small measured area and to pass the energy through it, and a detector capable of measuring I and I_o . Suitable complete instruments can be very complicated, particularly when designed for use in the ultraviolet, and they have been considered in detail elsewhere (Walker, 1956, 1958). We will only mention at the appropriate place certain special designs which help to reduce errors in measurement, particularly in the staining procedures.

In interference microscopy the following basic equation is used (Davies, 1958):

$$m = \frac{\Delta_w \cdot a}{\chi} \text{ grams} \quad (3)$$

where Δ_w refers to the optical path difference (o.p.d.) and is equal to $(\mu_c - \mu_w) \times l$ (cm.) where μ_c and μ_w are the refractive indices of the specimen and medium (water), respectively. This is the parameter which corresponds to absorbance and is measured directly in the interference microscope; χ is defined as being 100 times the specific refractive increment, that is the increase in refractive index which occurs with a unit increase in concentration. The specimen thickness need not be known [as in equation (2)] but if it is, the concentration of dry substance (gm. cc.⁻¹) can be calculated from

$$c = \frac{\Delta_w}{l\chi} \text{ gm. cc.}^{-1} \quad (4)$$

The value for χ at 546 m μ has been fully discussed by Davies (1958), and it is shown that most biological constituents have values which vary only within a narrow range, and that therefore refractive index can be used as a measure of dry mass.

A. Errors in Measurement

Errors in these techniques can be divided into two categories. First, there are those inherent in the process of measurement, which are primarily due to the use of a microscope and to the heterogeneous structure of cells. Second, there are those errors which are peculiar to individual kinds

of measurement. The latter will include variations of k or χ and questions of the specificity and stoichiometry of stains and will be considered in more detail in Section III. In particular we would like to divide between the two categories the causes for failure of Beer's law, since there seems to be some confusion about the proof that it holds in cells, as is shown by the paper on protein staining with mercuric bromophenol blue by Mazia *et al.* (1953) and its criticism by Glick (1953). First, instrumental inaccuracies, like stray light or errors due to specimen heterogeneity, will cause a departure from the linear relation between concentration and absorbance, since the percentage error from these causes increases as the measured absorbance increases. This type of error is not necessarily due to concentration of the chromophores, which may still have the same absorptivity as they do in dilute solution. Such a nonlinearity can be detected as a difference in the shape of absorption curves, which have different maximum absorbances, provided that they are plotted logarithmically. Secondly, the chromophores may change their absorptivity (k) due to molecular interaction at high concentrations, and if this change is unaccompanied by a change in shape or in the position of the absorption peak, it will not be detected by comparing absorption curves. The proof of the validity of Beer's law will then require an independent measurement of concentration, which is almost impossible in cells. The best that can be achieved is to demonstrate an agreement between the ratio of photometric and biochemical measurements over a range of cell types, as was first done by Ris and Mirsky (1949) for the Feulgen reaction. Such comparisons do not eliminate the possibility that changes in absorptivity occur at high concentrations, but they do show that the differences in k in the different cells are not so large as to preclude comparative measurements.

Instrumental errors have been featured to a greater or lesser extent in all reviews of these cytochemical techniques, and a guide to these reviews may be helpful. Caspersson (1936), in his pioneer paper, is mainly concerned with the physical basis of ultraviolet microspectroscopy, particularly as it concerns image formation and the shape of biological objects, and in a later book (Caspersson, 1950) he has summarized the work of the Stockholm school. Nurnberger's (1955) review contains among other things tables of amino acid analysis of proteins, which are discussed in relation to microspectroscopy. Davies and Walker (1953) have discussed in detail the theoretical bases of the errors in measurement due to the microscope and the specimen. Walker (1956) has considered the ultraviolet microspectroscopy from primarily a practical and technical viewpoint, from which the various types of apparatus are discussed. In a shorter article, Walker (1958) is concerned with

simpler apparatus which can be made quickly in the laboratory but also discusses in some detail the variations in the absorptivity of DNA. Swift (1955) and Swift and Rasch (1956) have reviewed techniques for the cytochemical determination of nucleic acids and in particular discuss the Feulgen reaction, which has also been discussed by Leuchtenberger (1958). The physical bases of interference microscopy have been considered in very great detail by Davies (1958), and some of the practical ways of using the method have also been discussed by Davies and Deeley (1957) and by Mitchison *et al.* (1956).

If, therefore, the reader wishes to understand thoroughly the reasons for inaccuracies in these various techniques he should consult at least the references cited. It is however inevitable as well as necessary that sources of error should be mentioned, and we will try in particular to compare the magnitude of error in the various types of measuring technique we are here considering.

1. Nonparallel Light

As high a numerical aperture as possible is required in both condenser and objective if we are to resolve the detail in the specimen and to pass sufficient energy through the specimen to allow accurate measurement. This means that a correction should be made for the increase in the average path length within the measured volume, which will depend on both the effective numerical aperture of the optical system and the refractive index of the specimens. Blout and co-workers (1950) calculated this correction for absorption measurements and gave a graphical representation of the error for certain values of refractive index and numerical aperture (see also Walker, 1956, for a replot of this in a more convenient form). A similar expression for the measurements of the optical path difference has been given by Davies and Deeley (1957). In measurements of stain this error can be neglected provided only comparative measurements with the same numerical aperture of condenser are attempted. In both absorption and interference measurements where absolute values are required, correction must be made, and it will also be needed if measurements on living and fixed cells are merely compared. Owing to the low refractive index of the living cell (~ 1.35) (Barer, 1956), a 10% error in measuring absorbance with a 1.0 numerical aperture condenser will not occur unless $A > 0.65$, but for a fixed cell this error will only be reached when $A > 0.95$.

It is sometimes helpful to define the area of a structure seen in the microscope as the "projected area," that is, as the area projected along the microscope axis. It is easy to see that with converging illumination the measurement may include material outside the projected area. The

degree of imprecision will depend on simple geometry and will be greater for small thick objects than for large, thin ones. A related source of error, which however also occurs with parallel light, is the difficulty of separating the absorption due to one structure from that due to others lying above or below it.

2. *Distributional Errors*

One of the major differences between microspectroscopy and conventional spectroscopy is that cells, of course, have structures, which cause variations in the absorption and refractive index within the measuring field. This results in considerable errors, which may conveniently be divided into three categories.

a. Errors due to absorption heterogeneity. If the absorbing material is unevenly distributed within a measured area, the absorbance measured will always be less than the true value. The magnitude of this effect depends on the absorbance differences and is only negligibly small if these are less than about 0.1. The effect has been particularly important in the history of the measurement of stains, where it has been common to use the so-called aperture or plug method, in which the whole or a substantial part of the nucleus was included within the measuring area. This practice was severely and justifiably criticized by Glick *et al.* (1951), and since then accounts of two good methods of overcoming this difficulty have been published.

Since the percentage error rises with increasing absorbance difference, its presence can be detected as a distortion of the contour of the absorption curve. If, therefore, two wavelengths are selected for any particular stain such that in a homogeneous object without error the absorbances have a known ratio (usually 2 to 1), then measurements at these wavelengths in the presence of error will show a departure from the expected ratio from which the true values can be calculated. This is the basis of the two-wavelength method of Ornstein (1952) and of Patau (1952), which is now widely used.

This method is applicable to any stain for which it can be shown that the absorption spectrum is unlikely to be affected by its concentration. It is not easy to apply to naturally occurring absorption, since variation in the ratio may be also due to variations in composition. It has the disadvantage that, in order to achieve reasonably high accuracy in the corrected result, the two measurements need to be accurate within at least 1% (Ornstein, 1952). This may be difficult to achieve, particularly when either measurement must be made in a wavelength region in which the absorbance is varying rapidly and in which, therefore, small changes in the position of an absorption maximum may introduce considerable error.

Another method of reducing distributional error is to reduce the size of the measuring area, as was done by Caspersson (reviewed in 1950) in his measurements of ultraviolet absorption and by Walker and Yates (1952) in their measurements of the total amount of ultraviolet absorbing substances in living nuclei. An important development of this method, which is particularly adaptable to measuring stain, was introduced by Deeley (1955), who used a mechanical scanning device which allows the energy reaching each small element of the image to be individually measured. These absorbance measurements are then automatically integrated to give a value for the total absorbance within a field. Consecutive measurements of the same field, with and without the specimen, thus give a measure of the total amount of stain in the specimen, which is unaffected by gross heterogeneity of material at the focus of the microscope. This instrument has been used for a number of investigations (e.g., Richards *et al.* 1956; Atkin and Richards, 1956) and may be applied to any staining procedure in which the measured body is separated by a short distance from other stained structures. It is thus not immediately applicable to ultraviolet measurements of parts of whole cells.

A third rather ingenious method of decreasing this error is also attributable to Ornstein (1952), who suggested that a chemical estimation of the amount of silver in the photographic image could be used to obtain an accurate measure of the amount of stain. Ornstein has used this method and the two-wavelength method in a very precise study of DNA in the sperm of a species of grasshopper, where it might be expected that those with the male and those with the female chromosome complement would have different amounts of DNA. A variant of this technique has been developed by Mendelsohn (1958) to measure gallo-cyanine stain in isolated nuclei of rat liver cells before and after ribonuclease treatment.

None of these methods, except the two-wavelength, can eliminate distributional errors due to particles which are below the resolution of the microscope and also have a high absorbance, since there is a range of particle size between the largest dimension at which the chromophores behave statistically, as in solution, and the minimum area resolvable in the microscope. Whether appreciable error can be attributed to particles within this range depends on the absorptivity and concentration of the chromophores; Stokes (see Davies and Walker, 1953) has calculated that the percentage error is ~ 116 times the absorbance of the particle, and it can be shown that 0.1μ thickness of dry solid DNA has an absorbance of 0.3, giving about 33% error. In practice there is a considerable amount of protein, perhaps water, and other constituents which will reduce the absorbance of the particle and the error. As already

mentioned in Section I, this is a limiting factor in the choice of highly absorbing dyes for quantitative staining reactions.

It seems, however, that, at least with the measurements of gallo-cyanine stain, there is no appreciable error due to particles below the resolution of the microscope since directly comparable measurements on the same cells by the scanning and two-wavelength methods (see Fig. 1) have given excellent agreement (Mendelsohn and Richards, 1958).

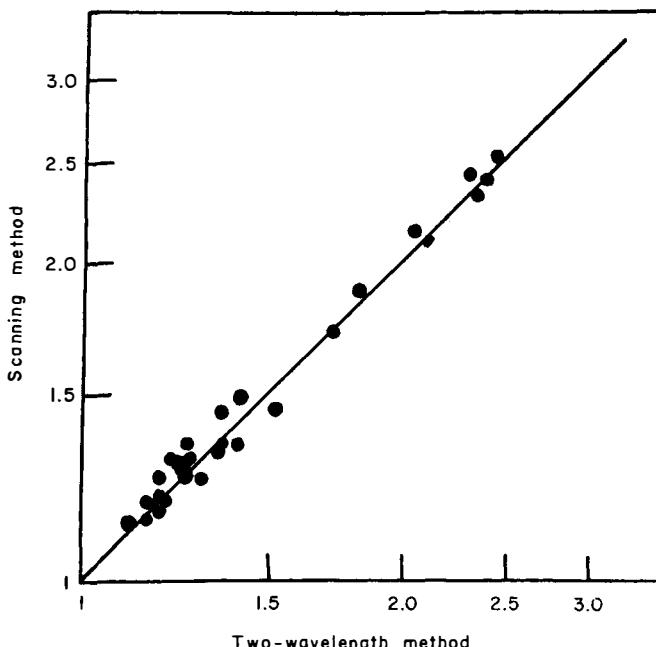


FIG. 1. Comparison of the results of measurements by the two different methods for overcoming distributional error in visible microspectrophotometry: 30 cells on the same slide were measured successively by the two-wavelength method and the scanning method (gallo-cyanine stain). From Mendelsohn and Richards, 1958.

In interference microscopy the effect of heterogeneity is to reduce the contrast of the interference band system within the specimen compared to that of the background (Davies, 1958). The error will therefore vary depending on the method of measurement and will be least if the displacement of the bands is measured using as the reference point the intensity at which the phase angle (ϕ) is 90° . This is difficult without extra equipment, and more serious errors can be expected if the method of setting on minima ($\phi = 0$) is used, or if intensity differences are

measured in the even-field method. Davies states that the loss of contrast will be negligibly small only when the optical path differences are less than 0.01λ or the over-all o.p.d. is less than 0.1λ . It is of interest that 0.25μ of dry protein has an o.p.d. of 0.1λ .

b. Errors due to particles being out of focus. Stokes has also calculated the expected error when an absorbing particle is completely out of focus and finds the percentage error to be similar to that for absorption heterogeneity, namely $\sim 116A$. This error is potentially more serious since the upper limit set for A by the resolution of the objective is greatly increased. The two-wavelength method will, however, correct this error, and the cell-crushing procedure of flattening cells (Davies *et al.*, 1954) can be used with the scanning method already described. Fortunately many living cells exhibit little heterogeneity and fixed cells are often considerably flattened, and it appears that it may be only in very thick heterogeneous objects like chromosomes at metaphase that this error will be large. In interference microscopy out-of-focus objects produce errors similar to those caused by absorption inhomogeneity, and the same means of mitigation and the same limits apply.

c. Nonspecific light loss or scatter. This error is caused by light being scattered outside the collecting aperture of the objective as the result of changes of refractive index within the measured area and of molecular scattering. Measurements of absorbance will therefore be too high. Scatter can be greatly reduced by immersing the specimen in a medium of the same refractive index, which is easily accomplished for fixed cells in the visible spectrum but is much more difficult in the ultraviolet.

Various expedients have been tried for the ultraviolet region, such as the use of triple-distilled glycerol to swell the proteins (Casperson, 1950), chloral hydrate saturated with zinc chloride (Köhler, 1904; Rudkin and Corlette, 1957b), methacrylate (Swift, 1955), or dried poly-glutamic acid (Walker, 1958). None of these is completely successful in all instances. The chief difficulty is in measuring the amount of scatter near the absorption peak of the nucleic acids. The method often used by spectroscopists (e.g., Beaven and Holiday, 1952) is to extrapolate to shorter wavelengths the apparent absorption curve obtained from wavelengths where protein absorption is not expected. There is some uncertainty in this procedure, since changes in refractive index will occur on the long wavelength side of the general protein absorption below $240 \text{ m}\mu$. More recently Rudkin and Corlette (1957b) have suggested measuring the light scattered with a form of dark-field illumination. This is a most interesting idea and it would be most useful to compare both methods, although it seems that the dark-field method is also subject to uncertainty due to the absorption of the incident and scattered light.

In interference microscopy it is usually undesirable to reduce refractive index changes and thus remove scatter, and indeed it is often essential to measure in water, when path differences will be largest. Scatter has the effect of reducing the maxima of the interference patterns without altering the minima, so measurement by setting on a minimum will be accurate. When using the photometer eyepiece it also is possible to obtain a true value by measuring retardation under light- and dark-field conditions and averaging the results (Davies and Deeley, 1957).

3. Glare in the Microscope

Glare, caused, for example, by unwanted reflections at optical surfaces and on mechanical mountings, is an insidious disability in all optical instruments and can cause serious errors. In microscope glare a uniform intensity is usually spread over both specimen and background; this causes a reduction in measured absorbance which is greatest for high absorbances. It can be detected by measuring the apparent absorbance of small opaque objects like iron spheres. This measurement can then be used to correct others when the same optical system is used (Davies and Walker, 1953). Glare has no systematic effect on the band method of measuring in interference microscopy, but a reduction in o.p.d. measurement will occur with an even field. Glare may be reduced by illuminating only the small area being measured, providing suitable stops in the microscope and carefully blacking all illuminated parts of the mountings.

4. Molecular Orientation

The experimenter should be aware that orientation of the chromophores in absorption measurements (Commoner, 1949; Pollister and Swift, 1950) and birefringence (Faust and Marriman, 1955) in interference microscopy (particularly with the Baker-Smith microscope) may cause error. Therefore, it is necessary to test new material in which such effects are suspected, although they seem to be absent from most biological specimens.

B. Model Systems

Although we have deliberately avoided discussion of most instrumental errors, certain general tests which involve model systems should be mentioned. It is possible to make a suspension of small spherical droplets 1–30 μ in diameter, whose density matches that of the surrounding liquid. Measurements of the absorbance at the center due to substances dissolved in the droplets should then bear a linear relation to the diameter.

For the ultraviolet, King and Roe (1953) used droplets of naphthalene dissolved in nonane, which were suspended in aqueous glycerol, while Walker and Deeley (1955) used ethylene dichloride instead of nonane. Mitchison and associates (1956) found that celloidin spheres were most suitable for testing their integrating interference microscope, but they also describe in detail several other systems. A linear correlation, for example between thickness and absorbance up to absorbances of 1.5, indicates that the apparatus is substantially free of instrumental errors. It does not of course eliminate the possibility of all forms of distributional error.

Another useful test, particularly of integrating instruments, is to use a uniform population of mammalian sperm (e.g., ram) and to measure these cells under various conditions and with more than one cell in the field (see for example Deeley, 1955).

C. Summary on Errors

It appears that errors of measurement are least likely to occur in staining procedures, in which well-tried methods are available for overcoming distributional error and the refractive indices of cell and medium can be matched. In the ultraviolet region, difficulties remain and care is required in planning the experiment and in preparation of the specimen. In interference microscopy, selection of the best method of measurement may eliminate certain errors, but the conflicting demands due to the presence of several sources of error may make this difficult.

In practice these specimen and instrumental errors must be considered in relation to the other sources of inaccuracy, such as variations in k or χ , which will be considered in the next section. There is little point in reducing the percentage error due to one cause to much less than half that due to any of the others.

III. INDIVIDUAL TECHNIQUES

A. General Remarks

In the preceding section an account was given of the over-all similarities in quantitative methods, with particular reference to errors which result from the use of the microscope as part of the measuring instrument. In this section we will consider in detail individual procedures with the emphasis both on the kinds of substances which may be investigated and on the general reliability of the technique. The final section is devoted to the biological results, and in it we will attempt a summary of the contribution that quantitative cytochemical techniques have made to biology.

Each of the three main methods which we are considering has different limitations regarding the classes of chemical compounds which may be studied. Measurements of naturally occurring absorption can be made only in the spectral range between the near infrared ($700\text{ m}\mu$), where low optical resolution prohibits most cellular measurements, and the near ultraviolet at about $240\text{ m}\mu$, where the general absorption due to the peptide linkage begins. Within this range the number of absorbing compounds is confined first to the nucleic acids and similar nitrogen-containing compounds like nucleotides, pterins, coenzymes, and adenosine triphosphate (ATP); second, to proteins containing the amino acids, tyrosine, tryptophan, and phenylalanine; and third, to special colored substances like the heme compounds, flavones, and anthocyanines. In addition certain simple substances like catechol and ascorbic acid, which may occur in quantity in some cells, also absorb in this region.

The photometric measurement of fixed and stained cells has hitherto been confined almost exclusively to nucleic acids and proteins. The special limitation of these methods is in the necessity of discovering the selectivity and of proving the specificity and stoichiometry of each stain or color reaction, but provided these conditions are satisfied quantitative measurements on other groups of substances like carbohydrates and lipids should be possible.

Interference microscopy is confined to measuring total dry mass and is therefore most useful in the study of this aspect of growth and in providing a convenient parameter in the cell with which measurements of individual substances can be compared. The usefulness of the method may be extended by selective extraction, but the all-inclusive nature of the measurement of dry mass will make it more difficult to prove the specificity of the procedure. Although a different physical principle is used, interference microscopy produces results comparable to those obtained by the older X-ray method of measuring dry mass (Davies and Engström, 1954), which is, however, confined to fixed cells.

B. Measurement of Naturally Occurring Absorption

We have already mentioned a number of reviews of the ultraviolet absorption method, but reference should be made also to Blout (1953), who mentions most of the biological results obtained with the method up to 1951, to Glick (1953), and to Kurnick (1955).

Caspersson in 1936 laid the theoretical foundations of microspectrophotometry, and his group has used the method in the characterization of nucleic acids in cells and in demonstrating the correlation between RNA and protein synthesis in many different tissues. In recent years considerable improvements have been made in the complicated appa-

ratus which is required to overcome the many difficulties in the method, but this very complexity of instruments has limited the number of workers engaged in the field.

1. *The Variation of Absorptivity*

Apart from the many sources of error due to instruments, there remains a general problem in measurements of absorption which must be made in the poorly defined environment of the biological cell. The shape or contour of the absorption curve of a substance is not fixed, but may vary owing to a large variety of causes (Scott, 1955). The most important of these from our point of view are the degree of polymerization or of denaturation, changes of pH which will cause ionization of the constituent groups, and increase of concentration which may result in molecular interaction.

Such changes of absorptivity (k) have been most fully worked out for DNA. The molar absorptivity of polymerized DNA *in vitro*, expressed on the basis of a gram-atom of phosphorus $\epsilon(P)$ (Chargaff and Zamenhoff, 1948), is 6800 (Beaven *et al.*, 1955). This increases to about 9000 when the DNA is measured at high or low pH, but in addition this treatment causes a denaturation of the molecule so than on returning it to a neutral pH the $\epsilon(P)$ does not return to 6800. Complete degradation of the DNA into its nucleosides will cause the $\epsilon(P)$ to increase to 10,800, and each of these nucleosides shows characteristic changes of its absorption spectrum if the pH is altered. Other factors such as salt concentration may affect the rate of change of absorptivity of DNA as the pH is reduced, and different methods of denaturation, such as heating to 100° C. (Thomas, 1954), will give the expected increase in absorptivity. It is therefore essential at least for *in vitro* studies to know not only the pH at the time of measurement, but also the previous history of the preparation.

Blout and Asadourian (1954) reported that DNA at high concentration (over 0.045%) does not obey Beer's law, but Svensson (1955), using a more elegant method of measuring small path lengths, reported that RNA did not deviate from Beer's law at a concentration of 4%. Since the nitrogen bases are situated inside the DNA in the double helical structure and since RNA may be a single chain (Fraenkel-Conrat, 1954), this is exactly the opposite of what may have been expected. It is not easy to see what form of interaction between the bases could cause the reported deviation for DNA, and it would be useful to repeat Svensson's measurements on DNA.

The cytochemist must consider, therefore, how far these large changes of absorptivity may affect his measurements of DNA in the cell. There

are at least two possible means of doing this: he can compare the amount of DNA per cell, obtained by microabsorption measurements, with the value given by bulk biochemical procedures. This has been done by Leuchtenberger *et al.* (1952) for citric acid isolated nuclei, by Walker and Yates (1952) for living sperm and erythrocyte nuclei, and by Nurnberger (1955) for rat liver cells. These results gave quite good agreement and, at least within the errors of both types of measurement, they show that no gross effect due to the chemical environment occurred in these cells.

Another approach is to measure the amount of DNA in a living and a fixed cell and then to follow the changes of DNA during those chemical procedures which are known to alter absorptivity *in vitro*. Only uniform populations of cells without RNA can be used easily for this kind of experiment, and one of us (Walker, 1957) has investigated the integrated absorbance of DNA-protein in mammalian sperm heads. In these cells, in contrast to the expected 30% increase for DNA *in vitro*, only a small change (< 10%) occurred when the pH was decreased in the cells fixed by freezing substitution. This smaller change may be due to the stabilizing effect of the protein, since acid hydrolysis of the sperm head results in the DNA being much more sensitive to pH changes. Treatment with H₂O at 100° C., which, besides denaturing DNA, may also be expected to break the DNA-protein bonds, causes the absorptivity to increase in the sperm heads.

Owing to its much more labile nature, the absorptivity of RNA has not, until recently, been so fully studied, although it has long been known that depolymerization also causes about 30% increase in absorptivity (Tsuboi, 1950; Magasanik and Chargaff, 1951), and Gordon and Nurnberger (1955) have shown that RNA can be obtained with an even lower absorptivity than Tsuboi's original preparation. Since there is evidence that RNA is a single-chain molecule (Fraenkel-Conrat, 1954), this is probably due to association between the adenylic and guanylic groups, of which the former (and probably the latter) shows the hypochromic effect when polymerized (Felsenfeld and Rich, 1957).

In proteins the position is even more complex; tyrosine shows marked changes of absorptivity with pH, while tryptophan does not (Beaven and Holiday, 1952), and they also have evidence that tyrosine changes its absorptivity on denaturation. The presence of certain anomalies in the absorption spectra of proteins is indicated by the inaccuracies which occur when nucleic acid:protein ratios are calculated from the ratios of absorbance near the nucleic acid and near the tyrosine and tryptophan absorption peaks. Walker (1956) calculated the nucleic acid:protein ratios from Moberger's (1954) data and found a wide

discrepancy between the ratios obtained from dry mass and from absorption measurements of only the nucleic acid and those obtained from absorption measurements of both components.

Further, Davies (1954) showed that the $A_{265}:A_{280}$ ratio for the nuclei of chick fibroblasts is the same as for pure DNA and that the nucleoprotein form of curve only occurs after fixing the cell. Walker (1957) found that while the absorption spectra of living, freeze-substituted, and formalin-fixed sperm heads all showed the DNA form of absorption curve, a change similar to that found by Davies occurred in the nuclei of mouse tissue culture cells. This may be due to the very much smaller proportion of protein in the sperm head (1-1.5:1; Davies *et al.*, 1957) compared to the tissue culture nucleus (7:1; Richards and Davies, 1958). The evidence indicates that this change in absorption cannot be entirely due to an increase in scatter, and it seems that a change in the absorption spectra of proteins occurs on fixing.

Fortunately, interference microscopy is a much better way of determining the total protein of the cell if the amount of nucleic acid and other substances is known. On the other hand, these changes of absorptivity of protein may provide information about the molecular state in the living cell which it will be difficult to obtain in other ways.

The possibility of quantitatively investigating the chemical constituents of the living cell is one of the great advantages that measurements of natural absorption share with those made with the interference microscope. Unlike the latter, however, ultraviolet radiations may damage living cells and care must be taken in designing both the apparatus and the experiment so that radiation damage does not affect the results. For this reason it is often best to make only one measurement on each cell.

C. Staining Methods

Of the color-producing techniques, which may be loosely called "staining methods," there are two different classes; the first is dye-binding, in which there is electrostatic attraction between the dye and substrate, and the second is the color reaction, in which substrate and reagent combine to form a colored compound. To illustrate this distinction, we may consider the binding of the dye naphthol yellow S (Deitch, 1955) by basic proteins as compared with the colored compound formed when the colorless Feulgen reagent (leucofuchsin) combines with the aldehyde groups formed during the hydrolysis of DNA. This colored compound is different from the basic fuchsin, from which the reagent was prepared, as is shown by its absorption spectrum (see Fig. 2).

There are disadvantages in both types, but these are perhaps greater in dye-binding since other ions with charges of the same sign as the

dye can compete for sites on the substrate. Such interference is much less likely in the color reaction, in which only certain chemical groups are involved.

In living cells nucleic acids are combined electrostatically with certain proteins, and it is therefore difficult for dye-binding to occur unless the basic dye is present in a sufficiently high concentration to allow it to compete with the basic amino groups of the protein (Michaelis, 1947);

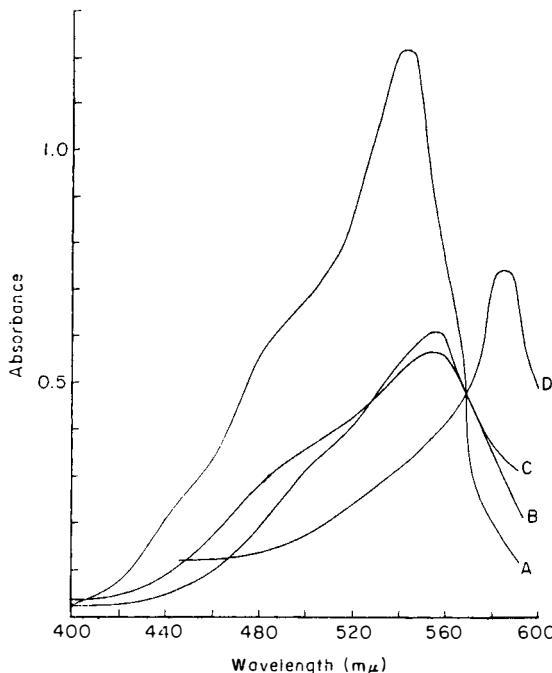


FIG. 2. Absorption spectra in solution. A. Basic fuchsin, 0.001% solution of solid purified according to Yarbo *et al.* (1954). B. 10cc. leucofuchsin (0.001% solution) recolorized with 1 cc. of 1% acetaldehyde. C. 10 cc. leucofuchsin (0.001% solution) recolorized with 2 cc. of approximately 1% formaldehyde and diluted three times. D. DNA-Feulgen complex in solution. Purified DNA hydrolyzed in N HCl at 60° C. until dissolved and then reacted with Schiff's reagent for 2 hours.

but this high concentration will cause denaturation and killing of the cell. In fixed cells, nucleic acids bind basic dyes with their phosphate groups and proteins normally bind acid dyes with basic amino groups, although at high pH's the carboxyl groups of proteins can compete with nucleic acids for basic dyes.

Denaturation during ordinary fixing does not necessarily make all

dye-binding sites available. For example, the bonds between nucleic acids and histones must be broken before histones can be stained with fast green (Alfert and Geshwind, 1953; Bloch and Godman, 1955a), and conversely the incubation of fixed sections with dilute solutions of protamines and histones abolishes or reduces their basophilia.

An additional complication is the possibility of adsorption of dye due to forces other than electrostatic attraction, such as Klotz (1946) has suggested for certain anionic dyes and proteins; the degree of adsorption is influenced by the size of the dye molecules or ions (Klotz and Urquhart, 1949). Furthermore, some basic dyes are metachromatic, that is, the color of the dye is dependent on the nature of the substrate. Thus, under suitable conditions, azure B stains DNA green-blue and RNA red-purple (Flax and Himes, 1952). An excellent review of this subject has been made by Schubert and Hamerman (1956), and Singer (1952) has written a more general account of such factors as pH, dye concentration, and temperature, which affect dye-binding.

Most color reactions for components of cells have been derived from standard analytical techniques in chemistry, just as the most successful, the Feulgen reaction, was developed from the Schiff test for aldehydes. We might expect, therefore, that the specificity and stoichiometry of the color reactions might be fully understood. That this is not so, is due to the complexity of the cellular environment when compared to a solution, and, in particular, to the fact that the availability of the reactive groups may be controlled by neighboring substances. This could be because the sites are inside a complex molecule or because one substance may modify the unmasking of reactive sites on another molecule. The latter seems to occur when heavy metal fixatives are used with the Feulgen procedure; this causes a change in the shape of the time-hydrolysis curve (Di Stefano, 1948) which could be explained by the stabilizing effect of this fixative on the histone, which in turn prevents the continued disintegration of the DNA.

In the discussion of individual procedures which follows, the reactions will be classified on the basis of their substrate.

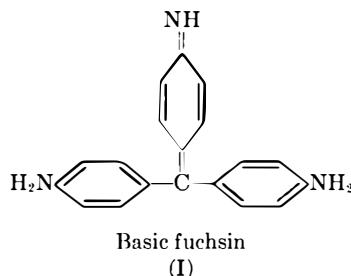
1. Methods for DNA

a. *The Feulgen reaction.* This reaction has been so widely used that many reviews have been required both for the results obtained with it and the nature of the reaction itself (Stowell, 1945; Swift, 1953; Lessler, 1953; Kurnick, 1955; Swift, 1955; Leuchtenberger, 1958). Even so, no satisfactory account of its chemical basis has yet been given. It has been so widely used because it can give a relative measure of the quantity

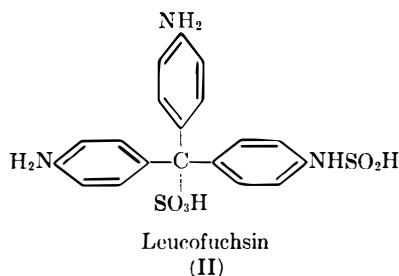
of DNA in a cell, but its justification has largely depended on the existence of a fairly constant amount of DNA in nuclei with the same number of chromosomes. Such a justification, as Swift (1955) has commented, contains an element of circular reasoning. Indeed, as anyone who has played with the reaction will know, the intensity is subject to so many variables that it defies all but the most ardent who attempt to standardize it.

i. Chemical basis. Briefly, the acid hydrolysis of DNA preferentially removes the purines, which allows the adjacent deoxyribose sugar rings to form aldehyde groups. These react with the decolorized Schiff's base (chiefly pararosaniline) to give a colored compound. The latter process is often called regeneration, but this is a bad term since the color is different from that of the original colored basic fuchsin. The Feulgen reagent has been prepared from the colored base by several methods, so that it differs in the details of its pH, SO₂, and dye content. Variation can also arise because the commercial types of basic fuchsin are mixtures of related dyes, of which only the chief is pararosaniline.

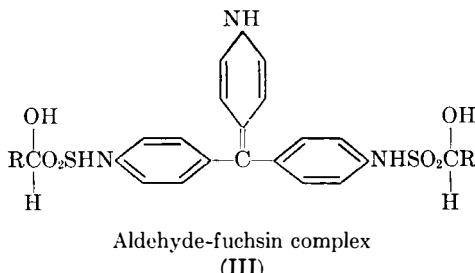
According to Wieland and Scheuing (1921), the dye pararosaniline or basic fuchsin (I)



is converted by sulfurous acid to leucofuchsin (II),



which is colorless owing to the loss of the quinonoid structure. The leucofuchsin couples with the aldehyde groups (III) to give a purple color as the quinonoid structure re-forms.



This purple color has an absorption spectrum which depends on the kind of aldehyde to which it is coupled (Fig. 2); thus formaldehyde- and aceteldehyde-fuchsin have an A_{\max} at $555 \text{ m}\mu$, compared with DNA-fuchsin at $580-585 \text{ m}\mu$. The shape of the absorption curve of DNA-fuchsin seems however to remain constant in a variety of different cells and even after removal of nuclear protein (Kasten, 1956, 1957).

ii. Specificity. After extraction with purified DNAase, nuclei fail to give a Feulgen reaction and equally RNAase does not affect the color produced (McCarty, 1946; Catcheside and Holmes, 1947; Brachet, 1947). The presence of aldehyde groups in hydrolyzed DNA is indicated by the effect of chemical blocking agents, such as semicarbazide and hydroxylamine, which prevent color development (Brachet, 1946; Lesser, 1956). The failure to stain RNA is not due only to its much greater lability during acid hydrolysis, since ribose sugars stain very much less than desoxyribose sugars (Overend and Stacey, 1949; Overend, 1950).

The Stedmans (see for example, Stedman and Stedman, 1947a, b) suggested that the DNA-Feulgen complex was diffusible in the nucleus and acted as a basic dye staining acidic proteins in the chromosome. Although this view is now unacceptable, the idea stimulated several other groups of workers to reinvestigate more carefully the nature of the Feulgen reaction (e.g., Ely and Ross, 1949).

iii. Stoichiometry. As with most color reactions, the proof that the reagent couples quantitatively with the substrate is much more difficult than proof of specificity. Among the first to examine this problem in Feulgen's method were Widström (1928) and Caspersson (1932); the latter showed that DNA and leucofuchsin only combined stoichiometrically *in vitro* up to a DNA concentration of 1.5% and then only when proteins were absent. More recently Lesser (1951, 1953) has re-examined the problem, using films made of DNA dissolved in gelatine. He found the expected linear increase of the absorbance when the proportion of DNA was within the range 0.2 mg. to 1 mg. per milliliter, but above this value the slope of the curve changed. This shows that in these conditions proteins do not affect the reaction provided that the proportion of DNA is below about 0.06% by weight.

Our own measurements (Walker and Richards, 1957) on dry DNA films were designed to test the ratio between the amount of color developed and the ultraviolet absorption of the hydrolyzed DNA. They do show that at any one hydrolysis time this ratio remains constant independent of the *thickness* of the DNA film. They do not, of course, indicate how this ratio is related to that for DNA-fuchsin in solution or in cells, but, at least, the concentration of the DNA is more likely to approximate to that in fixed cells in DNA films than in the models mentioned above.

Sibatani (1953) found that adding histone to the hydrolyzed DNA markedly alters the absorption spectrum of the DNA-fuchsin complex. But this, as Sibatani admitted, has little relevance to the situation in the cell nucleus, and indeed Kasten's (1956, 1957) extensive work on many cell types has indicated little variation of the absorption maximum of the DNA-fuchsin.

How far, then, do the model experiments described have any bearing on the stainability of cells? Clearly, acid hydrolysis will break DNA-histone linkages, but on the other hand the DNA will still reside in an environment of hydrolyzed proteins, which may be similar to that in Caspersson's and Lessler's experiments. One approach (Swift, 1955) to this problem is to measure the amount of DNA in nuclei of widely different volumes; if it can be assumed that the nuclei are all diploid and have the same amount of DNA, then a constant measurement of Feulgen stain indicates that there is a linear relation between stain and substrate over the concentration range studied. In the diploid mouse cells which Swift measured, such a linearity was found up to a concentration of 0.3 gm./cc. Our own view, which is supported by some experiments to be discussed in the next section, is that the protein actually linked to the DNA can modify the Feulgen reaction, but this appears to affect chiefly the coupling of fuchsin to hydrolyzed DNA.

Superficially at least, the earlier experiments seem to invalidate the whole technique as applied to cells, whose DNA concentrations are likely to be much greater than 1 or 2%, but this fortunately did not discourage attempts to measure Feulgen staining quantitatively (Stowell, 1947; Ris and Mirsky, 1949) which led to results of great biological interest (Swift, 1950a, b). The paradox remains, however, between the apparent consistency of the biological results and the uncertainty about the stoichiometry of the technique. This is underlined as we come to consider the detailed variations which are possible in the two main sections of the procedure—hydrolysis and stain coupling.

iv. Hydrolysis. This is usually carried out with normal HCl, but other acids have been used, notably perchloric (Di Stefano, 1952; Woods,

1957), trichloroacetic (Bloch and Godman, 1955a; Bern *et al.*, 1957), phosphoric (Hashim, 1953), and nitric acids (Itikawa and Ogura, 1954). There is also a recent report (Barka, 1956) that bromination will release aldehyde groups in the same way as does acid hydrolysis.

At least two processes occur during hydrolysis: the purines are first removed, because of the lability of the purine-sugar link, and the adjacent deoxyribose ring can then form an aldehyde group, which will later combine with the leucofuchsin. If only this process occurred, then the amount of color per unit of DNA would rise to a maximum at which it would remain, but some of the sugars with their aldehyde group are also removed from the molecule and are found in the hydrolysate (Ely and Ross, 1949). The result is the typical time-hydrolysis curve with its marked maximum, which is presumably the result of the comparatively rapid removal of purines and the much slower removal of sugar aldehydes, with the consequent breaking up of the DNA molecule.

In such a scheme the number of aldehydes available after a given hydrolysis time would be constant only if the two processes just outlined continued at the same relative rates, independent of cell type, or if the first finished before the second began. The latter view was held by Di Stefano (1948) on the basis of his measurements of the ultraviolet absorption during hydrolysis. More recent knowledge of the absorptivities of the purines and pyrimidines (Beaven *et al.*, 1955) do not support this interpretation, and there are, as we have said, strong indications that some aldehydes are lost before the time of hydrolysis which gives the maximum stain.

Walker and Richards (1957) studied the relationship of the amounts of stain and of integrated ultraviolet absorbance to the time of hydrolysis in ram sperm. These cells are particularly suitable, since they contain no RNA and show very little light scatter. It was possible to measure the absorbance at $263\text{ m}\mu$ (due mainly to hydrolyzed DNA) and at $560\text{ m}\mu$ (due to Feulgen stain) in the same cells after different times of hydrolysis. Those experiments showed that after an initial increase due to denaturation, ultraviolet absorbance decreases as the bases are lost. In the meantime the absorbance at $560\text{ m}\mu$ increases to a maximum before decreasing. If it could be assumed that all the fall in the ultraviolet absorbance was due to loss of purines, then we could calculate that the time of hydrolysis, at which all purines were removed (in this experiment 12 minutes) was after the stain maximum had been reached. Chromatographic evidence suggests that pyrimidines are also lost during this earlier period, as was shown by Woods (1957) for sporogenous tissue of *Lilium* and also by Ornstein (personal communication). This has also

been confirmed in our laboratory by Mobbs (unpublished) for ram sperm and thus shows that in these cells there are still purines present long after optimum hydrolysis.

The effect of hydrolysis on the pyrimidine part of the molecule is to remove base and sugar together by disrupting the bonds through the phosphate group (Tamm *et al.*, 1953), thus breaking one strand of the DNA molecule. Owing to the double-helical nature of the molecule and also possibly to the presence of investing protein, it is probable that the sugar-phosphate linkage must be broken at two fairly near positions before a part of the molecule containing several sugar residues can be washed out of the cell. It might be expected that the closeness of these breaks would be affected by the type or quantity of protein present as well as by the order of the purines and pyrimidines. There are in fact several references in the literature (see Swift, 1955) to different optimum times of hydrolysis, and this may be their explanation. On the other hand we have not found any evidence of this variation in the several animal tissues which we have measured. In the latter therefore the synchrony between purine and pyrimidine removal must be good, but this is certainly a possible source of error when small differences of staining are measured after, for instance, different experimental procedures.

v. *Stain coupling.* Swift (1955) has described the factors, other than time of hydrolysis, which alter the degree of staining in tetraploid nuclei of the mouse, fixed in formalin. They are the pH and concentration, not only of the stain but also of the SO₂ rinse, and, to a lesser extent, the time of staining. The amount of stain almost doubles when its pH is raised from 0.8 to 3.6, but it nearly halves if the concentration of the bisulfite in the stain is increased from 0.3 to 10.0%. Staining time had only a small effect, since most of the stain had coupled after 5 minutes and there was no further increase after an hour. The concentration and pH of the SO₂ rinses also caused small but definite effects. We have also found (Walker and Richards, 1957) that a second wash with SO₂ water of sections which had been stored in glycerol at 2° C. removes some of the coupled stain to an extent which was greater with longer times of hydrolysis. These may be stained sections of the DNA molecule, which have become more labile during storage and are eluted under acid conditions. All these factors indicate that standardization of the Feulgen reaction will be difficult, if only because the SO₂ concentrations of both stain and rinses change rapidly with time.

We have recently found another interesting effect of pH of staining (Richards and Walker, in preparation); in the previous paper (Walker and Richards, 1957), we showed that the amount of stain nearly doubles

as the pH was increased from 1.6 to 3.0, and that this was independent of hydrolysis time. In mouse sperm and liver cells which had had identical treatments, we found a rather different position (Fig. 3). The pH of staining had little effect on the amount of stain in the sperm, but, on the other hand, the amount of stain in both diploid and tetraploid liver cells doubled as the pH was increased from 1.25 to 2.9. The effect of

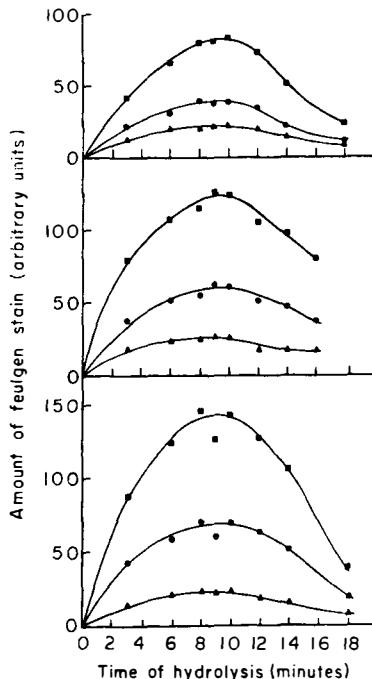


FIG. 3. Differential effect of pH on Feulgen staining at various times of hydrolysis on sperm heads and somatic nuclei (liver) of the mouse. Top, pH 1.3; middle, pH 2.4; bottom, pH 2.9; ■ = tetraploid; ● = diploid; ▲ = haploid (sperm). Coverslips for pH's 1.3 and 2.9 were hydrolyzed in pairs back to back. The ratio of tetraploid to diploid nuclei remains constant at 2:1, but for the extreme values of pH of staining, the ratio tetraploid:diploid:haploid stain values changes from approximately 4:2:1 to almost 8:4:1, as the absolute amount of stain is greater in somatic nuclei the higher the pH but remains fairly constant for sperm heads.

this has been to maintain the expected 1:2:4 ratio for the stain in haploid, diploid, and tetraploid cells only at the low pH. At the high pH this ratio is nearer 1:4:8.

We do not think this detracts in any way from the DNA constancy hypothesis, but we do think it demonstrates the great caution necessary before sperm cells can be used as standards for the control of stain

value in other cells. The cell which might be expected to be the best biological standard for DNA, has proved to be a bad cytochemical standard for the Feulgen reaction, and the procedure which gives the largest amount of stain provides the most inaccurate result. It seems that in this instance we have a good example of a protein effect and the implications concerning possible differences between sperm and somatic cells are of considerable biological interest.

We must now return to our paradox on the results and the chemical basis of the Feulgen technique. It is quite clear that only relative measurements should be attempted, and then only on cells which have received identical treatment, but naturally, *suitable* cells of known DNA content can be used as a standard with which measurements of other comparable cells can be converted to absolute units. With good instruments there is no doubt that highly accurate measurements can be made (Deeley *et al.*, 1954, 1957; Mendelsohn and Richards, 1958). Even if measurements have not been very accurate, results are usually self-consistent and thus support the view that the Feulgen reaction is not only specific but also stoichiometric, if all experimental conditions are identical.

This is quite satisfactory until we come to consider the exceptions and anomalies which have been reported in the literature. Moreover, it is these which are really important, since results like those listed by Leuchtenberger (1958) for various cells with different degrees of polypliody can be inferred from ploidy alone. We have (Richards *et al.*, 1956) previously underlined the three conditions which must be satisfied before an exception to the concept of DNA constancy can be proved. These are that no DNA doubling is occurring, that the chromosome or chromatid number is known, and that errors in measurement, either due to the instrument or to the stain chemistry, are absent.

It is this last condition which is so difficult to satisfy, and nothing we have already written about this reaction can eliminate the possibility of, for example, protein effects in many of these results. It does indicate however that there are two tests which should be applied to anomalous measurements: measurements should be made after staining at two pH values and the possibility should be eliminated of any difference in the shape of the time-hydrolysis curves for the experimental and control cells. It may also be desirable to check the absorption curve of the stain, since small shifts in absorption maximum can introduce considerable error.

b. Dye-binding methods for DNA. The Feulgen color reaction has been used for the quantitative estimation of DNA with such apparent good effect that there has been little incentive to develop other methods.

For this reason, except for the careful work of Kurnick (1954 and earlier) on methyl green, there have been very few papers dealing with the standardization of dye-binding methods for use in photometry.

i. Methyl green. This dye has been used in histology for almost eighty years, usually as part of the mixture with pyronine, which was first suggested by Pappenheim (1899). The success of this mixture in differentiating cell structures was eventually explained by Brachet's (1940) finding, with the aid of specific enzymes, that methyl green stained DNA green and pyronine stained RNA red. Subsequently Pollister and Ris (1947) attempted to measure the ratio of DNA to RNA by comparing the ratio of the absorbances of the two dyes, which can be easily done since the peak of the absorption curve of methyl green is at $630\text{ m}\mu$, while that of pyronine is at $550\text{ m}\mu$. They had to confine their observations to a comparison of ratios of the two types of stain in different tissues, since nothing was then known of the stoichiometry of the stains.

In 1947, also, Kurnick suggested that the selectivity of the two dyes was due to the way their binding capacities altered with the degree of polymerization rather than to the type of the nucleic acid. This suggestion tended to explain the observation made by Unna (1902) and later repeated by Pollister and Leuchtenberger (1949) that hot water abolished the capacity of chromatin (DNA) to bind methyl green. Kurnick (1950) later showed that hot buffer at pH 4.1 or hot acid caused a large decrease in the amount of methyl green bound to DNA in solution. On the other hand, while heating cell nuclei in 1 N HCl caused a loss in stainability, heating them to 60° C . at pH 4.1 caused no loss, and indeed treatment with cold dilute HCl caused a twofold increase in the stain. The latter was explained by Kurnick as being due to the removal of competing histone.

It soon became clear, however, that degree of polymerization alone, that is, the molecular weight, could not explain the specificity of methyl green for DNA. Kurnick and Mirsky (1950) found that one molecule of the dye was bound by ten phosphoric acid residues, and this figure was later modified to thirteen by Kurnick and Foster (1950), who used a better method of testing the stoichiometry and a slightly different dye. It therefore seemed probable that quite small molecular weight fragments should combine with the dye and that the loss of dye-binding capacity should result only from breaks between a pair of binding sites. Subsequently, much more work was done on the denaturation of DNA, which can be detected by increase in absorptivity (Thomas, 1954) and which occurs before there is much loss in molecular weight as detected by loss of viscosity and by changes in sedimentation rate or dialyzability.

Kurnick (1954), therefore, reinvestigated the binding of methyl green to DNA, after heat treatment or after deoxyribonuclease (DNase); DNase caused a sharp decrease in molecular weight as indicated by a fall in viscosity of 50%, but only reduced the amount of stain bound by 4%. If, however, a similar fall in viscosity is produced by heat, then the bound stain falls by 16%. In addition, Devreux *et al.* (1951) found that an X-ray dose which reduced viscosity by 20%, caused a 30% loss of stainability.

We must assume therefore that the molecular weight or state of polymerization of the DNA does not by itself affect the binding of methyl green to DNA. There must be some additional factor, presumably the molecular configuration of the molecule, which alters stainability and that, as might have been expected, different treatments disorganize the molecule to different degrees. But since all these treatments cause an increase in absorptivity (as $\epsilon(P)$), it is not essential for DNA to remain undenatured if it is to be completely stained. On this view, then, DNA can be denatured, depolymerized, and disorganized, but only if the latter occurs, will methyl green-binding be prevented.

Turning now to the staining of cells, Kurnick (1952) proposed a quantitative cytochemical technique, in which competing histones are first removed with acid and the staining is carried out at pH 4.1, to avoid, as he then thought, depolymerizing the molecule. Alfert (1952) questioned the whole basis of methyl green staining put forward by Kurnick and suggested that it is the degree of protein competition that determines the binding capacity of DNA for methyl green and that in RNA all the binding sites are blocked by protein. In support of this view, he presented some measurements which certainly indicate that methyl green stain is unreliable as a quantitative method. On the other hand, his measurements always gave a constant methyl green:Feulgen ratio, and it is unlikely that similar errors in stoichiometry should occur in both methods, as Alfert himself pointed out. To this criticism Kurnick (1955) replied that Alfert had not used the procedure which Kurnick had found necessary to prevent disorganization of the DNA and to remove the competing histone. There may be, however, some substance in Alfert's criticism, since Bloch and Godman's (1955b) work show that the cold HCl treatment does not remove all the competing protein groups and that it is only after acetylation of the amino groups that methyl green staining shows the same distribution in the same cells as does the Feulgen reaction.

This work of Bloch and Godman is also of great interest since they were able to show that protein blocking occurred only in the nuclei which had the diploid amount of DNA, as determined by Feulgen meas-

urements, and that it did not occur in some of the nuclei actually synthesizing DNA. They interpreted this as showing that during the first part of interphase the chromosomes were in a heterosynthetic stage, producing new proteins, and that later they went into an autosynthetic stage in which they duplicated themselves. Since there is good evidence that histone is normally bound to DNA and therefore likely to interfere with methyl green staining, it is also possible that their results indicate that, during DNA synthesis, the histone is much more loosely bound to the DNA or that, possibly, a more labile enzymatic protein is bound to the phosphate groups.

In these experiments, the cells were treated with ribonuclease to remove any possibility that RNA may be stained, as Alfert (1952) has reported in the cytoplasm of formalin-fixed cells. It is difficult to understand why this should occur, as there is increasing evidence that RNA has not a molecular structure similar to DNA, and in view of the sensitivity of methyl green to the molecular configuration of DNA.

It seems very desirable that more quantitative measurements should be made on methyl green stained preparations both after acetylation, when the results can be compared with those from Feulgen measurements, and without removing protein, when the measurements may give interesting new information on protein-DNA binding. Care will still be needed to prevent disorganization of the DNA molecules in the fixing of the tissues and in the various subsequent procedures for removing competition from proteins.

ii. Gallocyanine-chrome alum. Photometric estimations of DNA using this basic dye were reported by Swift (1953) and Mendelsohn (1957), and in addition Sandritter *et al.* (1954) have investigated the stoichiometry of its binding to RNA. Only a fixed amount of stain is bound, so that it is not possible to overstain and differentiation is unnecessary. The cytological picture for stained nuclei after ribonuclease treatment and the photometric measurements mentioned above seem to show a better correspondence to Feulgen stain than do those of other bound dyes.

Stenram (1954) investigated the specificity of this stain for nucleic acids, following the suggestion of its quantitative use by Einarsen (1954), but was unable to demonstrate that the stain was specific for DNA after RNAase digestion. Certainly competitive effects are present before RNA is removed, since Mendelsohn (1957) finds that the amount of dye bound to isolated nuclei *increases* after treating with RNAase.

2. Methods for RNA

As more information is accumulated about the important role of RNA in the cell, both in protein synthesis and in the transfer of genetic

information, so the need for a quantitative staining technique for RNA has increased. The cytochemical detection of RNA is fairly simple with basic dyes and ribonuclease, as Brachet (1953) has indicated (see also Taft, 1951). Even so, as he points out, the type of fixation, the possibility of eluting the RNA from the cells, and the purity of the enzyme are all of great significance.

The main difficulty in devising a suitable quantitative method for RNA seems to be due to its great instability and possibly to its heterogeneity, since it is known to occur in different fractions in nucleus and cytoplasm and it may be that these will react differently.

It will bind basic dyes, such as pyronine, azure B, and toluidine blue, but there is considerable risk of interference by other substances in the cell, and it is not generally considered that these methods can be used quantitatively. Ultraviolet absorption methods, in which RNA can be distinguished structurally or chemically remain at present the most promising way of investigating this important substance quantitatively, although Swift and his group have used azure B quantitatively.

a. *Azure B.* The binding of this dye to nucleic acids was investigated by Flax and Himes (1952), who found that it stains DNA orthochromatically and RNA metachromatically and that there is a considerable difference between the absorption curves. Swift *et al.* (1956) have made interesting attempts to measure RNA in the chromosomes, nucleoli, and cytoplasm of a number of cell types. They checked the specificity with RNAase and they state that absorption-curve analysis indicates that Beer's law holds, but they do not give any details in this review. The method must be regarded as in an early stage of development, but it could be useful provided that interference from other substances, particularly protein, could be controlled.

3. Methods for Proteins

Both color reactions and dye-binding methods have been suggested for proteins, which are quantitatively the most significant constituents of cells. None of the available dye-binding methods are able, however, to separate them more precisely than into histone and non-histone types, but the color reactions can be more specific and include methods for specific amino acids and for sulfhydryl groups. Quantitative measurements have been attempted with most of these, but with one of them—the diazonium reaction of Barnard and Danielli (1956)—measurements are only now being attempted; nevertheless the method is so promising that we will consider it here.

a. *Color reactions. i. Millon's reagent.* This is one of the oldest tests for proteins (Millon, 1849), and it was applied to tissue sections by Bensley and Gersh in 1933, but the first attempt to measure the colored

products in cells were not made until 1947 (Pollister and Ris, 1947; Ris, 1947). The reaction was shown by Folin and Ciocalteu (1927) to be specific for tyrosine and tryptophan *in vitro*, with which it seems to form colored nitroso compounds. The reagent contains mercuric and nitrate ions in an acid solution, and the end products are referred to as tyrosine and tryptophan "mercurials," each of which has a distinctive absorption curve. Both of them have absorption maxima at 360 m μ , but only the tyrosine mercurial has a high absorptivity in the visible region at 480 m μ . This, as Pollister and Ris (1947) suggested, would enable the ratio of tyrosine to tryptophan to be obtained. In addition, the method can be used to distinguish histone from nonhistone proteins, since the former is soluble in a Millon reagent containing sulfuric acid but not in one containing trichloroacetic acid. Pollister and Ris used this method, after suitable calibration procedures, to obtain relative amounts of histone and nonhistone protein in the nucleoli of the pollen mother cells of maize.

As a cytochemical procedure, this reaction has two serious drawbacks: first, it is so vigorous that it leaves the cell with a poor cytological appearance and, second, it has little color in the more easily accessible visible region, although the absorption peak around 350 m μ is much higher. Most measurements (Pollister and Ris, 1947; Ris, 1947; Leuchtenberger, 1950) have been made at 365 m μ , but recently the reaction has lost favor in the face of other better and less drastic techniques. It is interesting to note that a coupling reaction for tyrosine has recently been proposed (Lillie, 1957) which, it is claimed, gives a similar color distribution as that of the Millon reagent, but with greater intensity and clarity.

ii. The diazonium color reaction for histidine. This method of obtaining a color reaction from certain specific amino acids has been recently described by Barnard and Danielli (1956), although earlier attempts were made by Mitchell (1942) and Danielli (1947, 1950). The basis of the method is to react the amino acids with tetrazotized dianisidine and then to enhance the color by coupling it further with, for example, β -naphthol. Under these conditions the tyrosine, tryptophan, and histidine of the tissue proteins could be expected to react, but if the tissue has been previously treated with the blocking agent, benzoyl chloride, complete blockage does not occur, instead color remains in the nucleus. This color is stable and intense, provided that the tissue has been fixed by freeze-drying and that aqueous media have been carefully avoided until after benzoylation.

Careful studies, in which the colored product has been precipitated in bulk and studied by infrared spectroscopy, have shown that, after benzoylation, only histidine reacts. Chemical fixatives give only a poor

reaction, and from this and other evidence it has been concluded that part at least of the histidine is protected during benzoylation bonding to another substance. The close similarity between the distribution of the histidine and Feulgen colors in nuclei indicates that this other substance may well be DNA, and that the color reaction therefore shows the presence and number of the histidine-DNA bonds. This may be of great biological importance and the results of measurements of the amount of color developed under different conditions of cell growth are awaited with great interest.

iii. *The Sakaguchi reaction for arginine.* The method of estimating arginine in solution, developed by Sakaguchi (1925), has been used as a qualitative technique in cytology by Serra (1944a, b, 1946), Baker (1944, 1947), and Thomas (1946). Briefly the technique is to cause the protein in a smear or section to react with a mixture of sodium hypochlorite and a coupling agent in approximately 1% sodium hydroxide. Various coupling reagents have been proposed: Sakaguchi originally employed α -naphthol but later (1950) substituted 8-hydroxyquinoline, which was also used on tissues by Warren and McManus (1951) and by Carver *et al.* (1953). The most suitable agent seems, however, to be 2,4-dichloro- α -naphthol (Bell, 1952), which was employed by McLeish *et al.* (1957) in a method for the quantitative determination of arginine groups.

This quantitative estimation has several difficulties: the color, besides being rather weak in normal preparations, fades after staining to a degree that depends on the mounting medium. Carver *et al.* (1953) used Permount containing 0.02% aniline, but McLeish *et al.* (1957) found that glycerol with 1% sodium hydroxide was better. A second disadvantage is that the availability of arginine groups appears to depend largely on the type and the length of the fixing procedure. McLeish (personal communication) has measured the amount of stain in isolated nuclei from calf thymus, which had been fixed in several of the common chemical fixatives, and has found with all of them that the intensity of stain varies with time of fixation. Third, it is probable that the strong alkalies used both during the reaction and in rinsing will remove a variable amount of protein.

It seems, then, that much more needs to be done in standardizing the procedure before the results can become useful, but the importance of measuring basic proteins may well justify the effort.

iv. *Color reactions for sulfur-containing proteins.* Both Bennett (1951) and Barrnett and Seligman (1952) have developed color reactions for sulfhydryl groups of proteins. The latter authors' method, which gives a much more intense color and better specificity than Bennett's, consists of reacting the sulfhydryl groups with 2,2'-dihydroxy-6,6'-dinaphthyl

disulfide at pH 8.5. This gives an insoluble colorless compound with the protein which can be then coupled to tetrazotized o-dianisidine to give an intensely colored product. The method was later used by Barrnett and Seligman (1954) to investigate the distribution of sulfhydryl and disulfide groups in various tissues. With this method, Cafruny *et al.* (1955) and Teiger *et al.* (1957) have made quantitative photometric measurements of these two groups, which they distinguished by the difference in staining in normally treated sections and in those which had been reduced with thioglycolic acid or 2,3-dimercapto-1-propanol. The latter workers had to use blank unstained sections as controls in their measurements, which suggests that light scatter was high, but it seems that it may be a very useful technique, especially in view of the reviving interest in sulfhydryl groups in relation to cell division (see, for example, Swann, 1957).

There is a recent review of these methods by Bennett and Watts (1958).

b. Dye-binding methods for total protein. Recently, several acid dye-binding procedures have been published. Superficially, there is not much to distinguish between them other than in their color and absorptivity, although it might be expected that the size of the dye molecule may alter the ease with which it can bind to certain sites. In fact, with some there are interesting possibilities of distinguishing different types of proteins or amino acids, but none have been used extensively for quantitative measurements and it is difficult at present to judge how useful they may be.

i. Mercuric bromophenol blue. Devised by Durrum (1950), this procedure was modified by Mazia *et al.* (1953) to make it more useful in cytochemistry. The dye has a very high absorption peak at $610\text{ m}\mu$ and can therefore only be used in thin sections. These workers attempted to check that Beer's and Lambert's laws held for this dye in sections, but as Glick (1953) pointed out and as we have discussed earlier on page 98, their method is not satisfactory as a test for Beer's law. They also found by means of model experiments that one dye molecule coupled with about one in ten of the basic amino groups present. This reaction is also interesting in that basic proteins bound the dye in the absence of mercury, unlike other groups which required the mercuric ion before they stained. It may, therefore, be possible to distinguish basic from other proteins, although as is usual with these reactions other substances such as nucleic acid can compete for the dye.

ii. Naphthol Yellow S. Deitch (1955) has described some careful experiments on this dye, which she selected because its absorption peak ($435\text{ m}\mu$) does not overlap that of the Feulgen-DNA complex; in this way total protein, after Feulgen hydrolysis, and DNA can be measured

in the same nucleus. Naphthol yellow has also been used by Vickery (1940) for testing for proteins *in vitro*. Deitch has shown that the dye probably binds to basic amino groups in tissues in a quantitative way, and that, in addition, the terminal basic groups of lysine could be selectively blocked and thus allow the ratio of these lysine groups to the content of dibasic amino acids to be obtained.

Removal of RNA has no effect on naphthol yellow stainability, but removal of DNA by DNase causes an increase in the amount of dye bound. It is of considerable interest that DNA after Feulgen hydrolysis can still bind enough protein groups to block about 25% of the total dye-binding sites. In her measurements on cells, Deitch measured only changes in absorbance per unit area and it may be that the changes reported should be corrected for changes in volume, but in general it seems that the method could be useful, provided the limitations common to all dye-binding methods are accepted.

iii. Fast Green. This is the selective dye-binding method for basic proteins of Alfert and Geschwind (1953), in which the nucleic acids are first removed from the tissue with trichloroacetic acid and the cells are stained with 0.1% fast green at pH 8. At this pH the stain is almost entirely confined to the nucleus, but in contrast the whole cell is colored after staining at pH 2.2. Deoxyribonuclease treatment, but not ribonuclease, can be used instead of the acid and gives a similar stain distribution.

Model experiments with known proteins on filter paper indicated that the dye-binding at the higher pH is correlated with a high isoelectric point of the protein rather than with the presence of specific basic groups. In particular, edestin, which contains a high percentage of arginine, does not stain at pH 8. It seems, therefore, that this method is selective for histonelike proteins, which have a preponderance of basic groups.

Bloch and Godman (1955a) have introduced a useful modification which allows both Feulgen and fast green staining to be measured in the same cells. They have substituted trichloroacetic for hydrochloric acid in all stages of the Feulgen procedure and then, after measuring the Feulgen stain, have continued the treatment with trichloroacetic acid to remove the remainder of the DNA. The cells can then be stained with fast green.

D. Mass Determinations

I. Interference Microscopy

Although interference microscopes are not novel instruments, it was not until 1950 that Dyson (1950) and Smith (1950) separately designed two practicable microscopes, which were based on beam-splitting by

semireflectors and by birefringence, respectively. Soon after this, it was realized independently by Davies and Wilkins (1951, 1952) and by Barer (1952) that these microscopes could be used to measure, in biological preparations, dry mass, which is related to the optical path difference (o.p.d.) between the cell and its (aqueous) environment. The method depends, as we have mentioned, on the fact that the specific refractive increments (α) of substances in cells varies over only a limited range. Hence the product of o.p.d. and projected area of the object is basically nonspecific.

Many designs of interference microscopes have been developed (see Barer, 1956), and the comparative merits of the two most generally available commercial models have been discussed by Davies (1958). These comments on microscopes occur as part of more general reviews of interference microscopy which these authors have written (see also Barer, 1955; Davies, 1956). We shall attempt to discuss only two topics, namely the general significance of mass measurements in cytology and the constancy of the specific refractive increments; for a much more comprehensive account of the whole procedure, the above references should be consulted.

a. The measurement of mass. Growth can be defined in several ways: increase in size or volume is one; increase in mass is another; and, in addition, increase in complexity could also be used, although this is much more difficult to measure. Any of these definitions may be perfectly satisfactory for a whole multicellular organism, but on the cellular level obvious problems arise because the cell can increase its size simply by uptake of water. Dry mass is therefore a more satisfactory parameter, since its increase will often give a measure of the production of new protein. It does not of course show the appearance of a different protein, and for this purpose the various specific staining reactions will be much more useful.

Dry mass may also provide a convenient value, to which measurements by the more specific techniques may be referred. It is most desirable to have such a reference for measurements on sections in which it is otherwise difficult to control the thickness of the material. In this way the relative concentrations of substances in cells can be legitimately compared in different structures. Unfortunately, owing to the necessity for a second comparison beam in most interference microscopes, it is difficult to make measurements in the middle of a section. Isolated cells or nuclei can of course be measured, but these can often be obtained as whole structures and, depending on the problem, it may not be so essential to know the total dry mass.

Returning to the measurement of total protein, there are, of course, other substances in cells; for example there is about 13% by mass of DNA in the nucleus of a mouse cell. This amount may not be very

large compared with total nuclear proteins, but it can be measured, if necessary, in fixed cells in two ways. Either the unwanted substances may be specifically extracted or they can be measured by other cytochemical techniques and the amount subtracted from the total dry mass. The former method is usually less satisfactory, since, apart from uncertainty due to the specificity of extraction, the difference may be small and an accurate instrument such as that made by Davies and Deeley (1957) will be required. An additional difficulty is that extraction may cause a small alteration in χ (see Section III, D, I, b). The subtraction method is the better, provided the results of the specific technique can be converted into units of mass, as was done by Richards and Davies (in preparation) in measurements of DNA and dry mass of nuclei.

Changes of concentration in cells which may reflect their physiological activity should be measured only in living cells, since, after fixing, all the components are precipitated in the solid state with a small quantity of water, whose amount probably has little significance. Such a distribution of concentration in living cells may be determined with the interference microscope (Davies, 1958), but in those instances when the variations within the structure are not large it is probably simplest to use the phase microscope to match the refractive index of the cell and the medium (Barer and Ross, 1952; Barer and Joseph, 1954, 1955a, b), who found that media of known refractive index, containing albumin, did not harm the cells investigated.

b. *Variations in χ .* By definition (see page 97) χ is equal to 100α , where α is the specific refractive increment. Values for χ have been obtained by many workers for a large number of pure substances which occur in cells, and these have been listed by Barer and Joseph (1954), Davies *et al.* (1954), and Davies (1958). In brief, the values obtained for pure proteins in solution ($\chi = 0.18$), vary only slightly and for dilute solutions of nucleic acids they vary from 0.16 to 0.20 (best values 0.18–0.20). In dilute solutions of nucleoproteins, e.g., viruses, χ varies from 0.17–0.19. More serious discrepancies occur in lipids. ($\chi = 0.14$), in carbohydrates ($\chi = 0.13$ –0.14), and in certain electrolytes. In the latter, χ is 0.115 for a dilute solution but falls to 0.079 in the crystal. The available measurements are generally for dilute solutions at concentrations, naturally, much below that found in the nearly dry fixed cell. It is also true that χ for gels of proteins, such as gelatin, and of nucleoproteins, such as tobacco mosaic virus, are both 0.15. In view of this, Davies (personal communication) has been measuring values of χ for protein crystals, for example those of β -lactoglobulin, and has found there is no serious change from the value in dilute solution.

Although there is no doubt that χ is much less affected than is

absorptivity by environmental factors such as pH, temperature, and ionic strength, it has not yet been shown that small changes do not occur; these may be of great importance when measurements by extraction are attempted. In particular we might expect that the gross changes in physical state which occur during fixing and during some extraction procedures would alter its value. It does not seem, therefore, that interference microscopy can be used for accurate measurements of the amounts of substances which comprise only a small fraction (< 10%) of the total cell or nuclear mass without much more information about small changes in χ .

It is equally true that the apparent absence of gross errors in χ and the less serious errors in measurement, which may be expected (see Section II), make interference microscopy much the best way of measuring total dry mass and total protein.

2. Absorption of Soft X-Rays

A detailed account of this method for determining the mass of biological structures is beyond the scope of the present article (and of its authors), but since it employs an essentially microscopical procedure a brief description is necessary. Since its first development in Sweden it has been employed in only a few places mainly because, in common with ultraviolet absorption techniques, it demands apparatus outside the normal scope of most present biological laboratories.

The measurement of the absorption of soft X-rays and its use for historadiographic analysis and mass determination in biological objects was first described by Engström and Lindström (1949, 1950). A recent review of the method was given by Engström (1956) and a survey of the use of the technique mainly in nonbiological fields is given by Cosslett *et al.* (1957).

In the technique of Engström and Lindström the specimen, usually a smear or section of fixed material, is photographed in a fairly parallel beam of soft (2-13 Å wavelength) X-rays. Since a vacuum is necessary in the camera the specimen must be dry, and this clearly limits the application of the technique. Because the X-ray beam is parallel, however, no error arises from parts of the specimen being out of focus, unlike other microscopical techniques. X-rays cannot easily be focused, and therefore the specimen has to be placed in contact with the film so that the absorption image is in fact a shadow at unit magnification. It is essential that good contact between specimen and the fine-grain film be obtained, since, although focusing effects are virtually absent, considerable error can arise from scattering of the radiation by the specimen. Calibration of the absorption image is by means of a reference "wedge"

system made of thin colloidin or nitrocellulose films (having an X-ray absorption range similar to that of the specimen) which is exposed simultaneously with the specimen. The X-ray image of the specimen and the wedge is then enlarged about 200-500 times, and photometric determinations are made on the enlarged image.

In the absence of accurate knowledge of section thickness, the data obtained refer to the total dry mass per unit projected area. Total amounts of substance can be obtained only for structures such as whole cells. The wavelength of the radiation used is, of course, considerably shorter than in the other techniques already discussed, but unfortunately it is not possible to benefit from this by increased resolution because the resolving power is limited by the grain size of the film.

The quantitative use of the technique requires a knowledge of the average mass absorption coefficient of the specimen. Unlike its analogous factor, χ in interference microscopy, this varies considerably with both the wavelength and the substance. In order to obtain a reasonably accurate average value it is necessary to obtain independent data about the quantitative chemical composition of the specimen. Difficulties might arise in exceptional cases, however, where local high concentrations of an element having a high absorption coefficient occur, unless these could readily be detected by a characteristic X-ray spectrum. It is also desirable to avoid using fixatives which deposit heavy metals in the specimen and so artificially increase its X-ray absorbance.

As we have already noted, there is good agreement between measurements by X-ray absorption and interference microscopy on the same or similar objects (Davies *et al.*, 1953; Davies and Engström, 1954). The resolution of the interference microscope is about 0.25μ as for any light microscope, and in recent years the resolution of the X-ray method has been improved from 1μ to a value which approaches that of the light microscope (Engström, 1957). In other respects also the two methods are similar: both have to contend with errors due to inhomogeneous distribution of absorbing material and other errors present in photometric procedures.

It is apparent that the X-ray method will find increasing application in the studies of biological structures, especially in historadiographic chemical analysis and also in microtechniques of X-ray diffraction.

IV. CONCLUSIONS

In the previous sections we have considered the biological and technical justification for quantitative microscopical methods, as well as the individual techniques themselves. It remains for us to touch on two other topics: the relation of these methods to others available to the

cytologist and cytochemist and our own views on the achievements and prospects of quantitative cytochemistry.

At the beginning of this chapter we mentioned that one of the principal disadvantages of quantitative cytochemistry is the difficulty of checking results by any method, in which we do not have to make assumptions regarding the intracellular environment. In practice, only in a few instances have cytochemical and biochemical techniques been compared on uniform populations of cells which may be studied by both methods. It is, however, in studies of heterogeneous populations that quantitative cytochemical techniques are most essential, and it is therefore necessary to consider other procedures which may confirm and amplify cytochemical results and which in turn may be illuminated by them.

The most important of these is autoradiography (see Chapter 5); this powerful method is subject to uncertainties of a quite different kind, compared to those we have been considering, and it usually provides a measure of the rate of incorporation of a chemical group. In some instances the results from the two methods are directly comparable and show good agreement. An example is the rate and time of DNA synthesis of chick nuclei in tissue culture as demonstrated by ultraviolet absorption and Feulgen staining (Walker and Yates, 1952) and by tritium-labeled thymidine (Firke and Verly, 1958; see also Firke, 1958). Such agreement increases our confidence in both methods; but, on the other hand, discrepancies between the two approaches may be more revealing. For example, the apparent increase of DNA during the formation of a puff in a salivary chromosome of *Rhynchosciara* is, it seems, very much less when measured by ultraviolet microspectrophotometry (Rudkin and Corlette, 1957a) than when measured with tritium-labeled thymidine (Ficq and Pavan, 1957).

Other useful methods are those developed by Edström and Hydén (1954) and by Edström (1956) for the measurement of extracted RNA and for microelectrophoresis. These methods rely on the ultraviolet microscope for making the final measurement; but since the material is extracted and measured as a uniform area in a structureless matrix, errors due to inhomogeneity of the specimen are eliminated. In addition individual nucleotides as well as nucleic acids can be investigated with the electrophoresis method.

Many of the methods which Glick discusses in Chapter 6 are reaching or, in some instances, have already reached the level of sensitivity of the microscopical techniques; this is often achieved by using the very sensitive fluorometric methods of estimation (Robins, 1957). There is little doubt that many other methods which have been developed in

biochemistry and allied fields will soon be applicable to a single mammalian cell.

Before considering the future usefulness of photometric methods in this situation, it is perhaps desirable to look at the contributions these methods have made in the last ten years to our understanding of the chemistry of the cell. In the development of the DNA constancy hypothesis, Feulgen staining showed how the existence of polyploidy in rodent liver (Ris and Mirsky, 1949) could explain the high average value for DNA which had been obtained by biochemical procedures (Mirsky and Ris, 1949).

Later, Swift (1950a, b) demonstrated the existence of amounts of DNA in diploid dividing cells which were intermediate between the amounts in diploid and tetraploid cells and that this was probably due to DNA synthesis in these cells. Walker and Yates (1952) confirmed the interphase synthesis of DNA by measurements both of ultraviolet absorption and of Feulgen stain in nuclei, of which the time of their previous division had been observed. They also showed that in living cells, which were actively growing, there was a large amount of ultraviolet absorbing substances, probably nucleotides and RNA, which did not occur in living erythrocyte nuclei or in sperm heads. Other workers (e.g., Pasteels and Lison, 1950) reported DNA synthesis during telophase, and, although particular results may be questioned, it is not impossible that DNA synthesis can occur during different fractions of interphase (cf. Hornsey and Howard, 1956).

Variations in the amount of DNA have also been studied extensively in various abnormal and pathological cells (e.g., Leuchtenberger, 1957), and accurate measurements of Feulgen stain have been used to count chromosomes in ascites tumor cells of mice (Richards *et al.*, 1956). Such measurements can be employed, not primarily to find out more about DNA, but to study cell populations in, for example, human tumor cells (Atkin and Richards, 1956). This may be a most rewarding aspect of cytochemical research.

More recently the interferometer microscope has enabled us to follow the growth of a single cell during more than one cell generation (Mitchison, 1957), and to measure the average rate of increase in mass of cell nuclei (see Davies, 1958). As more methods have been developed, very useful information has been obtained by combining several techniques, and this too, is likely to provide stimulating results. An example of this is the work, already mentioned, of Bloch and Godman (1955a) and also of Alfert (1955) in which the relation of DNA to histone is studied in a growing nucleus.

Although the DNA constancy hypothesis is generally accepted, there

have always been workers who have held that the amount of DNA could vary, depending on the metabolic state of the nucleus. Govaert (1957) has recently written a paper, supporting this view, which contains most of the references to this side of the argument. Unfortunately, at least some of the early work on which the variability hypothesis has been built could be criticized on technical grounds, and this has led many workers to ignore all evidence which might indicate real DNA variability. Our own view is that this may exist, but that proof of the presence of exceptions is difficult and that in most instances the careful measurements required have not been attempted. The problem remains a most interesting one and may well yield important information about the functioning of DNA in the chromosomes.

Although cytochemical measurements have played no small part in reviving and enhancing interest in the nucleic acids, we must admit that, at present, the most fruitful ideas are coming from the physical and chemical study of cellular extracts or from microorganisms. One reason for this is that investigation has been mainly concerned with the basic properties of living things—gene action and protein synthesis—and very much less research is being done on cell growth and differentiation. It seems that cytochemical techniques which rely on microscopical measurement, including microchemical methods, will be essential in studying the processes which occur when cells stop growing and differentiate or when they stop producing one kind of protein and start dividing again. It may be that the methods which we have discussed are not sensitive enough to detect the small quantities of substances which may be all-important; on the other hand, we have seen that they are often only too sensitive to changes in the intracellular environment. The resolution of this, our initial paradox may indeed be the best hope of discovering more about the chemistry of those cells which differentiate as well as grow.

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CHAPTER 5

Quantitative Microchemical Techniques of Histo- and Cytochemistry

By DAVID GLICK

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I. INTRODUCTION

Microchemical techniques of quantitative histo- and cytochemistry are usually counterparts of well-established macrotechniques which have been scaled down for use on histologically or cytologically defined samples. There are occasional exceptions in which a principle that had not been employed previously in macrowork is applied to microchemical measurements, e.g., the Cartesian diver in micromanometric analysis. The precision and accuracy of the microchemical techniques are generally about the same as those of their macroparents, since principle, concentrations of reagents, etc., are changed little if at all. Volume reduction is the essential innovation, and the microequipment can provide an accuracy similar to that of the usual macroapparatus.

The kinds of biological sample particularly amenable to investigation by current quantitative microchemical methods are microtome sections of tissue or structures dissected out by manual manipulation, whole large cells such as protozoa, ova of marine invertebrates, and cells in nervous tissue, or isolated portions of large cells. Reliable quantitation still demands the sacrifice of a degree of localization in many instances.

To deal with the present subject within the limitations of a single chapter necessitates confinement of the treatment to chief considerations of approach, principle and area of application, and omission of details of design and manipulation of apparatus, methodological procedures, etc. Accordingly, this chapter will be concerned with the chief considerations of the isolation and preparation of the biological sample, measurement of its amount, and its analysis by titrimetric, spectrophotometric, flame photometric, fluorometric, gasometric, dilatometric, and microbiological techniques.

II. PREPARATION OF SAMPLES

The main requirement in the preparation of the sample is that it provide a histo- or cytologically defined portion of material that can be adequately analyzed by one of the available microchemical techniques. To this end several modes of procedure are employed, each with its own field of application and limitations, and these will be considered.

A. Centrifugal Separations

Isolation of cell particulates or intact cells by differential centrifugation in a medium of fixed density, or by centrifugation in a density

gradient, usually yields quantities of sample that can be analyzed by macromethods (Anderson, 1956), and this subject is discussed by Allfrey in Chapter 8 of this volume. However, there are some cases in which centrifugal separation is employed to segregate parts of a single large cell or relatively few whole cells of a given type, and microchemical procedures are of great value for work on such samples. For example, the well-known studies of E. N. Harvey (1933) and E. B. Harvey (1936) on centrifugal isolation of morphological components of individual ova of *Arbacia punctulata* have been followed up by Holter and co-workers, who used protozoa as well as ova to obtain parts of cells for microchemical investigations (Holter and Linderstrøm-Lang, 1951); Holter (1954). Examples of particular types of cells isolated in a density gradient are given by Ottesen (1954) for blood lymphocytes and granulocytes, and by Glick *et al.* (1956) for peritoneal mast cells. The earlier method of flotation on albumin solutions of Ferrebee and Geiman (1946), Vallee *et al.* (1947), and Fawcett *et al.* (1950) is a similar procedure that has been used by these authors, respectively, for separation of parasitized erythrocytes, leucocytes, and malignant cells. The advantages in the use of solutions of high molecular weight substances for isolation and study of intracellular structures was pointed out by McClendon and Blinks (1952). It should be added that it is also advantageous to employ solutions of albumin or other high molecular weight substances for work on intact cells. The gentle disruption of tissue by pressing through graded screens to obtain a high percentage of whole cells was described by Kaltenbach (1954).

Although it has not yet been adapted to small samples, the counter-streaming centrifuge of Lindahl (1956) is of interest in this connection, and it has already been applied to the isolation of ascites tumor cells (Lindahl and Klein, 1955) and the concentration of eosinophile leucocytes (Lindahl and Lindahl, 1955). This centrifuge permits separation of particles of the same density but of different sizes, and it operates by counterbalancing centrifugal force by the streaming of the suspension in the centripetal direction. A density gradient which aids separation of the particles is produced by lowering the temperature of the inflowing suspension.

B. Chromatographic Separations

In addition to the properties of size, density, and shape which are utilized for centrifugal separations, physical and chemical surface properties are being exploited for cell and particulate isolations. The successful use of column chromatography for the separation of active particles of the viruslike agent of chicken tumor I by Riley (1948) led him and his collaborators to extend the technique to the separation of the micro-

copically visible cytoplasmic granules of the Harding-Passey and S 91 mouse melanomas (Riley *et al.*, 1949), and then on to an improved procedure for the segregation of cytoplasmic components from normal and tumor tissue (Riley and Woods, 1950). Columns of diatomaceous silica (Celite) were used in this work.

Concentration of human blood platelets has also been effected by adsorption (Freeman, 1951), and even bacteria have been chromatographed (Maruyawa and Yamagata, 1955). Albertsson (1956) investigated this technique further, using the intact and disrupted algae, *Chlorella pyrenoidosa*, bacteria, and spinach chloroplasts. He was able to obtain separations of the cells, chloroplasts, cell walls, and starch grains on calcium phosphate columns eluted with phosphate buffer, and also by partition with the system, water-phosphate buffer (pH 7)-polyethyleneglycol 4000 (Carbowax), which gives two liquid phases at certain concentrations.

C. Microdissection

This subject is treated in detail by Kopac in Chapter 7, but it should just be mentioned here that microdissection, with or without the aid of a micromanipulator, has been used to furnish samples for microchemical analysis not only from cells already isolated in nature, but from tissues as well, e.g., dissection to separate individual cells from nervous tissues as carried out by Lowry (1953), Lowry *et al.* (1956a), and Hydén (1955). McCann (1956) used trypan blue as an intravital dye to distinguish proximal from distal tubules in the microdissection of the dog nephron to obtain suitable analytical samples for studies of enzymes, lipid, and water. Such aids should materially increase the use of microdissection.

D. Microtomy

For single smaller cells common in tissues of higher organisms, the technical limits of microdissection sometimes are exceeded and a number of cells are usually needed for a suitable analytical sample. When nature provides groups or layers of one kind of cell in a tissue, microtomy can be used to furnish samples which allow correlation between chemical analysis and cell type and number. Unfortunately, the number of tissues in which this natural grouping occurs is small, but for retina, adrenal cortex, gastric mucosa, etc., this technique has been used to advantage. Microtome sections also provide preliminary samples from which morphologically defined portions can be removed by microdissection. The sampling and sectioning technique of Linderstrøm-Lang and Mogensen (1938) for preparation of fresh-frozen sections employed a rotary micro-

tome in a cryostat cabinet and a device to keep the sections flattened out. Subsequent modifications by Coons *et al.* (1951), Lowry (1953), and Grunbaum *et al.* (1956) have proved valuable. The unit of Richards and Jenkins (1950) is useful for the elimination of static electricity during sectioning. A convenient innovation is the motor-driven microtome devised by Brattgård and Hydén to permit free use of both hands in the cryostat cabinet (Hydén, 1955). Hallén (1956) discussed the sharpening of microtome knives and their qualities and presented some improved procedures which he devised. A general review of microtomy was given by Gettner and Ornstein (1956).

E. Freeze-Drying

For some microchemical work it has been found useful to freeze-dry the samples as an aid in storage and preparation for analysis. Vacuum dehydration of fresh-frozen microtome sections, which have been held at subfreezing temperatures has been used routinely to provide samples for microanalysis ever since this procedure was employed by Anfinsen *et al.* (1942) for histochemical studies on the retina (e.g., Lowry, 1953, Grunbaum *et al.*, 1956). It is also quite often feasible to embed frozen-dried tissues in paraffin and employ the deparaffinized sections for chemical analysis. Doyle (1954, 1955) and Doyle and Liebelt (1955) found that esterase, peptidase, and phosphatases in tissues prepared in this fashion lost no appreciable activity. They found that on storage of the paraffinized tissue at 5° C. for at least two years full enzyme activity was maintained. Frozen-dried tissue that is not embedded can retain practically the full activity of many enzymes for long periods of storage at deep-freeze temperatures (e.g., see Lowry *et al.*, 1954b; Strominger and Lowry, 1955). It has been known for a long time that fresh-frozen undried tissues can be stored at low temperatures for considerable periods without loss of many enzyme activities. Some more labile enzymes and substances might deteriorate on storage in the frozen state, or freeze-drying, or embedding and de-embedding. Therefore, it is wise to check the effect of the treatment employed on the particular substance or activity in the given tissue if there is any doubt. One of the most recent of the general reviews on freeze-drying is the excellent one of Bell (1956). Many more freeze-drying devices have been designed for blocks of tissue than for cut sections, and these are discussed by Gersh in Chapter 3 in this volume.

III. MEASUREMENT OF AMOUNT OF SAMPLE

Since this subject has been discussed earlier (Glick, 1949, p. 423; Holter and Linderstrøm-Lang, 1951), only a summary and more recent

advances will be given. Included in the references just cited are considerations of the techniques for determining cell or granule number, volume, weight, reduced weight, and chemical constituents such as protein nitrogen and deoxyribonucleic acid (DNA).

A. Cell and Particle Counting

Mechanization of cell and particle counting has been given special emphasis recently, and several commercial units for counting by photoelectric scanning are now available. A thorough treatment of the theory, equipment, and application of counting by photoelectric scanning was given by Lagercrantz (1952), and a comprehensive review of the general subject was given by Eränkö (1955, p. 74). Scanning devices which have been designed for absorption of other measurements can also be used for counting (e.g., see Deeley, 1955; Tolles, 1955; Caspersson, 1955; Bourghardt *et al.*, 1955; Hydén and Larsson, 1956; Mellors, 1956; Larsen, 1957).

B. Measurement of Volume

In addition to the older procedures of microscopic projection, measurement of areas and calculation of volumes from known thicknesses, the simple point-sampling methods of Chalkley (1943) for volume percentages of morphologic units and of Chalkley *et al.* (1949) for volume:surface ratios, as well as the line-sampling method of Uotila and Kannas (1952), are noteworthy. These have been discussed by Eränkö (1955, p. 57).

Scholander *et al.* (1952) and Lumsden and Robinson (1953) employed another procedure for measurement of volume. Live protozoa were flattened to a known uniform thickness and then the area was measured. The former authors with simpler equipment obtained a reproducibility of 6% for *Paramecium* and 10% for *Chaos chaos*, while the latter achieved a precision of 1-3% standard deviation, corresponding to 0.5 m μ l., for *Chaos chaos*.

It should also be pointed out that a discussion of caryometric methods was given by Bucher (1954), and a preliminary announcement by Carpenter and Lazarow (1958) of an integrating micrometer stage for the microscopic measurement of the volume of tissue components has just appeared.

Measurement of thickness is required to obtain volume in certain instances, and various procedures employed to determine thickness have been given most recently by Lange and Engström (1954), Brattgård (1954), Galbraith (1955), Hallén (1956), and Merriam (1957). The optical apparatus of Hallén which incorporates a measuring microcator

should be particularly useful, and it could be employed in some of the techniques described by the other authors.

C. Measurement of Weight

Instrumental improvements in the measurement of weight in the microgram and submicrogram range have been forthcoming. The original fish-pole balances of Trevan (see Eränkö, 1955, p. 39) and of Lowry are still used with only minor modifications. The latter quartz fiber instrument commonly employed has a sensitivity of about 0.01 μg . (Lowry, 1953), but with finer fibers sensitivities of about 0.0001 μg . are possible (Lowry *et al.*, 1956a). Loads of only a few hundred times the sensitivity can be accommodated, thus several of the balances with different capacities are required if weighings are to be made over a broader range. Noteworthy among a variety of newer designs are the quartz-fiber torsion balance of Carmichael (1952) and of Rodder (Micro-tech Services Co., Berkeley, California); the latter with a total capacity of 4 million times the sensitivity is commercially available for sensitivities from 0.1 to 0.001 μg . An announcement has just appeared of a quartz beam balance sensitive to 0.05 μg . and accurate to 0.1 μg . which has a capacity in the milligram range (Czanderna and Honig, 1957).

An important new development in microbalance design has come from the College of Ceramics at Alfred University where Dr. T. J. Gray and Dr. T. Jennings produced rugged electrical balances with sensitivities of 0.05 μg . for 0.5-gm. loads, 0.1 μg . for 1-2-gm. loads, 0.5 μg . for 5-gm. loads, and about 1.0 μg . for 10-gm. loads. These capacities are much greater than usually required for histochemical work, but the more sensitive instruments, in particular, could be used to great advantage.* A preliminary description of these instruments was given in a review on microbalance techniques by West *et al.* (1957), but the writer has been informed that this is being revised for future publication.

The writer is unaware of any new material on the use of the Cartesian diver balance and the measurement of reduced weight subsequent to the reviews of the subject by Holter and Linderstrøm-Lang (1951), and Holter *et al.* (1956).

D. Measurement of Protein

After the report of Brüel *et al.* (1946) on the Kjeldahl determination of total nitrogen in samples containing 0.1-1.0 μg . nitrogen, with a precision of 0.005 μg ., a number of modifications of this method have appeared, the most recent of which seems to be that of Boell and Shen

* The balances may be purchased by private arrangement with the individuals mentioned.

(1954). Kjeldahl procedures, as well as colorimetric reactions for amino acids liberated by hydrolysis, are sufficiently involved to render them less suitable for the rapid analysis of many microsamples than the equally precise dye-binding methods. Thus Nayyar and Glick (1953, 1954) employed the quantitative precipitation of protein by bromosulfalein at a pH of about 2, followed by photometric determination of the excess dye in an alkaline medium. These measurements were carried out with a standard error of 0.9 μg . of protein nitrogen, corresponding to 1.4%. The same principle employed with other dyes has also been used by Bohle and Fischer (1953), Plum *et al.* (1955), and Kingsley and Getchell (1957).

E. Measurement of DNA

The increasing use of the DNA content of tissue as an indicator of cell number is evidenced by the work of Misen and Petermann (1952), who measured DNA and counted cells in mouse spleen, Albert *et al.* (1953), who investigated the technique of counting rat liver cells in connection with DNA analyses, and especially Thomson *et al.* (1953), who studied the DNA content of the cell nuclei of different rat tissues, particularly the liver. For a background and general discussion of the constancy of DNA per cell nucleus, see Vendrely and Vendrely (1956), who have given the latest review of the subject at this writing; see also chapter by Mirsky and Osawa (Vol. II, Chapter 9) in this treatise.

IV. TITRIMETRIC TECHNIQUES

A. Apparatus

A great impetus to the development and use of microchemical methods in histo- and cytochemistry was initiated in 1931 by the work of Linderstrøm-Lang and Holter at the Carlsberg Laboratory, Copenhagen, who employed titrimetric methods extensively at first, since these were the most generally applicable at that time. Their burettes were graduated on the glass stem in 0.2- μl . divisions, and over the subsequent years many modifications were described both for this type of burette and for the micrometer screw type of Scholander, in which the readings taken are those of the advance of a piston which pushes out the standard solution from a reservoir or syringe (Click, 1949, p. 255). In addition to the Carlsberg Laboratory equipment, titration apparatus commercially available at present includes the designs of Gilmont (1948, 1953), Grunbaum and Kirk (1953), Lazarow (1950), and a modification of the gasometric apparatus of Natelson (1951).

B. Electrometric Titration

Electrometric microtitration, used for the measurement of chloride in histochemical work, was reviewed earlier (Glick, 1949, p. 281), but recently additional studies have appeared. Shaw (1955) reported a method for the analysis of sodium, potassium, calcium, and magnesium in 1- μg . amounts by precipitation of the ions with specific reagents, conversion to the chlorides, and electrometric titration. Standard errors of 1-2% were obtained, except for sodium which gave 2-4%.

Ramsay *et al.* (1955) described two methods—one for volumes down to 0.2 μl . by which 1 μg . of chloride can be determined with a standard deviation of less than 1%, and the other for volumes down to 0.5 $\text{m}\mu\text{l}$. which is capable of measuring 0.1 $\text{m}\mu\text{g}$. of chloride with the same precision. The former is the usual method in which the titration is carried out by adding silver nitrate from a burette, but the latter involves addition of silver ion by electrolysis of the silver electrode. This electrode is in series with a condenser, and the charge developed on the latter when a current is passed through the system to liberate silver ions is used as a measure of the amount of chloride titrated. The voltage across the condenser is a function of the amount of silver ion liberated, and as the chloride concentration falls the voltage decreases, 240 millivolts being taken as the end point. This particularly ingenious technique should find extensive application.

C. Applications

In spite of the numerous variations in microtitration equipment over the years, the analytical range and precision have changed little, except for the nonburette electrometric technique of Ramsay. With the advent of commercial photoelectric apparatus, a marked increase in the development and use of spectrophotometric and fluorometric methods has occurred, with the result that titrimetric procedures no longer hold their former position of prominence. Greater sensitivity, speed, and technical ease are chiefly responsible for this shift, and the trend can be expected to continue unless a radical new departure such as that of Ramsay is made more generally applicable. The sensitivity of present burette microtitrimetry usually does not permit work on samples smaller than whole microtome sections or biological units of comparable weight or volume, and of course this is a serious limitation for many studies. Nevertheless, within this limitation the technique has been quite useful in certain areas as is evident from such studies as those of Gregg and Ornstein (1953) on explant systems and the reactions of gastrulating amphibians to

metabolic poisons, of Patterson *et al.* (1949) on the increase of peptidase during growth of the larval salivary gland of *Drosophila melanogaster*, of Glick and Stecklein (1956) on cholinesterase in the rat adrenal, of Malmgren *et al.* (1955) on proteolytic enzymes of ascites tumor cells, of Lansing *et al.* (1948) on calcium fractions in mouse epidermis, and of the Carlsberg Laboratory workers on a wide variety of studies, reviewed by Linderstrøm-Lang (1952).

V. SPECTROPHOTOMETRIC TECHNIQUES

A. Apparatus

Even when colorimetry was still dependent on visual comparison, attempts were made to adapt this technique to analysis of the micro-samples presented by nature. Thus, in 1933 Richards and co-workers developed a clever system of capillary-tube colorimetry for studies on 1- μ l. samples of glomerular urine (Glick, 1949, p. 195), and at about the same time microcuvettes were introduced for use with standard macrocolorimeters, e.g., the Zeiss 0.2-ml. cuvette for the Pulfrich step photometer.

A variety of microcells have been designed for use with modern spectrophotometers, but for histochemical work employing a light path of 1 cm. the writer favors the Lowry-Bessey cell with the Beckman instrument for volumes down to 50 μ l. (Glick, 1949, p. 216). For volumes down to about 5 μ l. with a 1-cm. light path, the capillary cell of Holter and co-workers with a microscope photoelectric colorimeter, modified slightly by Malmstrom and Glick (1951), proved particularly serviceable. In order to extend spectrophotometry on volumes of liquid of the order of 5 μ l. to the ultraviolet and to afford greater technical ease, Glick and Grunbaum (1957) adapted the Lowry-Bessey cell by fitting it with a Teflon or Kel-F insert containing a capillary hole for the sample. This required the use of two matching pinhole diaphragms to reduce and collimate the incident light beam in the Beckman instrument, a precision cell-positioner to align the cell to the fine beam, and the photomultiplier attachment to increase the response for an adequate signal.

B. Quantitation of Staining Reactions

As already stated, one of the gravest limitations of these quantitative microchemical techniques is their inability to deal with the finer structures in tissues and cells. One approach to chemical quantitation with finer localization is the use of histochemical staining followed by extraction and measurement of the color. That the amount of color is a quantitative measure of the substance or activity to be studied has been

proved in very few instances to date. Some of the factors which plague this procedure are nonspecific color uptake, incomplete reaction in non-surface regions, and nonstoichiometric staining reactions. However, in special cases such difficulties have been circumvented and serviceable methods have been developed, e.g., Glick and Nayyar (1956) modified the procedure of Defendi and Pearson (1955) for the analysis of succinic dehydrogenase by staining with a tetrazolium dye which is quantitatively reduced by the enzyme system to a colored monoformazan that can be extracted and measured. The validity of such a method depends on the demonstration that equivalent results are obtained for the same kind of sample by an independent method of established reliability. For the case in question, the validity was demonstrated by comparisons to analyses performed by the nonstaining direct microchemical method of Jardetzky and Glick (1956). The study of Glick and Nayyar (1956) was applied to the rat adrenal, whereas Bahn and Longley (1956) used essentially the same method for an investigation of mercurial diuretic effects on the rat kidney.

In a recent study by Hiraoka (1957), it was shown that the fuchsin in the Feulgen stain can be extracted from tissue to give a quantitative measure of DNA. Ohba (1956) reported poor correlations between chemical analyses of cholinesterase in regions of the human liver and deposition of stain by the myristoylcholine method of Gomori.

C. Edström's Technique

A clever technique was elaborated by Edström (1953a) for the spectrophotometric analysis of RNA in single nerve cells. The tissue is fixed in Carnoy's solution, embedded in paraffin, sectioned at $70\text{ }\mu$, de-paraffinized, and microdissected to obtain the cells. These are then individually digested with ribonuclease, extracted, and the extract is placed on a cellophane strip $25\text{ }\mu$ thick and $40\text{ }\mu$ wide which absorbs the liquid and retains the solutes within a $100\text{--}150\text{-}\mu$ length. The strip is photographed with ultraviolet light ($257\text{ m}\mu$) and the photograph is subjected to densitometry for the measurement of the absorption. From the extinction data and the absorbing area, the amount of RNA is obtained. The cell volume is measured so that the average RNA concentration in the cell can be calculated. Large and medium-sized cells from the motor anterior horn of the rabbit contained 10^{-9} to 10^{-10} gm. of RNA, and the measurements were made with an accuracy of about 10^{-11} gm. Further work on this application of the technique was reported by Edström (1956a), and Brattgård *et al.* (1957). A modification with ten-fold greater sensitivity has just been developed, Edström (1958).

Edström (1953b, 1956b) went on to modify his technique for the

analysis of individual mononucleotides by separating them from the digest by electrophoresis on a cellulose thread (15μ diameter) before photographing with the ultraviolet light. This procedure, which has an accuracy of about 7%, was applied by Edström and Hydén (1954) to analysis of the ribonucleotides of single motor anterior horn cells from the rabbit.

D. Applications

The applications of the spectrophotometric techniques are widely diverse, as illustrated by such studies as the architectonic distribution of enzymes in the motor and visual cortices in the monkey (Robins *et al.*, 1956), topical distribution of proteases in mouse tumors (Sylvén and Malmgren, 1955), esterase distributions in epithelial and lymphatic tissues of the rabbit appendix (Doyle and Liebelt, 1955), carbohydrate changes during early stages in the growth of *Vicia faba* root tip cells (Jensen, 1955), analysis of material from individual orifices of human sebaceous glands (Schmidt-Nielsen *et al.*, 1951), and chemical differentiation during amphibian embryogenesis (Løvstrup, 1955), to mention just a few from the recent literature.

VI. FLAME PHOTOMETRIC TECHNIQUES

An excellent review of the principles and applications of flame photometry and spectrometry, given by Margoshes and Vallee (1956), provides an authoritative background and source of much detailed information on the subject. The extension of the more commonly used flame photometric techniques to the analysis of sodium in millimicro-liter volumes of biological fluids was carried out by Ramsay (1950) with an integrating flame photometer and special ancillary apparatus designed by Ramsay *et al.* (1951). A description of equipment that enables the simultaneous determination of sodium and potassium in samples of this size followed shortly (Ramsey *et al.*, 1953), and Ramsay (1952) applied this equipment to a study of the fluid excreted by the Malpighian tubules of *Rhodnius*.

In principle, Ramsay's technique involves measurement of the millimicroliter sample in a silica capillary pipette, transfer of the sample to a platinum wire on which it is dried, mechanical injection of the wire into an air-coal gas flame, passage of the light into a spectrometer from which the doublets of sodium and potassium pass through separate slits in a mask to separate photomultiplier tubes, and recording of the currents generated after appropriate electronic treatment. The method has a standard deviation of $7 \mu\mu\text{g}$. sodium and $17 \mu\mu\text{g}$. potassium with samples near the lower limits of detection, i.e., $40 \mu\mu\text{g}$. sodium and $60 \mu\mu\text{g}$.

potassium. With larger samples the standard deviation is about 3% for both elements. In a test case, interferences from other substances in the sample were reduced to about 6% by the addition of an excess of ammonium phosphate (Ramsay *et al.*, 1953).

Bott (1957) announced that, by means of a modification of the equipment used in Ramsay's laboratory, with samples down to 2–20 $\mu\mu\text{M}$ of sodium and potassium she obtained standard deviations of 0.1–0.2 and 0.2–0.5 $\mu\mu\text{mols}$ for sodium and potassium, respectively. Of course the error was less with larger samples. Volumes of 0.2 $\mu\text{l.}$ of diluted serum, urine, and kidney tubule fluids were used for the simultaneous determination of the elements.

The flame photometer attachment to the Beckman Model DU spectrophotometer was used by Glick *et al.* (1955) to measure potassium in microtome sections of tissue, and this method was applied by them and Swigart *et al.* (1956) to studies on the adrenal. Kingsley and Schaffert (1954) also employed the Beckman apparatus for the determination of sodium, potassium, and calcium in small volumes of serum and urine, but they did not analyze tissue. They used organic solvents to enhance the analytical sensitivity. A modification of the Beckman instrument for sodium and potassium analysis was described by Solomon and Caton (1955), who hope to be able to study microliter volumes of perfusate from single kidney tubules in *Necturus*.

The adaptation of flame photometry to the microsamples provided by nature is still rather new. The usefulness of this approach, impressive for the beginnings already made chiefly in the analysis of sodium and potassium, might be extended to include calcium, magnesium, and even other elements, by the use of a multichannel flame spectrometer such as that developed in the laboratory of Dr. B. L. Vallee (Margoshes and Vallee, 1956, p. 395). The simultaneous analysis of various elements in a single sample by a rapid and automatic process offers advantages that should lead to an increased application in histo- and cytochemistry.

VII. FLUOROMETRIC TECHNIQUES

A. Apparatus and Applications

Fluorometric techniques are being adopted increasingly for microchemical analysis in histo- and cytochemistry, chiefly because of their exquisite sensitivity and relative simplicity. The main impetus to the development of the fluorometric methods arose from the work of Lowry and co-workers (1954a) who designed an adapter for the Farrand fluorometer to hold sample tubes of 2 mm.-bore for volumes of about 10 $\mu\text{l.}$ By the techniques described (Lowry *et al.*, 1954a, 1956a, b, 1957),

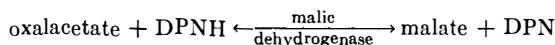
studies which were concerned mainly with enzymes were carried out on single nerve cell bodies, regions of the retina, etc.

Another microfluorometric technique was described by Holter and Marshall (1954) in which a photomultiplier tube was used with a fluorescence microscope to measure the fluorescence of the sample in a capillary tube placed on the stage of the microscope. In their application of this procedure, fluorescein-tagged proteins were supplied to amebas and the ingestion and fate of these proteins were followed in individual organisms.

B. Principles of the DPN and TPN Technique

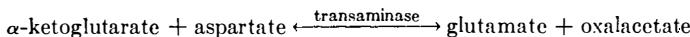
The principles on which Lowry and his group based much of their admirable fluorometric work can be outlined as follows:

Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) do not fluoresce, but they do develop fluorescence with strong alkali, and they are destroyed by weak alkali. DPNH or TPNH do fluoresce but they develop ten times greater fluorescence after oxidation to DPN or TPN and treatment with strong alkali, and they are destroyed by hydrochloric acid. Thus in the reaction:



the enzyme activity can be determined, either by measuring disappearance of DPNH by loss of fluorescence, or by the formation of DPN as indicated by its fluorescence after strong alkali treatment. In the latter case residual DPNH must first be destroyed by acid. Disappearance of DPNH might also be measured by first destroying any DPN present with weak alkali and then oxidizing the DPNH in strong alkali to develop fluorescence. Furthermore, if excess enzyme and appropriate coenzyme is furnished, oxalacetate or malate can be measured by making either of them the limiting factor in the reaction.

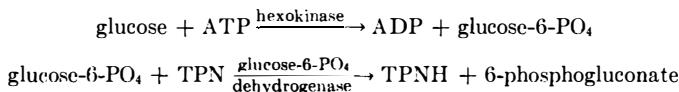
By coupling this reaction to another such as:



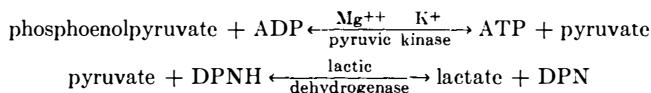
which provides one of the reactants, oxalacetate, for the other reaction, compounds and the enzyme in the latter reaction can be also measured. For instance, to measure the transaminase activity it is only necessary to supply adequate amounts of α -ketoglutarate, aspartate, DPNH, and malic dehydrogenase, so that the oxalacetate produced enzymatically will be consumed quantitatively in the malic dehydrogenase reaction. Then measurement of the DPN formed will provide a measure of the oxalacetate produced by the transaminase. Similarly, α -ketoglutarate can

be determined if sufficient aspartate, transaminase, DPNH, and malic dehydrogenase are provided, etc.

Additional examples of how enzymes, substrates, cofactors, and products of reactions can be determined by measurement of DPN, TPN, DPNH, or TPNH, are given in the coupled reactions:



and



VIII. GASOMETRIC TECHNIQUES

Those gasometric techniques which had been developed for histo- and cytochemical purposes up to 1947 were described in detail by Glick (1949, p. 313). Subsequently, several more reviews have appeared, the latest of which is the excellent one by Holter *et al.* (1956). Therefore, the present discussion can be considerably abbreviated.

A. Apparatus

To recapitulate briefly, the two chief techniques in vogue have been the volumetric, in which changes in gas volume in a small reaction chamber are measured at constant pressure by following the position of an indicating drop in a capillary tube, and the manometric, in which the changes in gas pressure in a small reaction vessel are measured at constant volume in a device such as the Cartesian diver.

Of the numerous designs of gasometric apparatus that have been described, the one that seems to have been adopted most widely for the kind of work under consideration is the Cartesian diver in one form or another. The original apparatus, first described by Linderstrøm-Lang in 1937, has a total volume of about 1–10 μl . and changes in gas volume of 0.1–1.0 $\text{m}\mu\text{l}$. can be detected. Thus, a sensitivity of about 1000 times that of the common Warburg apparatus is achieved, and work can be carried out in which gas changes down to 10 $\text{m}\mu\text{l}$. per hour occur (Holter, 1943).

Zeuthen (1943) designed a capillary diver with a gas volume of 0.04–0.11 μl ., having a sensitivity of 10–20 times that of the original diver, which can follow gas changes of 0.2–2.0 $\text{m}\mu\text{l}$. per hour with an error of about 0.02. For special respiration studies Zeuthen developed additional diver apparatus; i.e., the frog egg diver for relative measure-

ments with a sensitivity of about 0.1 μl . (Zeuthen, 1946); the stoppered diver for both relative and absolute measurements, particularly on dividing marine ova, with a sensitivity of around 0.04 μl . for divers having a gas volume of about 1 μl . (Zeuthen, 1950, 1953a), and a sensitivity of the order of 0.2 $\mu\mu\text{l}$. for the smallest divers having a volume of 0.06 μl . (Zeuthen, 1955); and the ampulla diver with a fine manometer for both relative and absolute continuous measurements over extended periods on protozoa and other cells (Zeuthen, 1953b, 1955), sensitivities down to about 4 $\mu\mu\text{l}$. in relative measurements with a 1- μl . diver being obtained.

Scholander *et al.* (1952) elaborated still another unit in which a minute reference diver is utilized in the same chamber with the cell whose respiration is to be measured. This apparatus, like the smallest stoppered divers, has the remarkable sensitivity of 0.2 $\mu\mu\text{l}$., and it holds considerable promise for further refinement.

Adaptation of the Cartesian diver to measurements of cholinesterase in single megakaryoblasts and megakaryocytes was carried out by Zajicek and Zeuthen (1956), and Giacobini (1957) determined the activity of this enzyme in individual nerve cells by this technique.

Membrane respirometers had been described many years ago by Tobias and by Heatley (Glick, 1949, p. 340 and p. 399), and recently another form of this type of apparatus was devised by Turner (1956). In the latter, the lumen of the concave or recessed end of a capillary tube is sealed by a collodion membrane and a small reaction chamber is formed by fitting on a piece of glass cover slip above the membrane. Measurements are made of the pressure required to hold the membrane in its original null position, a Warburg manometer being employed to adjust the pressure. Any displacement of the membrane is detected by double-beam interferometry with monochromatic light. It is still too early to know how well this instrument functions in biological work. The earlier instruments of this type met with little favor.

Additional designs of microgasometric apparatus reported more recently are the respirometers for tissue culture studies of Prop (1954) and Grunbaum *et al.* (1955), the tissue respirometer of Cruickshank (1954), and the volumeter for photosynthetic measurements of Kok (1955). Increases in sensitivity of 10- to 100-fold in ordinary Warburg manometers can be attained by the use of hydraulic-leverage principles as described by Burk and Hobby (1954), and these principles might also be applied to magnify the sensitivity in other types of manometers such as differential, compensation, and Cartesian diver instruments. Holter *et al.* (1956) pointed out, however, that before such refinements can prove fruitful it will be necessary to increase the stability of the

more sensitive instruments now in use, since stability is their limiting factor.

B. Applications

In addition to those indicated in the preceding section, a few of the interesting newer applications of the gasometric methods are the following: metabolism of embryonic chick heart fibroblasts grown in tissue culture (Danes, 1955a, b); succinoxidase changes in the hearts of developing rat and chick embryos (Sippel, 1954); cytochrome oxidase in mitochondria during amphibian development (Boell and Weber, 1955); and the distribution of cholinesterase in the frog brain (Shen *et al.*, 1955).

IX. DILATOMETRIC TECHNIQUES

The earlier review of dilatometry as applied to histo- and cytochemical studies (Glick, 1949, p. 413), still covers most of the work that has been done since this technique was first adapted to such studies by Linderstrøm-Lang in 1937. The principle of this technique is that the isothermal changes in volume which accompany chemical reactions in liquid systems can be used in certain instances as measures of the extent of the reactions. In the adaptation with which we are concerned, a droplet of aqueous reaction mixture is suspended in a density gradient consisting of a nonaqueous medium. The volume changes are followed through the resulting density changes which cause a vertical displacement of the droplet that can be measured accurately.

The technique is limited to those reactions which cause a great enough volume change to furnish the required accuracy, and to those that neither contain nor produce substances soluble in the nonaqueous medium. Although Linderstrøm-Lang and his co-workers have been employing dilatometric measurements for their studies of protein structure, etc., the only applications to cytochemistry are the early studies of peptidase in the sea urchin egg and the measurement of reduced weight as a means of defining the amount of certain microsamples.

X. MICROBIOLOGICAL TECHNIQUES

Microbiological analysis is the technique of choice for the estimation of certain substances such as particular amino acids, B vitamins, and antibiotics which can be analyzed otherwise only by the much more difficult and less accurate methods of animal growth, etc. In addition, microbiological analysis is remarkably sensitive, although usually less accurate than chemical or physical methods. The only adaptation of the microbiological technique to work in histo- and cytochemistry seems

to be the study by Lowry and Bessey (1944) on riboflavin in the rat cornea. However, a few methods which might be applied to work on the microscale are those of Henderson *et al.* (1948) and Ågren (1949) for amino acids, and that of Løvtrup and Roos (1957) for DNA.

Microbiological analysis is currently being adapted to the histochemical measurement of certain B vitamins, for work on the adrenal, etc., in the laboratory of the writer with the collaboration of Dr. H. C. Lichtenstein, Dr. R. Twedt, and Mr. R. B. Ferguson; *L. arabinosus* 17-5 is being used as the assay organism, and with it methods are first being developed for biotin and pantothenic acid. Analyses can be performed on samples having microgram dry weights which contain micromicrogram quantities of vitamin.

XI. CONCLUSIONS

The ultimate goal of histo- and cytochemistry has been stated to be "the quantitative chemical analysis of the cell, its parts, and extracellular products, *in situ* and in the living state" (Glick, 1953). Microphysical and staining techniques have offered special advantages in dealing with the "*in situ*" aspect of this goal, but they have been rather limited with respect to the "quantitative chemical analysis," while the reverse has been true of the microchemical techniques. To approach the ideal, either the techniques will have to be extended beyond these limitations—the physical and staining yielding more quantitative chemical information, particularly of enzyme activities, and the chemical furnishing more information on the finer biological structures—or else the techniques will have to be brought to bear simultaneously. One can expect that both of these alternatives will be exploited, and that, at least to some degree, the techniques will eventually converge in the attempt to achieve the end desired.

Meanwhile, the future development of the use of quantitative microchemical techniques within this frame of reference will depend largely on two factors: refinements in analytical procedures to achieve reliable quantitation on still smaller morphological units, and improvements in methods which will provide such units for analysis. Progress in the former has, in general, outstripped that in the latter, and consequently it is not uncommon to find that the limiting factor in many investigations is the degree to which the morphologically defined sample can be obtained without influencing the localization or quantity of the substance or activity to be studied. Accordingly, emphasis on the development of sampling methods as one of the more immediate goals should prove particularly valuable.

Compared to the volume of work with the other techniques, much

less use has been made of the microchemical techniques. It would be well for histo- and cytochemists of all persuasions, including, of course, those who use microchemical techniques, to be alerted to the potential aid inherent in the techniques they do *not* employ, for surely the greatest progress will follow from combined efforts from many directions.

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CHAPTER 6

Micrurgical Studies on Living Cells

By M. J. KOPAC

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I. HISTORICAL

The history of micromanipulation represents an important cross section of all the research that has gone into the study of living cells. Almost from the time it was realized that cells are the structural units of plants

and animals, the possibility of microdissecting these cells was discussed. For example, Purkinje not only coined the word protoplasm, but he also stated, in 1844, that his next endeavor would be to construct an apparatus consisting of micrometrically movable forceps and shears and a stage equipped with suitable supports and rotators for the small and, often times, delicate objects encountered in the study of embryos especially during the first stages of development (cited by Florian, 1928).

H. D. Schmidt was the first investigator to design, build, and use a mechanical device for microdissection. Most of Schmidt's interesting observations were published in obscure medical journals. In one of his papers (Schmidt, 1869), he remarked that a manuscript and original drawings showing the microdissection of liver cells were lost in a fire at the Smithsonian Institution of Washington during the Civil War. Schmidt (1859) described his "Microscopic Dissector," an ingenious instrument consisting of three separate units each of which provided a three-way movement for a needle. With this instrument, minute dissections were performed on small fragments of liver tissue. Although his hand-ground, steel needles were large by present standards, Schmidt could tear individual liver cells, expose the cell contents, and even liberate the nuclei. Several of his drawings clearly show the size of the needles used and also the ripping apart of liver cells. Other drawings (Schmidt, 1870) show a partially torn human liver cell with the viscid cell contents drawn out as filaments by the needles. It is impossible to tell, however, whether or not these cells were alive at the time the dissections were performed.

McClendon (1906, 1909) described his "Mechanical Hand" with which he could remove the chromosomes from marine invertebrate eggs at the time of maturation. The Mechanical Hand, actually a mechanical stage with a vertical movement added, provided a three-way control for placing the pipette near the polar body and the underlying nuclear structures of the egg. After placing the pipette properly, the nuclear structures were sucked out with a simple mouth pipette. Despite the relative crudeness of the instruments, the operations were unusually successful since the enucleated starfish eggs, on insemination by starfish sperm, did not show any striking differences in development from the normal eggs.

Schouten (1901, 1905) and Barber (1904) independently developed novel methods for isolating individual bacterial cells and other micro-organisms. Both designed mechanical devices for controlling the micro-needles and micropipettes. Schouten used fine glass needles, often shaped like small loops, while Barber perfected the pipette method. An important innovation introduced by these pioneers was the hanging drop tech-

nique with a moist chamber which prevented the hanging drop from drying. The hanging drop containing the cells was suspended from the bottom surface of a thin cover glass which formed the roof of a box-like chamber lined with moist filter paper. The microneedles or micro-pipettes entered through an open end of the moist chamber and so could be brought to the cells in the hanging drop. The thin cover glass was the only obstacle between the cells and the objective of the microscope, and, thus, microoperations were made possible even under the highest magnifications. Essentially the same procedures are still used unless the inverted microscope is available, which eliminates several undesirable features of the hanging drop. The "lying drop" is used instead.

Barber's mechanical device, known as the Barber Pipette Holder, was one of the earliest types of micromanipulators with which microoperations on living cells could be performed. His complete directions (Barber, 1914) for making microneedles and micropipettes by hand are followed by most contemporary micrurgists. Barber also developed an ingenious method for microinjecting fluids into cells and also for extracting substances from cells. This procedure, however, has been replaced by more effective devices including the volumetric submicromanipulator (Kopac, 1953). Barber (1911) wrote that with a single microneedle, amebas could not only be successfully bisected but that the nucleus could be removed with little loss of protoplasm or apparent immediate injury to the ameba. The clean removal of the nucleus of an ameba is a beautiful example of microdissection and, as a classic experiment, should be mastered by every student of micrurgy.

Kite (1912) began his first microdissection studies on living cells. His investigations launched an important new approach to determine the physical properties of protoplasm (Kite, 1913). He pointed out (Kite, 1914a) that with this technique, the reality of chromosomes, nucleoli, and even mitotic spindles was established beyond doubt. Kite's experiments were made with the Barber Pipette Holder using microneedles drawn from hard glass tubing. Kite (1914b) stated that: "A new microdissection apparatus has been designed embodying the well known ball bearing principle. The three micrometer screws in this apparatus are made with an accuracy common to the best microtomes." No description was published; nor is it certain whether this instrument was ever built.

Kite's work showed unusual promise and his untimely death at the age of 37 was a blow to biological science. In the minutes of the American Society of Zoologists, there appeared the following statement: "During the brief span of his labors, George L. Kite showed special aptitude and an adequate preparation for the investigation of the problems which

lie in the field where zoology, chemistry, and physics meet. His loss is only partially repaired by the inspiration which the methods he developed and the results he attained are affording to the workers who have taken up the problems he relinquished."

One of these workers was Robert Chambers who collaborated with Kite in a study on the function and structure of the nucleus (Kite and Chambers, 1912). Shortly thereafter, Chambers began his own extensive investigations which have contributed, for over four decades, much to our knowledge of living cells and have also established him as one of the leading international authorities on experimental cell research.

Chambers designed a new micromanipulator and an effective method for microinjection (Chambers, 1922). Copies of his equipment are extensively used all over the world. The Chambers micromanipulator, a beautifully designed instrument, may be used, and even misused, for years without becoming utterly unserviceable. Only now is this instrument being extensively superseded by more modern and convenient micromanipulators.

Seifriz (1921) also carried on with Kite's techniques. Much of his work was on plant cell protoplasm with special emphasis on the colloidal properties of protoplasm. One of his earlier contributions (Seifriz, 1924) was a method for evaluating the elastic properties of colloidal systems made possible by inserting small nickel particles with a micropipette either into colloidal masses or into living cells and then measuring subsequently the magnetic forces needed to displace the imprisoned particles.

C. V. Taylor used microdissection methods to investigate the physiological aspects of protozoa. A classical study demonstrated that certain fibrillae in the ciliate, *Euplotes*, were responsible for coordinating movements of the locomotor organelles and thus functioned as a primitive neuromotor system (Taylor, 1920). Taylor also removed the micronucleus from *Euplotes* and showed that this structure was essential for reproduction, although organisms deprived of the micronucleus survived for several days. The micromanipulator and an "accurately controllable micropipette" which Taylor (1925) designed were excellent instruments and capable of great precision of movement and regulation.

The history of micromanipulation would be incomplete without including Tibor Péterfi, who coined the term, "micrurgy" (*mikros*, small; *ergon*, work) and used it to include microdissection, microisolation, microvivisection, and microinjection. In collaboration with Janse, Péterfi developed a micromanipulator and many accessories which, prior to World War II, were commercially available (Péterfi, 1924). Péterfi contributed much to improvements in micrurgical techniques as well as in

the application of these techniques to the study of living cells, especially those in tissue cultures (Péterfi, 1923a,b, 1927).

The era of Barber, Kite, Seifriz, Taylor, Péterfi, and Chambers has greatly enriched our knowledge of protoplasm, chromosomes, cell division, fertilization, extraneous coats, nuclei, mitochondria, and other structures and properties of both plant and animal cells. This illustrious group of investigators introduced the basic concept that living cells could be subjected to direct experimentation despite their small size. Their results leave a rich background for the future students of the cell. Because of their pioneering pathways, the future will be smoother for the younger generations of scientists. The micromanipulator that was designed by Chambers and so remarkably used by him climaxes this great era. Future work will progress because of this stimulating background, and also because better methods and instruments are being developed to delve still deeper into the manifold problems with which living cells challenge the investigator.

II. INSTRUMENTATION AND PROCEDURES

A. *Micromanipulators*

Since 1859 when Schmidt described his "Microscopic Dissector," well over 200 different types of micromanipulators have been described in the literature. Of these designs, relatively few have reached the status of commercial production. Most of them existed only as prototypes. Since 1931, there has been an increasing tendency to design micromanipulators which operate on the principle of the lever or "joy stick." There is no question that these instruments afford better maneuverability than the screw-operated instruments used heretofore. On the other hand, one should not confuse maneuverability with precision.

Several types of lever-controlled micromanipulators are now produced commercially. Emerson's (1931) instrument was the forerunner. de Fonbrune (1932) and Cailloux (1943) developed lever-controlled pneumatic micromanipulators. May (1953) designed a simple lever-controlled hydraulic micromanipulator. The sliding micromanipulators by Reinnert (1938) and more recently by Barer and Saunders-Singer (1948) are sliding-type instruments whose motion depends on direct application of movement by the hands without the participation of screws except for the vertical controls. An electric, lever-controlled micromanipulator for independently moving four needles or pipettes was designed by Bush *et al.* (1953). More recently, Leitz of Wetzlar engineered a lever-controlled micromanipulator which promises to be one of the most useful and versatile instruments of its kind (Seidel, 1957).

The Leitz micromanipulator is the author's choice of all currently available instruments. One adaptation in the author's laboratory (Fig. 1) includes four Leitz micromanipulators which may accommodate as many as eight microneedles or micropipettes, although ordinarily only four are used, one in each micromanipulator.

B. Microscopes and Accessories

Microscope stands have undergone considerable revision during the past decade. There has been an increasing tendency to focus with a

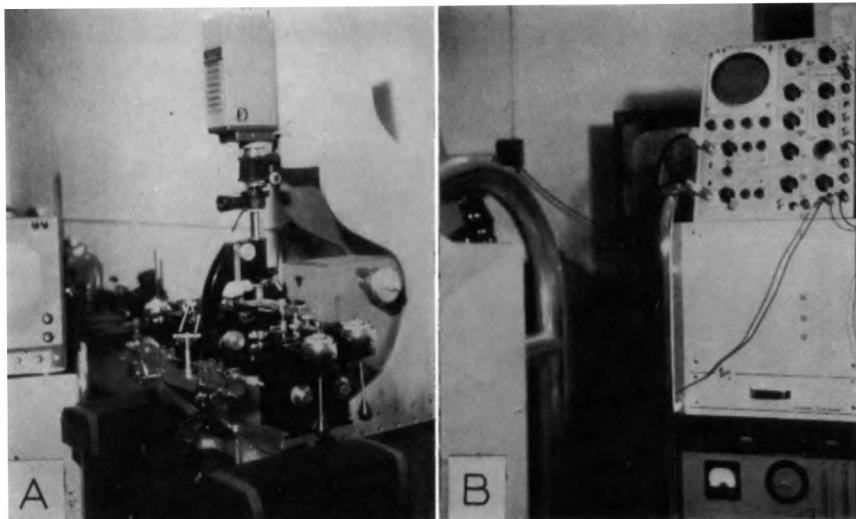


FIG. 1. Television-micromanipulator-oscilloscope unit.

A. Photograph shows the RCA Vidicon camera mounted on a special stand together with microscope, beam splitter and shutter, monochromator, four Leitz lever-control micromanipulators, and two semi-quantitative volumetric microinjectors. The monitor and control unit for the camera are seen in the left side of the picture. This equipment was used for transplanting the nucleoli shown in Figs. 2-5 and for obtaining the oscilloscope traces included with the same figures.

B. Photograph of Tektronix type 545 oscilloscope with 53/54C dual-trace preamplifier mounted on a Scopemobile truck in front of the Conrac 17" monitor. In the left foreground is the Scopemobile truck on which is mounted the monitor-control unit for the Vidicon camera. The photographs of cells shown in Figs. 2-5 were taken from the screen of the Conrac monitor with a standard Polaroid camera (model 95) equipped with a type 540, No. 1 close-up lens. The camera is mounted on a tripod in front of the Conrac monitor.

The photographs of oscilloscope traces are taken with the Aremac automatic recordoscope which is mounted directly (not shown in photograph) onto the oscilloscope. This camera is adjusted to photograph three traces (reduced to 0.7 actual size) on one frame. Type 44 high-speed panchromatic film is used in both cameras. Photographs from the video screen are taken with the exposure index set at 2 ($f/11$ at $\frac{1}{15}$ second) plus a half-stop neutral density filter. Oscilloscope traces are $\frac{1}{10}$ second exposures taken at $f/2.5$.

movable stage rather than with a movable body tube. This type of microscope stand is rather undesirable for micrurgical purposes. The stage should be permanently adjusted for vertical level, and focusing should be accomplished in the conventional way by moving the body tube up or down. The inverted type of stand is excellent, in principle, for micrurgy. Unfortunately, the production models have inverted optics but the mechanics are still more or less conventional. Because of this, the convenience of changing from one objective to another with a revolving nosepiece is completely lost. Perhaps, some day an enterprising and imaginative engineer will design an inverted microscope that is really inverted.

There have been significant improvements in optics in both objectives and oculars. Many objectives can now be obtained with long working distances and, at the same time, still have excellent optical properties. The wide-field oculars are extremely useful in micrurgy. Another innovation is the production of long-working distance phase contrast condensers which permit the use of moist chambers of considerable depth. Especially advantageous are the condensers which can provide almost continuous illumination ranging from bright field to phase contrast to dark field.

Unfortunately there have been no striking changes in the design of mechanical stages. In many instances, it would be desirable to have a finer control over the movement of the object by the mechanical stage so that the cells could be more precisely positioned in the field of the microscope. Mechanical stages have been constructed with controls on both sides—a convenient feature for micrurgy, since it is possible to move the object on the stage with either hand.

C. Microneedles, Micropipettes, and Microinjection

Most of the important information concerning moist chambers, microinjection equipment, the fabrication of microneedles and micropipettes has been fully described (Barber, 1914; Chambers and Kopac, 1950; Kopac, 1955b). It should be emphasized that the serious micrurgist should learn to make microneedles and micropipettes by hand. On the other hand, when the requirements for a given micrurgical problem are established, the use of needle-pulling machines (Livingston and Duggar, 1934; Alexander and Nastuk, 1953) and microforges is clearly indicated. The microforge developed by de Fonbrune (1949) is still one of the best.

D. Micrurgical Techniques

The essential procedures adaptable to micrurgical problems are fully covered in several reviews, of which the following are the most complete: Barber (1914), Chambers and Kopac (1950), Langeron (1949), Seidel (1957), Kopac (1955b), and de Fonbrune (1949). Useful informa-

tion may be found also in the technical bulletins supplied by manufacturers of micrurgical equipment. Information on the handling of cells especially for micrurgy may be found in the reviews by Chambers and Kopac (1950) and by Kopac (1955b).

E. Electronic Adaptations

Some of the more exciting developments, in recent years, are in the adaptation of various electronic devices to the microscope and, accordingly, for the micrurgist. One of these is the application of closed-circuit television to the microscope. The television micromanipulator (Kopac, 1955c, 1956a, b, 1957) is equipped with a monochromator and quartz or reflecting optics so that either visible or ultraviolet light may be used. The RCA Vidicon camera and control monitor have been augmented with a Conrac 17" auxiliary monitor (see Fig. 1). Aside from the possibility of using ultraviolet light for micrurgy, the television principle offers other advantages which are even more important (see also Williams, 1957). By connecting oscilloscopes with flexible delay circuits (Tektronix 545, for example) into the television chain, it is possible to select any one of the 525 horizontal lines comprising the video image and display this line on the oscilloscope screen as a densitometric tracing. At the same time, a pulse from the positive gate of the oscilloscope is fed into the viewer to blank out and thereby indicate the line selected.

The television-oscilloscope unit is valuable in studying the effects on cellular phenomena especially following the transplantation of subcellular particles. One example is the supravital staining of nucleoli with methylene violet (Bernthsen) prior to transplantation. Immediately after the nucleolus is transplanted, the preparation is illuminated with light of contrasting color (approximately 580 m μ). Any one of the scan lines passing through the nucleolus can be selected by the delay circuits and displayed on the oscilloscope screen. The size of the nucleolus can be increased or decreased on the video screen merely by changing the optical magnification. As many as 40 or 50 scan lines may pass through a large nucleolus (Lucké adenocarcinoma cell of frog kidney, in tissue culture) and, by serial or sequential scan-line selection, the profile of the nucleolus can be generated electronically. From the amplitude and width of the portions of the trace passing through the nucleolus, one may obtain information on the size and staining capacity of that structure. Changes in dye retention following micrurgical transfer of a nucleolus to a new environment can be instantly demonstrated by the changes in pulse amplitude (staining capacity) and the width (dye diffusion) of the traces as displayed on the oscilloscope screen. Metachromatic changes in the supravitally stained nucleolus may also be detected and estimated.

Although ultraviolet light may be used in television through the incorporation of the ultraviolet-sensitive Vidicon tube, there is the difficulty of avoiding radiation damage to living cells owing to the relatively high intensities of light needed for proper illumination. Montgomery *et al.* (1956) are adapting the flying-spot principle for ultraviolet illumination of living cells. Since there has now been developed a series of cathode ray tubes that emit ultraviolet light (peak emission at 258 m μ), this adaptation is an accomplished fact. These investigators (1957) have photographed HeLa cells under ultraviolet light (monochromatized at 265 m μ by interference filters). As shown by time-lapse photography, HeLa cells go through mitosis and cytokinesis even though exposed to ultraviolet light. Further modifications and improvements in both tubes and circuitry will permit ultraviolet micrurgy without the danger of inducing radiation damage in cells. Scan-line selection and display of selected lines on the oscilloscope can be as readily accomplished with the flying-spot microscope as is now possible with the present conventional television equipment.

III. APPLICATIONS OF MICRURGY TO THE STUDY OF LIVING CELLS

Over 5000 papers published since 1859 include some application of micrurgy. These may be classified into several categories such as: single-cell isolation, especially of bacteria, spores, and ascites tumor cell; microdissection studies including studies on the neuromotor system in *Euplotes*, virus inclusions, cell adhesiveness, enucleation of cells, stretching of chromosomes; microinjection of pH and redox indicators, salt solutions, enzymes, drugs, and oils; bioelectrical studies for determining action and membrane potentials; electrical resistance of protoplasm; properties of extraneous coats; mechanisms of cell division; cytochemistry with special reference to isolation of cell components; and subcellular transplantation.

With the exception of subcellular transplantation, one of the recent applications, most of the above topics have been adequately reviewed elsewhere and, accordingly, need not be reviewed again. Some of the selected reviews dealing with micrurgical applications are the following: Barber (1914); Chambers (1924, 1938, 1940, 1943, 1949); Chambers and Kopac (1950); Seifriz (1936); D'Angelo (1950); Duryee (1950); Duryee and Doherty 1954); Kopac (1940, 1943, 1950, 1955b); and Tyler *et al.* (1956).

IV. SUBCELLULAR TRANSPLANTATION

The more recent developments in subcellular transplantation will be reviewed extensively. The reasons for emphasizing this topic above all others are manifold: this topic represents a fairly complete cross section

of the many micrurgical studies that had to be made before subcellular transplantation became feasible; it represents the most recent applications to cell biology and potentially the most fruitful; and it requires the most advanced procedures, skills, and instrumentation.

A. Examples of Subcellular Transplantation

1. Removal and Transposition of Micronucleus in *Euplotes*

Taylor and Farber (1924) were the first micrurgists to remove a sub-cellular structure from a cell and replace it successfully. The micronucleus of *Euplotes patella* is essential to the life and proliferation of this ciliate. Organisms deprived of the micronucleus could not live more than a few days nor divide more than twice. In two separate animals, the micronucleus was drawn into a micropipette and returned within a short time to its original position. In each instance, the organism survived and formed vigorous colonies. Furthermore, the stained specimens from these colonies showed the presence of micronuclei.

It is clear that the micronucleus continued to live in the micropipette for a few minutes. It was undoubtedly surrounded by some cytoplasm or cytoplasmic residue and, in view of present experience, it did not come into contact with the external fluid environment. Taylor and Farber (1924) planned to transfer the micronucleus from one *Euplotes* to another whose micronucleus may or may not have been previously removed. It is not known whether these experiments were ever performed.

2. Transplantation of Cytoplasm

Duryee (1949) produced a typical radiation effect in the nuclei of nonirradiated eggs of *Triturus viridescens* by microinjecting cytoplasmic material obtained from X-irradiated eggs into the cytoplasm. The hyaloplasm was readily extracted with a micropipette and, when injected into another egg, the cytoplasmic material could be seen mixing with the host's cytoplasm. The injected mass when placed near the nucleus caused a temporary flattening of the nucleus. The amount of cytoplasm from an irradiated oocyte that would produce radiation damage in a normal nucleus was approximately $0.1 \mu\text{u.l.}$ ($100 \mu^3$). Changes in the nucleus, following transplantation of irradiated cytoplasm, began to develop gradually after 10 to 30 minutes. It is important to point out that injections of nonirradiated cytoplasm produced no visible nuclear damage. Some changes in the cytoplasm, following transplantation of irradiated cytoplasm, were also produced, but these were obviously more difficult to follow. Such changes were usually of the flocculation or coagulation types.

Daniels (1955) injected cytoplasm from normal giant amebas, *Pelomyxa illinoiensis*, into X-irradiated pelomyxas. The donor pelomyxas were supravitally stained with neutral red thus making it easier to follow, as well as certain, that cytoplasm and occasional nuclei were actually injected into the irradiated pelomyxas. As will be discussed later, it is always wise to "tag," wherever possible, the subcellular fractions to be transplanted into other cells.

Immediately after microinjection, in some instances, the stained protoplasmic bodies were clumped together in a coagulum. These granules, however, became free within 5 to 20 minutes and were randomly distributed within the cell. There was no indication that a vacuolar wall was formed around the protoplasm after it was injected into the X-irradiated cell. The mean survival times were significantly prolonged by injections of normal protoplasm. The amount of cytoplasm, ranging from one-fifteenth to one-tenth of the volume of the organism, was enough to allow occasional animals to live following moderate exposures to X-rays. Without such treatment, the irradiated pelomyxas would have died.

3. Transplantation of the Nucleus of the Ameba

Although the removal of the nucleus from an ameba was successfully performed by Barber (1911), many investigators since than have attempted to transplant the nucleus from one ameba to another, but without success. Comandon and de Fonbrune (1939) devised a procedure for accomplishing this operation. Their new procedure involved the pushing of a nucleus from one cell to another with a blunt needle. The donor and host amebas were held against one another with a hook-shaped microneedle, and this opposition prevented the nucleus from coming in contact with the external environment. Even momentary contact with any external aqueous medium instantly kills the nucleus. In these experiments, the donor and host amebas were of the same species, *Amoeba sphaeronucleus*.

Lorch and Danielli (1950; 1953a, b) and Danielli (1952) transplanted the nucleus from *Amoeba proteus* to *Amoeba discooides*, and vice versa. They reported that occasional clones were derived from cells with a grafted nucleus, thereby indicating the successful blending of heterospecific nucleus and cytoplasm. In the resulting clones, the cytoplasm seemed to dominate the cell type, that is, a cell consisting of *discooides* cytoplasm and a *proteus* nucleus resulted in organisms showing a nuclear size and cell forms characteristic of the *discooides* species. A *proteus* cytoplasm with a *discooides* nucleus produced amebas with forms characteristic of that usually associated with the *proteus* species.

Other examples of nuclear transplantation have been reported. Ord

(1956) and Ord and Danielli (1956a, b) used the technique of nuclear transplantation for localizing the action of nitrogen mustards or X-irradiation on *Amoeba proteus*. Their experiments showed that both cytoplasm and nucleus of the ameba were damaged by large doses of X-rays, with the nuclei being about 2.4 times more sensitive than cytoplasm. Furthermore, their results showed that a normal nucleus becomes damaged when transplanted into X-irradiated cytoplasm. Normal nuclei transplanted into amebas exposed to nitrogen mustards were also damaged. Death from cytoplasmic damage generally occurred within a few days, while death from nuclear damage was delayed up to 6 weeks, in some instances. On occasion, the damaged nuclei were even able to divide once or twice before death of the ameba.

At the present time, the most convincing and revealing experiments are those performed by Goldstein and Plaut (1955). In their ingenious experiments, a culture of *Amoeba proteus* was fed on *Tetrahymena pyriformis*, the latter being previously cultured in media containing radiophosphate (P^{32}). Through autoradiography, the accumulation of radiophosphate could be clearly demonstrated in the nuclei of the amebas. The radiolabeled nuclei were then transplanted to normal amebas, using the technique of de Fonbrune.

Autoradiographs of amebas fixed 5 hours after the operation showed that essentially all radioactivity was still localized within the transplanted nucleus. Little or no radioactivity was evident in the cytoplasm. Amebas fixed 12 hours or more after the transplantation, however, showed appreciable radioactivity in the cytoplasm with diminishing radioactivity in the grafted nucleus. These experiments were interpreted to indicate that radiolabeled RNA (ribonucleic acid), or some derivative of RNA, was transmitted from the transplanted nucleus to the cytoplasm. Prior treatment of the amebas with ribonuclease abolished radioactivity in both nucleus and cytoplasm, confirming the interpretation that the RNA fraction was involved in the shift from nucleus to cytoplasm. There was no transfer of radioactivity (presumably radio-RNA) from the cytoplasm to the nucleus.

Chambers (1954) devastatingly criticized the results claimed by Lorch and Danielli following the heterospecific transplantation of nuclei in the ameba. Chambers thought that the reason an ameba underwent division, following transfer into it of a nucleus from another ameba, was that a normal nucleus was already present in the host ameba and that the transplanted nucleus was ejected by the host ameba. The ejection of a damaged nucleus by the ameba is an inevitable and spontaneous event. Nuclei can be damaged by mechanical puncture or by injection

of noxious agents adjacent to the nucleus as well as by exposure to external media.

Furthermore, there is no question that amebas are frequently binucleate, and it might not be too difficult to overlook the presence of a second nucleus after the first one was removed. Such amebas even if deprived of one nucleus would survive since the second nucleus would remain functional and only one nucleus is necessary for the maintenance of the ameba.

Chambers (1954) argued on the basis of his own experiments that every time he got a nucleus transferred from one ameba to another, even of the same species, the sequelae were such that this nucleus was ejected. What Chambers observed is probably correct. If every precaution is not taken to prevent the nucleus from coming in contact with any known external medium, the nucleus will be almost instantly killed and would then be quickly ejected by the host ameba. With these precautions, however, a successful transplantation of the ameba's nucleus, by de Fonbrune's procedure, can be accomplished.

Unquestionably, the experiments by Lorch and Danielli (1953a, b) would be entirely convincing if the nucleus dominated the picture rather than the cytoplasm. Unfortunately, in these instances, the reverse was true. In support of these experiments, however, Danielli (1955) and Danielli *et al.* (1955) stated that a clone derived from a *proteus* nucleus transplanted into *discoides* cytoplasm behaved more like *Amoeba proteus* toward *proteus* and *discoides* specific antisera; that is, the antigens of the clone were similar to those of the nuclear species. Nevertheless, even this claim must be held with some reservation since the specific antigens as derived from cytolytic residue of amebas and used in the production of the antisera are not identified; nor have such responses of amebas to antisera such as toxicity, shape, or division rate been validated as antigen-antibody reactions. There could, therefore, be no certainty that a successful transplantation was accomplished.

On the other hand, the experiments by Goldstein and Plaut (1955) indicate clearly that a transplanted nucleus will survive in the host ameba's cytoplasm for periods up to 90 hours or more. Fortunately, in these experiments, the transplanted nucleus was labeled with radio-RNA and, accordingly, there could be no doubt which nucleus was implanted or which nucleus might have belonged to the host ameba. The mere fact that radiolabeled nuclei would remain in the ameba, following transplantation, was an excellent indication that such nuclei were not seriously damaged as a result of the operation.

It must be re-emphasized that the successful transplantation of a

viable nucleus from one ameba to another is extremely difficult and, unless scrupulous precautions are taken to prevent the nucleus from coming in contact with the external medium, the experiment will fail. Under such conditions, the transferred nucleus is irreversibly damaged and would be quickly expelled by the host ameba.

4. Transplantation of Amphibian Nuclei

Briggs and King (1952, 1953, 1955, 1957), and King and Briggs (1953, 1954, 1955, 1956) have successfully transplanted nuclei from cells of frog embryos into the cytoplasm of enucleated frog eggs, mainly of *Rana pipiens*. This was possible through their development of techniques which prevent the nucleus from coming in direct contact with artificial media. In this procedure, a cell containing the donor nucleus is drawn into a micropipette whose aperture is smaller than the diameter of the cell. Some of the cytoplasm is ripped off during the transfer, but enough cytoplasmic residue remains to protect the nucleus, at least temporarily, against the artificial media. Without such protection, the nucleus would be killed before transplantation could be completed.

It is noteworthy to report that the larger the cell containing the donor nucleus, the better were the chances of achieving successful transplantations. Accordingly, transplants of nuclei from blastula cells were more apt to be successful than were those obtained from the smaller gastrula cells. The results following successful nuclear transplants are discussed by Briggs and King in Chapter 13.

5. Sensitivity of Nuclei to External Media

One lesson that can be learned from nuclear transplantation experiments is the extreme sensitivity of the nucleus to external media. So far, no artificial medium has been devised that will permit nuclei isolated from amebas or amphibian cells to remain alive. In order to keep the nuclei viable they must be protected by an appreciable amount of cytoplasmic residue, and even then, the transplantation must be rapidly performed.

Some relevant information has been derived from microinjection studies. For example, the injection of nonphysiologic media or media that contain certain dyes (Monné, 1935) or noxious agents such as dinitrophenol against the ameba nucleus, *in situ*, damages the nucleus and invariably leads to the spontaneous expulsion of the damaged nucleus.

On the other hand, the injection of reasonably physiologic solutions against the nucleus rarely damages it (Kassel and Kopac, 1953, 1954). Yet the transfer of a nucleus with little, if any, adhering cytoplasm to

such media leads to drastic and irreversible changes even though the media may be completely tolerated on injection. There are striking changes in internal morphology, so that there is no doubt that the nucleus is altered and obviously nonfunctional. Transfer of nuclei from the ameba to less physiologic media produces even greater and more rapid changes. The best means of transferring subcellular particles from cell to cell is to avoid aqueous media. As will be mentioned later, any subcellular fraction can be protected from an injurious environment by surrounding it with essentially inert liquid or solid phases, such as fluorocarbon fluids and siliconized glass surfaces, which despite their non-physiologic composition seem to work unusually well for this purpose.

In all successfully executed nuclear transplants, the nucleus was either transferred by a micropipette or pushed from one cell to another. In many instances, especially in cells of vertebrates grown in tissue culture, the puncture of a nuclear membrane with either microneedle or micropipette leads to destruction of the nucleus. This was beautifully shown by Chambers and Fell (1931) in their studies on chick fibroblasts grown in tissue culture.

It is entirely possible that the survival of the ameba's nucleus following puncture depends on its physiologic or karyokinetic state. Thus, a resting nucleus might be less sensitive to puncture than a karyokinetically active nucleus, or conversely, although there is no published information on this point, as yet. Much research needs to be done in order to determine when a nucleus, in either amebas or other cells, is less sensitive to mechanical injury. Furthermore, the techniques of transplanting nuclei can undoubtedly be improved since most of them are still in the relatively crude stage of perfection.

B. Improvements in Subcellular Transplantation

The various difficulties encountered in subcellular transplantation have been extensively studied and certain improvements in procedure have emerged. One of the most important requirements for increasing the success of subcellular transplants is the elimination of water from the environment of the cells during micrurgy. For this, the fluorocarbon-transfer procedure was developed (Kopac, 1955a). Subcellular structures may be brought in contact with certain fluorocarbon fluids without danger of lytic or other drastic changes such as those that almost invariably occur when protoplasmic structures are brought in contact with water or various aqueous solutions. Certain fluorocarbon fluids such as Kel-F-I oil, a polymer of trifluoromonochloroethane, can be used because of their low surface tensions, reasonably high fluidities, extraordinarily poor solvent action, and other inert characteristics.

Further protection to the subcellular structures during removal from cells and subsequent transfer is provided by sealing the sample within the micropipette, at both ends, with small volumes of Kel-F-I oil. The micropipettes are siliconized to prevent coagulation of the cytoplasm or other cellular fractions and also to prevent the subcellular particles from sticking to the glass. The fluorocarbon seals protect the sample from chance contamination with aqueous media and subsequent injury.

Except for critical experiments, satisfactory transplantation of subcellular particles can be accomplished by maintaining the cells in their normal fluid environment. This is especially fortunate since cells growing in tissue culture are useful for transplantation studies. The important precaution is to prevent the subcellular particles from coming in contact with aqueous media, and this can be accomplished, in the main, by using fluorocarbon seals and siliconized micropipettes.

C. Transplantation of Nucleoli

Chambers and Fell (1931) made some micrurgical studies on nucleoli found in chick fibroblasts grown in tissue culture. They reported that the nucleolus can be pushed about and divided with a needle into smaller fragments. Duryee (1950) also performed micrurgical operations on the nucleoli in amphibian oöcytes. He stated that such nucleoli may be fused to form irregular large masses. Nucleoli could also be stretched and distorted by microneedles. At certain stages, the nucleoli of amphibian oöcytes were found to be attached to the *k*-thread of chromosomes, in that it was impossible to dissect them free without fracturing the chromosomes. Fragmentation of nucleoli was also described which involved the "breakdown of the nucleolar membrane with the escape or release of contained vacuoles or granules." Some excellent photographs of nucleoli being micromanipulated are published by Duryee (1950).

As discussed by Kopac (1956a, b; 1957), nucleoli can be removed with siliconized micropipettes from the nucleus of one cell and placed into either the nucleus or cytoplasm of another cell. Micropipettes with small apertures, 1μ or less, are suitable for removing pieces of the nucleolus. In a similar manner, the inclusion bodies which may develop as a consequence of viral infection in the nucleolus, the nucleus, or cytoplasm can be transferred with a micropipette to any part of the same or different cells.

1. Design of Experiments

A scheme for designing and recording experiments has been devised especially for transplanting nucleoli (Kopac, 1957). The transfer of a

nucleolus from the nucleus to the cytoplasm of the same cell is designated as transposition. The transfer of a nucleolus from one cell to another is termed transplantation. Many variations of transposition and transplantation experiments involving the nucleolus are possible, and each one may be precisely stated, using symbolic notation. The source and position of the nucleolus prior to the operation as well as its position following the operation are recorded.

It should be emphasized that the nucleus is frequently sensitive to puncture with a microneedle or micropipette as described by Chambers and Fell (1931). The nuclei of chick fibroblasts, in tissue culture, are easily damaged by mechanical puncture. In mononucleated fibroblasts, the puncture of the nucleus results in complete disintegration of the cell. This final event is first preceded by a coagulation of the nucleus and a spread of granular precipitation in the cytoplasm which leads to disruption of mitochondria. In binucleate cells, the irreversible coagulation of the damaged nucleus continues, but the disintegrative changes do not progress to destroy the cell. The intact second nucleus permits the cell to recover completely.

Thus, the transplantation of a nucleolus to the nucleus is one of the most delicate operations, and survival of cells following this operation is rare indeed. Extreme precautions must be made to prevent any external aqueous substance from entering the cell and nucleus at the time a micropipette is inserted into it. For these operations, it is necessary to perform the transplantation with cells in completely nonaqueous media. The transposition of nucleoli can be satisfactorily accomplished in either bi- or multinucleated cells, for even if the donor nucleus is destroyed, the cell with its remaining intact nucleus or nuclei will recover.

2. Transplantation and Transposition of Nucleoli

Several examples of transplanted and transposed nucleoli are shown in Figs. 2-5. The living cells for these experiments were those of the Lucké adenocarcinoma of the frog kidney grown in a fluid medium, in tissue culture. The transplantations by micropipette transfer were made with the television-micromanipulator-oscilloscope unit shown in Fig. 1. Approximately 12 hours after the nucleoli were transferred, the cells were fixed in cold methanol. Following the method described by Long and Taylor (1956), the cells were then stained with methyl green and Pyronine Y to bring out differentially both structures containing DNA (stained green) and those containing RNA (stained rose pink). The experimentally transferred nucleoli had colors that were identical to those seen in nucleoli *in situ*.

The photographic records of the nucleoli were taken directly from

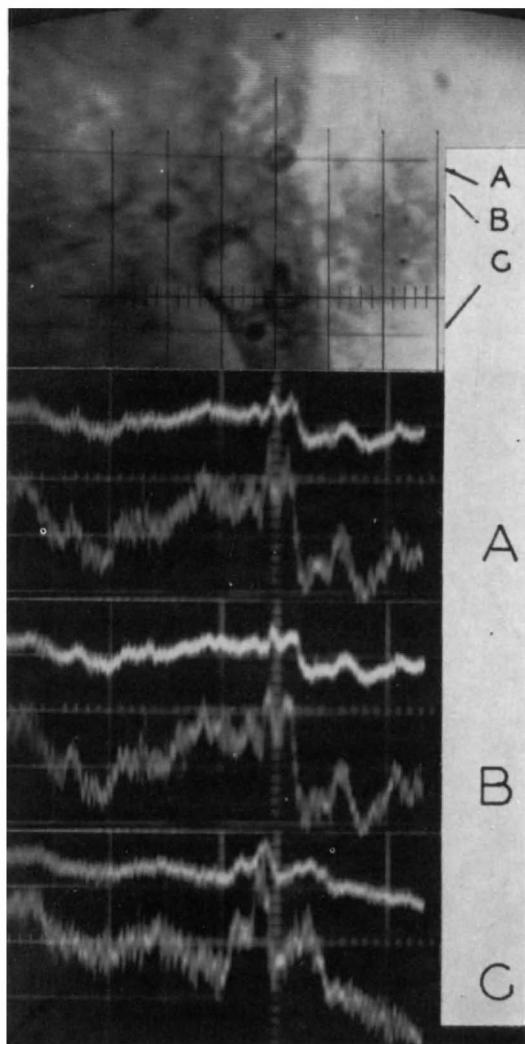


FIG. 2. Video picture of transplanted nucleolus.

Photograph from video screen shows a multinucleate cell (Lucké adenocarcinoma of frog kidney) in tissue culture, stained with methyl green and Pyronine Y. The video lines selected for analysis, which appear as black lines, are indicated by A, B, and C. The transplanted nucleolus is under intersection of vertical line 4 and horizontal lines A and B. Nucleolus *in situ* selected for oscilloscopic line analysis is located between vertical lines 3 and 4 and is transected by horizontal line C. The oscilloscope traces are shown below with the letters corresponding to the selected lines. Traces A and B are duplicates to show reproducibility of the line selection method and signal generated by that line. The upper trace in each pair (obtained with 53/54C dual-trace preamplifier) is set at low amplification while the lower line

the video screen with a Polaroid camera. All pictures of cells were either double or triple exposures so that the positions of all selected scan lines could be indicated. The monitor screen is ruled with seven vertical lines. The video lines selected for oscilloscopic analysis appear as black horizontal lines in the photographs. The oscilloscope traces, shown below the photograph, are positioned and the sweep time adjusted so that the key vertical portions of the traces register with the structures shown in the cell. This method of recording video images and oscilloscope traces is extremely useful and illustrates the large amount of data that can be derived.

The cell shown in Fig. 2 contained three nuclei, each possessing a central, prominent nucleolus. The transplanted nucleolus shows the formation of a small vacuole which is also indicated in the oscilloscope traces. This nucleolus is surrounded by a perinucleolar chromophobic corona which is shown not only in the photograph but also by the oscilloscope traces. Line c was selected to represent the nucleolus *in situ* with the highest density. This nucleolus also shows a perinucleolar chromophobic corona.

In contrast to Fig. 2, the transplanted nucleolus in Fig. 3 does not show a vacuole and its density is approximately equal to that of the nucleolus *in situ*. Both of these nucleoli are surrounded by chromophobic coronas, although the one surrounding the transplanted nucleolus is perhaps more striking. The line passing through the nucleolus *in situ* was selected to show its maximum density.

Another interesting experiment is illustrated in Fig. 4. Here the transplanted nucleolus has developed a large, prominent central vacuolar zone which is clearly indicated by both the video image and the oscilloscope traces. Trace C represents the maximum density of the nucleolus *in situ* while trace B shows the maximum density shown by any part of the

is amplified approximately 2 times higher. Positioning of traces and adjustment of sweep time permits registry of pulses with the structures shown in corresponding vertical segments in the photograph.

Note that the vacuole in the transplanted nucleolus appears as a sharp dip in both traces A and B (near center vertical line). The chromophobic corona surrounding this nucleolus appears as dips on both sides of the pulse generated by the nucleolus. The nucleolus *in situ* as indicated by trace C is smaller and shows a higher density than the transplanted nucleolus. It also shows a well-defined chromophobic corona, especially on the right side.

The cell was photographed (double exposure) to show positions of the two selected lines from the video screen with a Polaroid camera while the oscilloscope traces were recorded with the Aremac recordoscope, using type 44 film in both cameras.

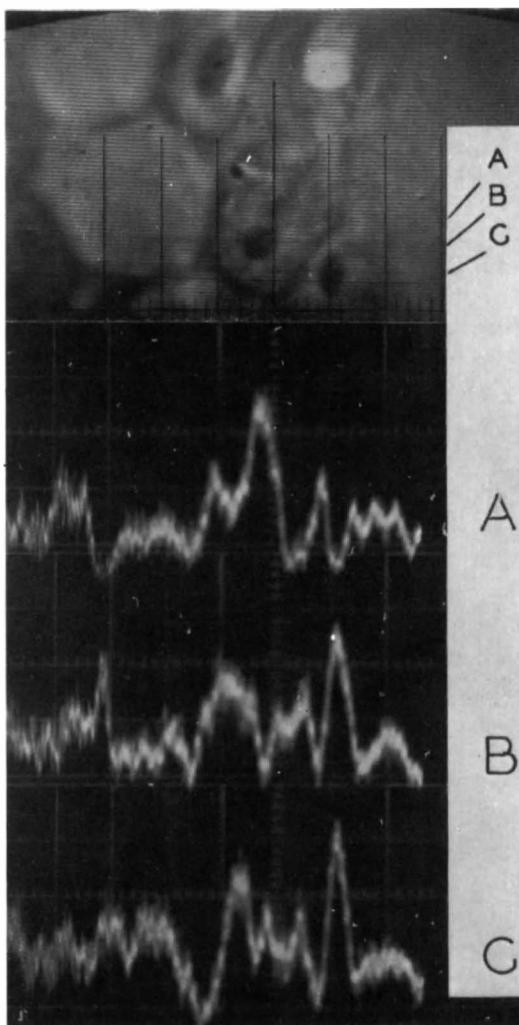


FIG. 3. Video picture of transplanted nucleolus.

Photograph from video screen shows a portion of a multinucleate cell (Lucké adenocarcinoma of frog kidney) in tissue culture, stained with methyl green and Pyronine Y. Transplanted nucleolus is located under vertical line 5 and is transected by two black horizontal lines B and C. The nucleolus *in situ* is located between vertical lines 3 and 4 and is transected by the black horizontal line A. The preamplifier was set at high amplification with sweep time and horizontal position of trace adjusted for registry with vertical portions of the video photograph.

The density of the transplanted nucleolus (traces B and C) is of the same order as the nucleolus *in situ* (trace A). Furthermore, there is no indication of vacuolization in the transplanted nucleolus. Both nucleoli are surrounded by prominent chromophobic coronas, indicated in the video image and by the sharp dips in the traces on either sides of the pulses generated by the nucleolar signal.

transplanted nucleolus. Both nucleoli are surrounded by chromophobic coronas.

Nucleoli, following transplantation and approximately 12 hours' sojourn in a new environment (cytoplasm of a different cell), may or may not develop vacuoles as shown by comparing the transplanted nucleoli photographed in Figs. 3 and 4, for example. It was impossible to tell whether or not the transplanted nucleoli had vacuoles before the operation, since the observations on the unstained living nucleoli were made with ordinary transmitted light. At the present time, there is no explanation as to why some nucleoli are vacuolated, following transplantation, and others are not. At least, the appearance of vacuoles in transplanted nucleoli, or the lack of such structures, cannot be the result of transplantation per se. Furthermore, the relationship between nucleolar changes before and after transplantation must also be studied in reference to the nucleolonema and pars amorpha as postulated by Estable and Sotelo (1951) and Estable (1955).

A transposed nucleolus and also the nucleus from which the nucleolus was obtained is shown in Fig. 5. The nucleus shows obvious signs of deterioration such as the appearance of a coagulated mass which has shrunk away from the original nuclear-cytoplasmic boundary. Also there is almost complete loss of staining of the nuclear residue by methyl green. The nucleolus, on the other hand, maintained its density although an incipient intranucleolar vacuole was developing as seen between horizontal lines b and c. The three lines selected show the regions of highest density possessed by the nucleolus. Trace C also includes a small portion of the intranucleolar vacuole. As indicated in the video image and by the traces, the nucleolus is also surrounded by a chromophobic corona. It is interesting to note that the nucleolus, even though in a new environment—the cytoplasm—has survived while the nucleus has not. Although not shown in the photograph, this cell contained several nuclei, which accounts for the fact that the cell and transposed nucleolus were able to survive the operation.

These are explorations designed primarily to test the practicability of the various transplantation procedures devised for this work. There is no question now that nucleoli can be successfully transplanted and, on basis of their pyroninophilic content, in a reasonably intact state. The main difficulty is to maintain the cells alive for periods ranging from several hours to several days so that enough time may be allowed for all possible changes to occur either in the nucleoli or in the cytoplasm of the transplanted cell.

The cell was photographed from the video screen on Polaroid type 44 film. This picture is triply exposed to show positions of selected lines, a, b, and c.

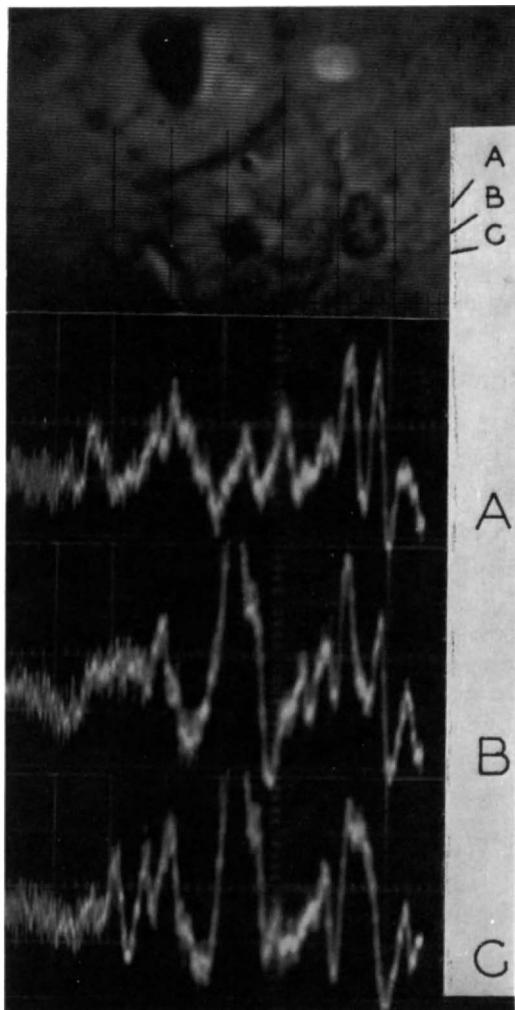


FIG. 4. Video picture of transplanted nucleolus.

Photograph from video screen shows one of several nuclei present in Lucké adenocarcinoma cell in tissue culture, stained with methyl green and Pyronine Y. The three video lines selected for analysis appear as black horizontal lines in the photograph and are labeled A, B, and C. The nucleus has a dense, centrally positioned nucleolus near vertical line 3. The transplanted nucleolus is located between vertical lines 5 and 6. This nucleolus shows a prominent central vacuolar zone and is surrounded by a well-defined chromophobic corona. The oscilloscope traces are shown below with the letters corresponding to the selected lines. The sweep time and horizontal positions were adjusted to register with the structures shown in the photograph. The single-trace preamplifier was set at high amplification.

The high pulse amplitudes in traces B and C represent the optical density of

There is as yet no explanation for the chromophobic corona which is frequently associated with nucleoli. It may be seen around nucleoli, *in situ*. It also develops around transposed or transplanted nucleoli. There must be some subtle and probably most interesting cytochemical changes that render nucleoplasm or cytoplasm unstainable by such dyes as methyl green, pyronine Y, hematoxylin, or eosin. These coronas can be seen in living cells by phase contrast optics and, accordingly, are not necessarily a fixation artifact.

V. FUTURE APPLICATIONS AND EXTENSIONS OF MICRURGY

A. *Tissue Cultures for Micrurgy*

The complete evaluation of biologic effects, following nucleolar or other transplantations, is contingent on the survival and maintenance of cells following the operations. Except for the more instantaneous responses of either nucleoli or the cytoplasm following transplantation, the only means of determining the action of nucleoli on the cytoplasm, or vice versa, is through the subsequent behavior of the modified cells. This is especially important since certain biologic effects may not appear until the next generation of cells. In any event, the possible effects of sub-cellular transplantations on subsequent generations of cells might be extremely interesting and should, therefore, be explored. In most instances, the approach to the maintenance of the cells is through modifications of tissue culture procedures.

Several methods for the production of clones from isolated single cells have been described (Sanford *et al.*, 1948; Likely *et al.* 1952; Kopac, 1955a), but none of these methods seems to be sufficiently practical to use where the cells have been subjected to prior micrurgy and hence are extremely susceptible to handling and other environmental factors.

Micrurgy and tissue culture have not, as yet, been fully blended.

the nucleolus *in situ*. The highest density possessed by the transplanted nucleolus is shown by trace *B*. Traces *A* and *B* show the profiles of the transplanted nucleolus as represented by two peaks which indicate the margin (high absorption) and central vacuole (low absorption). Trace *C* transects the lower margin of the transplanted nucleolus which also indicates considerable absorption. The maximum absorption by any part of the transplanted nucleolus is almost as high as that shown by the nucleolus *in situ*. The chromophobic corona appears on the trace as sharp dips on either side of the two peaks produced by the nucleolar margin (traces *A* and *B*). The nucleolus *in situ* also shows a chromophobic corona, but this is less symmetrical than the one surrounding the transplanted nucleolus.

The cell was photographed (triple exposure to show positions of selected lines) from the video screen on Polaroid 44 film while the oscilloscope traces were recorded on the same type film using the Aremac recordoscope.

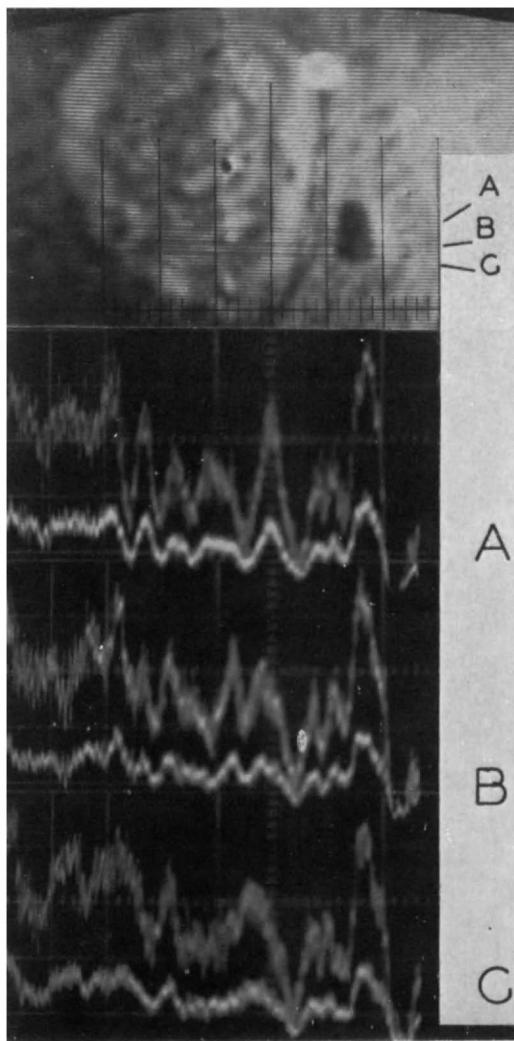


FIG. 5. Video picture of transposed nucleolus.

Photograph from video screen of a portion of a multinucleate cell (Lucké adenocarcinoma of frog kidney) grown in tissue culture and stained with methyl green and Pyronine Y. The transposed nucleolus is located between vertical lines 5 and 6 and is transected by three black horizontal lines, A, B, and C. The nucleus, from which the nucleolus was obtained, is the large, less dense mass positioned to the left of vertical line 5. This nucleus shows a large coagulated mass with loss of most of the methyl green staining material. The nucleolus, on the other hand, has retained its pyroninophilic material and shows a high density as indicated by the peaks of the pulses included in the three traces.

The traces were positioned and sweep time adjusted so that the key vertical

The deterring factor is the present unavailability of a satisfactory chamber that can be used simultaneously for micrurgy and tissue culture. An interesting attempt to solve this problem was mentioned by Crocker *et al.* (1956) using the oil cover slip similar to that devised by de Fonbrune (1949) through which a microneedle or micropipette can be passed. With this method, HeLa Cells could be studied, following enucleation, for considerable periods of time. Time-lapse motion pictures afford a convenient means of recording the observations.

Our approach now includes the development of several culture vessels which seem to satisfy all requirements for tissue culture, including both solid and liquid media, and also all requirements for micrurgy. An important feature is that this chamber permits micrurgy without unnecessary disturbance of the cells growing in the culture. Not only are the host and donor cells growing in a conditioned and hence favorable medium, but also further disturbance of the cells, following transplantation, is eliminated.

Donor cells, used for transplantation studies, must be flattened, since it is much easier to see, and thereby obtain, the desired structure from a flattened cell with a micropipette. The recipient or host cell, however, should be essentially spherical in order to present a better target in depth for implanting the subcellular particles. To satisfy these two requirements, the culture chamber should provide a means of culturing cells in both fluid and semisolid culture media simultaneously. In general, cells show a tendency to round up in fluid media, while solid media promote flattening. Spherical cells can be conveniently and harmlessly held, during the implantation of subcellular particles, with a modification of the elastimeter described by Mitchison and Swann (1954) and adapted for holding starfish eggs by Tyler *et al.* (1956). Another feature of the new chamber is that it provides two parallel glass surfaces and thus presents a reasonably satisfactory optical pathway for both phase contrast and television observations.

Even though single-cell isolation and culture is desirable, it is still

positions of the traces register reasonably well with the structures shown in the cell. The dual-trace preamplifier was used, with one amplifier set at a high level (upper trace) and the other at approximately one-third of the high level (lower trace).

Both Traces *B* and *C* show a suggestion of the vacuolar zone that is seen developing in the transposed nucleolus. Except for this structure, the nucleolus resembles the transplanted nucleolus shown in Fig. 3. This nucleolus is also surrounded by a chromophobic corona as seen in the video image and also by the sharp dips in the traces on either side of the pulse resulting from nucleolar absorption.

The cell was photographed (triple exposure to show positions of selected lines, *A*, *B*, and *C* which appear as black lines) from the video screen on Polaroid 44 film. The oscilloscope traces were recorded on type 44 film with the Aremac recordoscope.

impractical, especially with cells which have undergone extensive micro-surgery. Considerations must be given to the procedures devised by Puck and his associates (Puck *et al.*, 1956; Marcus *et al.*, 1956). With slight modifications of these methods recommended by Puck, small numbers of cells can be plated out in the micrurgical-tissue culture chambers. The recommended medium is fluid, and most of the cells become attached to the glass substrate and soon spread to form flat shapes. Viable cells begin to proliferate and form colonies, so that each colony may be considered as a clone. Since these cells, by proliferation, have demonstrated their capacity to survive and grow in the culture medium, they are in excellent shape for the transplantation procedures. Each cell in a colony will be closely related genetically at its neighbors and hence, *a priori*, one would expect more successful transplantations of subcellular particles than if the cells were not isogenic.

Furthermore, the cell that has been subjected to micrurgy need not necessarily be completely isolated from its neighbors since there are several ways to mark such cells (Kopac, 1955a). A small group of cells frequently forms a recognizable pattern that can be identified and subsequently reidentified. Morphologic changes in such cells, following transplantation of subcellular particles, can best be followed by time-lapse or sequence photography, as successfully utilized by Duryee (1956a, b).

Sterility, especially of the micropipettes, offers no serious problems at present. The micropipettes can be conveniently sterilized by short exposures to high-intensity ultraviolet light. The culture medium usually contains penicillin and streptomycin, in addition to other antibiotics, so that sterilization of micropipettes is not always required.

B. Microinjection Procedures

The removal of nucleoli from their normal sites presents special problems. One of these is a means of introducing suction at variable intensities over limited, although adjustable, volumes. This requirement, therefore, needs something in addition to the present facilities for micro-injection such as the micrometer-controlled syringes (Kopac, 1955b) or the volumetric submicromanipulator (Kopac, 1953).

One promising possibility now being tested is to combine the present methods (micrometer-controlled pistons) with a magnetostriction-driven piston for regulating both the volume range and speed of attaining such volume changes. The magnetostriction principle has been elegantly applied to control linear displacements automatically in the "Inch Worm Motor" by Airborne Instruments Laboratory. The problem involved in the removal of nucleoli is that slow suction must be applied, at first, to

get the nucleolus lined up with, and on its way into, the orifice of the micropipette. Then, at the right instant, suction must be applied vigorously in order to extract the nucleolus from its nuclear position while, at the same time, the total volume of material brought into the micropipette must be carefully controlled. An electrically controlled drive seems to offer the most promise, especially since drives of this type may be readily programmed for semiautomatic and even automatic operations.

C. Automatic Micromanipulator and Other Electronic Adaptations

A new four-piece micropositioner using servomechanism controls is now being constructed (Harris and Kopac, 1957). An automation control unit, which is feasible with servomechanisms, is included in the design, so that a number of micrurgical operations such as positioning of micropipettes with reference to a nucleolus can be programmed by punched tapes and hence automatically performed.

Included in the instrumentation is an entirely new inverted microscope incorporating, in addition to conventional methods of illumination, the flying-spot principle for both visible and ultraviolet light. Both the mechanical stage and fine adjustment are arranged for operation by remote control. The mechanical stage has now been completely redesigned to permit the same degree of fine movement as is built into the micropositioner. The fine adjustment through automatic servomechanisms programming will alternately maintain two levels of focus. One of these levels is on the positions of the micropipettes which are kept above and out of the cellular range until needed for removing or inserting a subcellular structure; the other level is focused on the cells.

Scan-line selection and electronic computing devices will be used with this instrument for analyzing changes in nucleoli and other structures and their physicochemical properties, following transposition or transplantation. Images as seen on the video screen may now be permanently recorded on magnetic tape for subsequent playback.

Mention should also be made of the application of computer and other techniques for the automatic recording of information that can be obtained from scan-line analyses. At the present time, these developments have been incorporated in the Cytoanalyzer by Tolles and Bostrom (1956) for the purpose of screening smears of exfoliated cells obtained from cervical fluids. The automatic handling of data as indicated in Figs. 2-5 is clearly a next and important step.

The micropositioners, mechanical stage, and focusing mechanism of the microscope can also be controlled manually, except that motion will be started, regulated, and stopped by push buttons rather than by levers, control knobs, or other schemes used heretofore. The magnetostrictive

volume controls for the micropipettes can be integrated with the micro-positioners for both manual and automatic operation.

D. Future Role of Subcellular Transplantation in Cell Biology

The various developments in micrurgical techniques which make subcellular transplantations feasible now permit a direct attack on the properties and functions of nucleoli. The nucleolar lesion which occurs frequently in neoplastic cells may be studied experimentally, as well as the inclusion bodies seen in cells of the Lucké adenocarcinoma of the frog kidney and in cells of human ovarian neoplasms. In either transposition or transplantation experiments, the cytoplasm of the same or different cells serves as a physicochemical substrate permitting certain reactions, albeit, of considerable complexity and subtlety. Especially highlighted is the interplay of enzymes of the nucleus and cytoplasm with the constituents and enzymes of the nucleolus.

Along with changes that may be induced in nucleoli following transplantation, other effects can be studied such as the changes the nucleolus may induce in the cell. These effects may be noted in the cytoplasm in terms of changes in nucleic acids, structure of particles, the identification of the substances in the chromophobic corona, etc. Thus, we have the basis for evaluating the "subcellular ecology" of intracellular structures. In addition, means are now available for establishing the probable role of the nucleolar-chromosomal complex as one of the basic regulatory mechanisms in the cell.

Although emphasis has been placed on nucleoli, the same experimental and theoretical procedures are directly applicable to a similar attack on the many problems involving the properties and functions of other subcellular particles. Of these, chromosomal transplantation, transplantation of cytoplasm from melanocytes to amelanocytes, transplantation of mitochondria from malignant to normal cells, and transplantation of nuclear and cytoplasmic inclusion bodies from cells of the Lucké adenocarcinoma to normal frog kidney cells, are a few representative examples.

The whole field of virus-cell interactions may be approached through new experiments especially since virus inclusion bodies or structures that may be associated with viruses can be transplanted to uninfected cells. The interaction of viruses and animal cells, especially in the instances where striking cellular effects may be induced, could very well be one of the most fruitful areas of future work, especially if combined with cytochemical and cytologic analyses.

It is reasonable to expect that any success in transplantation experiments will depend on the compatibility of the donor subcellular fraction with the host cytoplasm. This would imply that the donor-host cells

should be similar, thus recognizing the role of genetic constitution. Accordingly, one should expect more favorable compatibility in melanocytes and amelanocytes from the same tumor, in kidney cells and kidney carcinoma cells from the same frog, in amebas from the same clone, or in ascites tumor cells from single-cell sublines. The development of clonal strains of various cells, in tissue culture, as evidenced by the important work of Puck and his associates should simplify the problem of intercellular compatibilities of subcellular structures.

Finally, the potentials of transplanting subcellular fractions, together with the possibility of inducing cell changes, can add new horizons for the study of somatic genetics. No longer is one dependent on the chance inclusion of, or infection by, some subcellular particle. One may now deliberately take out a subcellular structure and place it precisely into another cell. The products of such research will then depend on the survival of the cell with its new structures. Much of the success in survival and propagation of the cell, following subcellular transplantation, will be enhanced with more precise procedures, as well as with the most modern instrumentation, for performing such operations. With these points in mind, there does not seem to be any limit to the future of experimental cell research.

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The television-micromanipulator assembly was constructed by my associate, Mr. Jack Harris, who has also helped in the design and is constructing the servo-mechanism-controlled micromanipulator and special inverted microscope. His skills as an instrument designer and maker are gratefully acknowledged. It is impossible to estimate how much micrurgy has been advanced through his efforts.

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CHAPTER 7

The Isolation of Subcellular Components

By VINCENT ALLFREY

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I. INTRODUCTION

A. Origins

Experiments on isolated or partially isolated cell components date far back into the previous century: Kölliker's studies of muscle cell granules were published in 1856; Engelmann's experiments on free chloroplasts appeared in 1881; and Miescher's preparations of nucleins from isolated nuclei were first described in 1869.

Yet, despite this earlier history, modern techniques of cell fraction-

ation, and the inspiration to study the biochemistry, function, and morphology of isolated subcellular components find their practical origins in the much more recent past. For the purposes of this review, it all began with the systematic isolation procedures introduced by Martin Behrens in 1932, with the isolation of mitochondria by Bensley and Hoerr in 1934, and the preparation of chloroplasts by Hill (1937) and by Granick (1938). These and subsequent investigations by Claude, Chantrenne, Dounce, Hogeboom, R. Jenner, Mirsky, Palade, W. C. Schneider, and by many others form the modern foundation for what has become one of the most useful and powerful techniques in cell biology.

In discussing the aims and methods of cell fractionation an attempt will be made to illustrate and document the discussion by references to the literature. It should be emphasized that such references were not selected with a view to completeness and that they may sometimes be given without emphasis on priority. A number of review articles have also been included to permit a rapid scanning of diverse fields and individual points of view. For further details on the structure and function of intracellular components the reader is referred to other chapters of this work, notably those by Mirsky and Osawa on the cell nucleus, Novikoff on mitochondria, Porter and Palade on the organization of the cytoplasm, and Granick on the chloroplast.

B. The Aims and Some Applications of Cell Fractionation Procedure

The aims of cell fractionation, although potentially as varied as the interests of the investigator, usually fall into one of three broad categories. The first of these general aims is the study of the activity, composition, and appearance of subcellular components.

The components studied may be the discrete, formed elements of the cell, readily defined in morphological terms and easily identified under the light microscope. Nuclei, chloroplasts, and mitochondria serve as examples of this class. On the other hand, the isolated cell fraction may be defined and characterized by its properties and behavior during the isolation procedure. Microsomes, "postmicrosomes," and "supernatant" fractions, for example, are defined largely in terms of their sedimentation behavior during the differential centrifugation of cell homogenates. Both classes of subcellular fraction have an important role in research on the elements of cell structure.

Despite the comparatively short history of the method, the results of such research already indicate the great power, scope, and utility of cell fractionation procedure. Studies of isolated nuclei include the demonstrations (1) that deoxyribonucleic acid (DNA) is localized in

the nucleus (Feulgen *et al.*, 1937); (2) that different somatic cell nuclei in a given organism have a constant amount of DNA per nucleus, the nuclei of germ cells containing half that amount (Boivin *et al.*, 1948; Mirsky and Ris, 1949); and (3) that DNA is essential to both protein and ribonucleic acid synthesis in the nucleus (Allfrey *et al.*, 1955).

Experiments on isolated mitochondria constitute one of the main applications of cell fractionation technique. The role of the mitochondrion in oxidative phosphorylation was first shown less than ten years ago, and the details of adenosine triphosphate (ATP) synthesis coupled to oxidative reactions are being worked out in large part on isolated fractions (Kennedy and Lehninger, 1949; Kielley and Kielley, 1951; reviews by Chance and Williams, 1956; Lehninger, 1955; Green, 1958; Lindberg and Ernster, 1954, 1957).

Studies of the "microsome" fraction, coupled with suitable tracer techniques, have shown its importance in protein synthesis in the cytoplasm (Borsook *et al.*, 1950; Hultin, 1950) and allowed the demonstration that amino acid incorporation into microsomal protein requires the presence of ribonucleic acid (Allfrey *et al.*, 1953). The use of the microsome fraction together with other components of the cytoplasm has permitted some of the most interesting advances in research on the mechanism of protein synthesis (Hoagland *et al.*, 1957; Keller and Zamecnik, 1956; Hoagland *et al.*, 1958.)

The uses of cell fractionation are not limited to animal cells; the study of the photosynthesis in isolated chloroplasts (Arnon, 1955) and of protein synthesis in broken bacterial cells (Gale and Folkes, 1953) and plant cell microsomes (Webster, 1956) demonstrate the wide applicability and range of the methods.

Isolated cell components are often used for studies of intracellular enzyme distribution. This aspect of cell fractionation includes a zone of controversy and is discussed below in some detail in connection with methods (Section II, C) and standardization procedures (Section V, B).

A second broad application of cell fractionation is its use in the isolation of substances which have a characteristic intracellular localization. For example, the preparation of deoxyribonucleic acid is often best achieved after a preliminary isolation of nuclei or chromosomes (Daly *et al.*, 1950; Emanuel and Chaikoff, 1957b). The same is true of histones and protamines (Daly *et al.*, 1951; Daly and Mirsky, 1955; Crampton *et al.*, 1955).

Similarly, it was shown that the mitochondria are a rich source of oxidative enzymes, that they contain all of the succinic dehydrogenase (Hogeboom *et al.*, 1948) and probably all of the cytochrome oxidase activity of the cell (Schneider and Hogeboom, 1950b). Liver mitochondria

dria have been used as a source of enzymes participating in fatty acid oxidations (Mahler, 1953) and of coenzyme A (Higgins *et al.*, 1950). Indeed the value of the isolated mitochondrion in these and related problems, such as the study of the cytochromes, can hardly be exaggerated.

Glycogen, zymogen, and pigment granules all constitute a natural storage depot for substances of great biochemical interest, and procedures for the isolation of such granules have an obvious role in the study of their contents (see Section IV, F).

A third function of cell fractionation is the use of the isolated cell components as adjuncts or standards in other studies. Mitochondria, for example, have been used as an ATP-generating system in experiments on amino acid incorporation by microsomes (Siekevitz, 1952; Webster, 1956). Isolated nuclei have been used as adsorbing agents for serum factors which appear in the course of the disease, lupus erythematosus (Holman and Kunkel, 1957), and as a source of nucleoprotein in the demonstration that immune reactions to skin transplants may involve a specific deoxyribonucleoprotein (Billingham *et al.*, 1956). Chemical analyses performed on isolated nuclei permit a standardization of the Feulgen staining reaction so that the DNA contents of single nuclei can be measured in the microspectrophotometer (Ris and Mirsky, 1949).

A final goal of cell fractionation is the integration of the separate activities of different isolated components with a view to understanding their interrelationships in the normal function of the cell. Comparative studies of isolated cell components, of mixed fractions, and of whole homogenates can only approximate this goal, but even the approximation carries with it a wealth of interest and future promise. The recent work of Zamecnik and his collaborators on protein synthesis provides a striking illustration of this procedure. In these experiments amino acid activation was obtained with a soluble enzyme fraction; the activated amino acids were then incorporated into the proteins of the microsomes (Keller and Zamecnik, 1956). This type of synthetic approach, in which separate activities become integrated to perform more complex functions, will serve as a model for studies of nuclear-cytoplasmic interaction, of regulatory or feed-back mechanisms in metabolism, and in many similar problems relating to the organization and function of the cell.

II. FACTORS INFLUENCING THE CHOICE OF ISOLATION PROCEDURE

The varied isolation methods to be considered in this review are "bulk" isolation methods; that is, they are capable of yielding sufficient amounts of a subcellular component to permit routine biochemical studies of its composition and function. Though the choice of an isol-

tion procedure is dictated and limited by the purposes of the experiment, some factors influencing the choice form the subject of the following discussion.

The ideal isolation procedure is easy to define; it is the method which yields the desired intracellular components as they exist in the cell, unchanged, uncontaminated, and in quantitative yield. Unfortunately, all cell fractionation techniques known at present fall short of this ideal, and some compromise becomes necessary. Methods which give quantitative yields often involve serious alterations in form, structure, or composition, and procedures which preserve the morphology often destroy activity and function. Purity and homogeneity of the product are rarely, if ever, achieved. Nevertheless, the fact remains that meaningful cell fractionations are possible, and their significance can be heightened considerably by an awareness of methods and their limitations and by selective discrimination on the part of the investigator.

A. The Selection of Tissue

The first problem to arise in any cell fractionation concerns the selection of source material. The choice is often fixed by the nature of the experiment, and existing isolation procedures may need modification to fit the material under investigation. Isolations are often carried out using a single cell type, such as nucleated erythrocytes, protozoa, algae, or bacteria. A number of methods exist for preparing whole cells from solid tissues and for the isolation of certain cell types from heterogeneous suspensions. (These are described below in Section IV, H.) Although the scale of these preparations is usually small, the advantages of free-cell suspensions as a source material and their value as "standards" in indicating the properties and behavior of the intact cell are considerable. However, most of the cell fractionation methods to be described in this review were developed using more complex material, namely the mixed cell populations of animal tissues.

Cells and tissues vary greatly in their contents and composition, in their fragility, density, and in many other properties, depending on the nature of their specialization. This specialization makes certain differentiated tissues ideal for particular isolations. The thymus, for example, is often used for nuclear isolations because the nuclei of thymocytes comprise (on the average) more than 60% of the mass of the cell. Similarly, the large number of mitochondria in liver cells makes this the tissue of choice for most studies of mitochondrial function. A knowledge of diverse biological materials and proper selection of the appropriate tissue often constitute the difference between a successful fractionation and an unsuccessful one, but in many cases the selection can only be

made on the basis of experiment. For example, the morphology of the pancreatic acinar cell and its high ribonucleic acid content immediately suggest its use as a source of "microsome" fractions, and much work on the ribonucleoprotein of the pancreas cell has already appeared (Allfrey *et al.*, 1953; Palade and Siekevitz, 1956b). Yet microsomes prepared from mammalian pancreas, unlike those of the liver, usually fail to incorporate amino acids *in vitro*. (This is probably due to the partial hydrolysis of their RNA by pancreatic ribonuclease.) This constitutes a serious limitation on studies of their activity, and experiments on microsome function *in vitro* have been largely limited to other tissues, despite the disadvantage of lower yields.

It should be pointed out that animal tissues are, in the main, heterogeneous cell populations and that subcellular fractions prepared from whole organs inevitably reflect this heterogeneity. A preparation of isolated liver nuclei, for example, shows all nuclear shapes and sizes observed in liver sections, reflecting variations in cell type, ploidy, and composition. Mitochondrial fractions also show a broad spectrum of size, composition, and activity. Thus one is always dealing with mixed populations, and chemical analyses of isolated fractions, apart from questions of purity, can only yield an average composition. However, the significance of the average as representative of the individual elements can often be demonstrated by auxiliary techniques, such as ultramicro-analysis or radioautography; this is discussed briefly in connection with standardization procedures (Section V, D).

Apart from showing a variety of cell types, tissues vary greatly in other ways which affect cell fractionation techniques. Animal organs differ in their content of connective tissue, blood, lymph, lipid, and fiber. The differences are obvious even when one compares the same organ in different animals; the changes in consistency and appearance of the liver in the mouse, rat, and calf are unmistakable. It seems a fair rule that where the increased size of an organ is reflected in a higher content of connective tissue, this usually leads to increased difficulty in breaking the cells and lower yields of subcellular components. Large amounts of connective tissue and fiber often pose a problem in cell fractionation, and methods of dealing with this problem are discussed below (Section III, C).

The isolation of subcellular components is often complicated by contamination of the desired fraction by red cells. The extent of such contamination can be appreciable. Rat liver, for example, contains 20 ml. of blood per 100 gm. of tissue (Harrison, 1953b), and in mice the blood volume is even greater, about 30% of the liver volume (Storey *et al.*, 1951). In liver and in kidney the problem is usually solved by a prior

perfusion of the organ, using saline, mixed salt, or sucrose solutions (Hogeboom *et al.*, 1952). [It has been observed that 0.15 M NaCl removes blood more completely than sucrose solutions (Philpot and Stanier, 1956).] A technique of rapid perfusion of the rat liver, permitting removal of the blood within 4 minutes, has been described by Anderson (1955a). In general, perfusion is readily carried out in organs whose blood vessels are easy to cannulate, but other organs (e.g., thymus) are more difficult to perfuse and erythrocytes must be removed in other ways. Fortunately, such tissues often contain little blood and contamination problems involving red cells do not arise. Some questions exist concerning the effects of perfusion on cells and intracellular structures, and it has been observed that alterations in mitochondrial form may be produced in the perfusion of rat liver (Anderson, 1956).

One of the most serious problems in cell fractionation is the rapid course of post mortem changes. A sensitive indicator of such change is the adenosine triphosphate level of the tissue. Experiments by Osawa *et al.* (1957) have shown that within 5 minutes after death the ATP level of the rat thymus is only half the original content. Similar observations on deoxynucleotides have recently been reported by LePage (1957). Liver glycogen falls to 60% of its initial value within 15 minutes after death (Torgerson *et al.*, 1952). Such changes in composition and metabolic equilibrium which inevitably occur on the death of the cell are usually compounded by subsequent steps in cell fractionation. Yet the changes are often reversible and do not interfere with studies of function. Mitochondria, thymus nuclei, and chloroplasts, after isolation, still retain their capacity for ATP synthesis. Microsome fractions and thymus nuclei, properly supplemented, still synthesize protein, and chloroplasts fix CO₂. However, there are unquestionably irreversible changes which occur when cells are broken and their internal order is destroyed. Some of these changes are discussed below; many others, still unknown, cannot be evaluated.

B. Problems of Breaking the Cell

There are numerous ways of breaking cells to produce suspensions of subcellular components; most of the methods are mechanical; others involve a chemical treatment or the use of specific enzymes. Since all fractionation procedures depend on the successful completion of this initial step, a brief discussion of aims and principles may be of value before the methods themselves are presented in detail.

Effective cell fractionations usually require that rupture of the cell membrane be achieved with a minimum of damage to the formed

elements of the cell. This aim can be most effectively achieved by working rapidly at low (but not freezing) temperatures to minimize autolytic changes and by using the gentlest of the available methods for breaking the cell. The latter requirement is often in conflict with the desire to obtain high yields of a subcellular fraction and the optimum conditions are then determined empirically, using more drastic techniques of cell fragmentation and selecting the method which leaves the highest proportion of the desired components intact.

When the components to be isolated are visible under the light microscope, the choice of method is best guided by frequent microscopic examination of the brei or broken-cell suspension. Here the use of phase contrast and the application of stains and counterstains can be of great advantage. In the preparation of submicroscopic particulates the electron microscope may be used both as a guide to the choice of method and as a means of identifying and establishing the species purity of the fraction isolated (see e.g., Novikoff, 1957; Kuff *et al.*, 1956; Palade and Siekevitz, 1956a, b). Such fractions are usually constituents of other, more complex structures in the intact cell, such as the endoplasmic reticulum, and their isolation clearly depends on techniques of breaking the cell which also fragment these structures.

All of the isolation procedures discussed in detail in this review make use of mechanical methods for breaking the cell. Where subcellular fractions are isolated in aqueous media, breakage is usually accomplished by the use of shearing forces, obtained in coaxial homogenizers and mixers with high-speed blades. In the nonaqueous isolation procedures cell breakage is the result of milling and grinding and depends on a combination of impact, maceration, and tearing of the cell between two grinding surfaces. These effects are obtained in ball or disk mills containing the tissue and an inert organic suspension medium (Section III, A and B).

Other mechanical methods include freezing and thawing, grinding with and without abrasives, osmotic bursting, and sonic or ultrasonic vibration. In a recent method minces or breis are forced through narrow orifices at high pressure, the dimensions of the orifice being varied to control the size of the particle selected. References to these and other methods are given in Section III, A, 4.

As mentioned above, the choice of method is usually empirical. In aqueous systems one aims for shearing forces which will rupture the cell membranes with a minimum of damage to intracellular particles. The results can often be modified by varying the pH and in other ways described below in connection with isolation media. In bacteria, yeast, and plant tissues more drastic grinding procedures are usually necessary.

On the other hand, suspensions of fragile cells (e.g., thymocytes, embryonic cells) can sometimes be broken simply by stirring at low speed or by passing the suspension through a narrow syringe needle.

C. The Selection of Suspension Media

Breaking the cell immediately introduces another problem in cell fractionation, namely the choice of a suspension medium. Ideally, this medium should so approximate the soluble phase of the cytoplasm that the cell particulates remain morphologically, structurally, and functionally intact. Such a medium has not yet been devised, and in practice all isolation media introduce more or less serious alterations in both structure and function.

The aqueous procedures of cell fractionation, despite their inherent advantages in many other respects (e.g., ease and rapidity of isolations) are subject to the criticism that they frequently alter the composition of the component isolated. Breaking the cell in an aqueous medium introduces two new problems. The first is the extraction and loss of water-soluble components from the cytoplasmic particulates and from the nuclei. The second problem is the transfer of substances between the soluble phase, particulates, and nuclei, and their retention by adsorption. The possibilities of both loss and adsorption of water-soluble substances are legion, depending on the composition of the medium, its pH, and the concentration of the suspension. These difficulties will be discussed below, but it should be emphasized that they need not invalidate aqueous isolation procedures; far from it: much of the most important information on the composition and function of nuclei and nearly all of the chemical knowledge of the mitochondrion were derived using aqueous systems. The aim of the following discussion is to emphasize the need for cautious interpretation of results, especially on intracellular enzyme distributions, and to illustrate techniques of verifying and standardizing the data obtained following fractionations in aqueous systems.

The best examples of loss and adsorption of water-soluble components during the isolation of subcellular structures are supplied by studies of isolated cell nuclei. The demonstration depends on the existence of an analytical standard, i.e., nuclei in which loss or adsorption has not occurred. Such nuclei are supplied by an independent, non-aqueous scheme of isolation (Behrens, 1938; Allfrey *et al.*, 1952; Dounce *et al.*, 1950). The steps in the isolation are as follows: (1) The tissue is rapidly frozen and lyophilized (this effectively minimizes the possibility of transfer of water-soluble substances within the cell). (2) The dry powder so obtained is ground in a ball mill to fragment the cells and liberate the nuclei. (3) The nuclei are then separated from the lighter

and heavier components of the ground tissue suspension by alternate sedimentations and floatations in cyclohexane-carbon tetrachloride mixtures of varying specific gravity (Allfrey *et al.*, 1952). Since these solvents do not extract proteins, peptides or amino acids, nucleic acids or nucleotides, water-soluble vitamins or minerals, nuclei obtained in this way retain their original water-soluble components. They have lost lipides, to be sure, but they may then be compared on a chemical basis with fat-free nuclei obtained by aqueous procedures and then extracted with lipid solvents.

What are the results of such comparisons? One of the most striking and unequivocal results was the finding that nuclei prepared from a variety of tissues in dilute citric acid solutions had lost much (at times, more than half) of their protein content. Similarly, it was found that liver nuclei prepared in sucrose solutions by several methods were devoid of certain water-soluble enzymes (adenosine deaminase and nucleoside phosphorylase) which were concentrated in liver nuclei prepared by non-aqueous methods (Stern and Mirsky, 1953; Stern *et al.*, 1952; Allfrey *et al.*, 1952). The latter result led to the conclusion that sucrose methods of isolation, in the absence of some form of additional standardization, give an equivocal picture of intracellular enzyme distribution. But it was pointed out at the same time that thymus nuclei isolated in sucrose-calcium chloride solutions were the equivalent of the standard non-aqueous nuclei in all respects examined: they had retained their soluble enzymes, proteins, and nucleic acids, and later studies (Osawa *et al.*, 1957; Allfrey and Mirsky, 1957a) showed they had even retained most of their nucleotides. The results of aqueous isolation procedures clearly vary with the nature of the tissue and are reliable only when checked against independent standards.

This conclusion was challenged when it was discovered that a DPN-synthesizing enzyme was localized nearly exclusively in mouse liver "sucrose" nuclei and that this enzyme could be solubilized when the nuclei were disintegrated by sonic vibration (Hogeboom and Schneider, 1952a). This was taken as evidence that the nuclear membrane acts as a barrier to the transfer of large molecules, a conclusion which, if true, would validate most enzyme studies on sucrose nuclei (provided, of course, that they were clean). This conclusion, though very desirable, has unfortunately not been substantiated by later experiments. The nuclear membrane of isolated sucrose nuclei, far from being impermeable to large molecules, is readily penetrated by deoxyribonuclease (molecular weight, 60,000). This was shown for rat liver nuclei by Anderson (1952) and for calf thymus nuclei by Stern and Mirsky (1953). The latter finding is of special interest because it demonstrates

that the retention of protein by thymus nuclei is not a function of the state of the nuclear membrane. Subsequent experiments on thymus nuclei show they are also penetrated by ribonuclease, by deoxyribo- and ribonucleic acids, and by polyadenylic acid (Allfrey *et al.*, 1957a). The particular enzyme discovered by Hogeboom and Schneider to be localized in, and retained by, liver "sucrose" nuclei is one which is probably adsorbed on the nucleic acid. This is indicated by its solubility when nuclei are extracted in strong salt solutions and its precipitability when such solutions are diluted (Hogeboom and Schneider, 1952b). This behavior would be expected for a DNA-protein complex.

There are undoubtedly other soluble proteins and enzymes which are retained by nuclei isolated in sucrose solutions. Some of these proteins may be retained, as are the histones, by combination with nucleic acid; others may be held in different ways. But the present evidence on the properties of the nuclear membrane in *isolated* nuclei does not warrant any optimism about its capacity to act as a barrier to proteins passing in either direction.

A further criticism of nuclei prepared in sucrose concerns the matter of their purity. In most tissues, cell fractionation techniques carried out in 0.25 M sucrose solutions give "nuclear fractions" which are always heavily contaminated by whole cells, connective tissue, and cell fragments. Enzyme measurements and composition studies of such impure "nuclear fractions" are of doubtful significance (except in cases where the data serve to demonstrate that all of a certain enzyme can be recovered in the mitochondrial fraction, and in the construction of activity "balance sheets," as described in connection with standardization procedures, Section V, B). Such "nuclear fractions" can, however, be further purified by layering techniques which take advantage of the differences in density between the nuclei and whole cells (Hogeboom *et al.*, 1952; Wilbur and Anderson, 1951). A more recent "sucrose" method which also selects nuclei on the basis of their density gives very clean nuclear preparations from the liver (Chauveau *et al.*, 1956). However, a comparison of calf liver nuclei prepared in this way with those made in nonaqueous media shows that the sucrose nuclei have lost their acid-soluble nucleotides (Mirsky, 1957). Further comparisons of aqueous and nonaqueous nuclear isolations will be given below in connection with standardization procedures. Save for rare exceptions, the nuclei isolated in aqueous media do not compare favorably with the standards. Yet their utility in many ways is unquestionable; as a source material for the preparation of DNA, histones, or protamines, in the preparation of isolated chromosomes, in measuring the DNA content per nucleus, and in studies of nuclear lipids, their value is considerable.

Problems of contamination and adsorption are not limited to isolated nuclei. Mitochondrial fractions as generally prepared in sucrose are subject to contamination by granules rich in hydrolytic enzymes, the "lysosomes" (de Duve *et al.*, 1955) or by adsorbed particles of the microsome fraction [it will be shown later that the extent of microsomal contamination can be estimated in several ways, including ribonucleic acid analyses and assays for glucose-6-phosphatase activity (see Standardization Procedures, Section V, A)]. Chloroplasts prepared in solutions of carbowax (a polymer of ethylene glycol) (McClendon and Blinks, 1952) also show contamination by soluble proteins, amylophosphorylase in particular (Stocking, 1956). Liver microsomes isolated after adding radioactive cytochrome c to the initial homogenate show appreciable adsorption of the radioactive protein (Beinert, 1951).

The problem of cross contamination is further complicated by the frequent redistribution of insoluble or bound enzymes which become solubilized or released from particulates during the course of isolation, either due to autolysis or to mechanical injury of the particulates. Autolytic changes can be minimized by working rapidly in the cold. The extent of mechanical damage depends on the isolation procedure. This has been carefully investigated by de Duve and co-workers for the "lysosome" fraction; injury of the lysosomes releases an acid phosphatase, cathepsins, nucleases, β -glucuronidase and other enzymes into the suspension medium (see de Duve and Berthet, 1954).

These examples were selected to illustrate the possibility of error in studies of isolated cell components. Many other examples which could be cited will not be included for lack of space. But the need for a critical, cautious interpretation of experiments on isolated cell fractions cannot be overemphasized. In most of the "four-step" cell fractionations known to the author, in which "nuclei," mitochondria, microsomes, and "supernatant" are prepared in sucrose solutions and analyzed at great length for soluble components, there is a discouraging lack of such critical awareness.

Apart from questions of extracting and adsorbing soluble enzymes, the nature of the isolation medium can affect the whole course of a fractionation and modify the function of an isolated component. For example, isolations carried out in salt solutions are usually unsatisfactory because of the clumping and agglutination of cytoplasmic particulates (Dalton *et al.*, 1949; Hogeboom *et al.*, 1948; Hers *et al.*, 1951). The introduction of sucrose solutions for cell fractionations by Hogeboom and associates (1948) was a great advance. Its advantages in mitochondrial isolations have made sucrose the medium of choice for most current work in the field. Recent observations on thymus nuclei show that

sucrose preserves both structure and function, and that saline solutions or phosphate buffers destroy structure, extract proteins, and inactivate synthetic processes. The role of sucrose and of other sugars in nuclear isolations is discussed in more detail in Sections III, D and V, C.

An important variable in isolations employing sucrose is the concentration of the solution. Hypertonic solutions (0.88 M) have been used in isolating mitochondria because high sucrose concentrations preserve their rodlike appearance (Hogeboom *et al.*, 1948). However, despite their improved morphological condition, such mitochondria are of little value in functional studies of oxidative phosphorylation, since they fail to synthesize ATP. For such studies mitochondria are prepared in 0.25 M or 0.44 M sucrose solutions. A similar dependence of functional activity on sucrose concentration has been observed in isolated thymus nuclei, where the optimal range is quite narrow; nuclei isolated in 0.25 M sucrose synthesize protein and RNA; those prepared in 0.4 M sucrose do not (Allfrey *et al.*, 1955, 1957a).

The ionic composition of the medium plays an important part in many fractionation procedures. For the isolation of nuclei in sucrose, a small amount of divalent cation is desirable to prevent swelling and gelation of the nuclei (Schneider and Peterman, 1950; Allfrey, 1954). Calcium is usually used. The presence of calcium is, however, a serious disadvantage if mitochondria are to be isolated from the same homogenate, because even small amounts of calcium inhibit oxidative phosphorylation. Indeed, the use of chelating agents (e.g., ethylenediaminetetraacetic acid) to bind calcium has been recommended (Slater and Cleland, 1952). Clumping and agglutination of cytoplasmic particulates may also result when divalent cations are added to sucrose isolation media (Hogeboom *et al.*, 1948).

The pH of the isolation medium also has an important influence on the nature and course of cell fractionations. Most preparations of cytoplasmic particulates are carried out at pH values near neutrality. Lower pH's may lead to clumping and agglutination of the particles and co-precipitation of components of the soluble fraction. Acidification to achieve these ends is sometimes deliberate; for example, the amino acid-activating enzymes which remain in the supernate after preparation of the microsome fraction have been precipitated by adjusting the pH to 5 (Hoagland *et al.*, 1956).

When cells are subjected to a powerful shearing force, as they are in coaxial homogenizers or high-speed mixers, the way in which they are fragmented is markedly influenced by the pH of the medium. In a neutral medium the cell membrane is broken first, and as the shearing process is continued, more and more nuclei are fragmented. In many

mammalian tissues, when the pH of the medium is lowered to 5, there is still no difficulty in breaking the cell membrane, but the nucleus is fragmented with great difficulty. At pH 3, the usual shearing forces will not break nuclei, though cells are easily broken (Mirsky and Ris, 1947).

Early methods for the preparation of cell nuclei, beginning with the work of Miescher, employed acid media. The use of citric acid by Stoneburg (1939) marked the beginning of modern isolations in acid media. Stoneburg used 4% (0.2 M) citric acid; Mirsky and Pollister (1946) reduced the concentration to 1% and 0.2%, and recent isolations by Dounce and co-workers (see Dounce, 1955) use very low concentrations indeed. Citric acid isolations have many advantages; they are fast, and they supply very clean nuclei in high yield from a variety of tissues. One of the disadvantages, the loss of soluble nuclear proteins, has already been mentioned. Another limitation on their use stems from the fixative effect of the acid. Since nuclei prepared in 1% citric acid are not fragmented, even after prolonged runs in the blender at high speeds, this precludes their use for subsequent isolations of chromosomes (Mirsky and Ris, 1947).

In alkaline solutions, nuclei tend to swell and dissolve. Even in the presence of divalent ions, pH's slightly above neutrality produce clumping and agglutination. Higher pH's induce more drastic changes leading to the dissolution of the nucleus and the extraction of much of its contents (Anderson and Wilbur, 1952).

In addition to its role in modifying the physicochemical properties of subcellular components, the pH of the isolation medium can have a profound effect on the nature and extent of the autolytic changes which accompany an isolation. In some tissues, the pancreas in particular, there are high concentrations of hydrolytic enzymes (including both proteases and nucleases) with pH optima in the neutral or alkaline range; this makes isolations in alkaline media correspondingly more difficult. (It has already been mentioned that pancreas microsomes exposed to ribonuclease usually fail to incorporate amino acids *in vitro*). In other tissues autolysis may be more extreme at acid reactions; most tissues, for example, contain nucleases and cathepsins with pH optima at 5 or below (Maver and Greco, 1949; Allfrey and Mirsky, 1952b). Autolytic changes can always be minimized by working rapidly at low temperatures. The pH of the medium offers another means of controlling and combating such changes. Another possibility which is frequently employed in isolating subcellular components is the use of specific enzyme inhibitors. Citrate, for example, is often used in preparing nuclei or chromosomes because it inhibits the activity of the "neutral" deoxyribonucleases.

The appearance and dimensions of nuclei isolated in sucrose vary in a complex way with changes in the pH and ionic strength of the medium (Anderson and Wilbur, 1952; Philpot and Stanier, 1956). Such nuclei tend to swell up and rupture in solutions which contain no electrolytes, and halos of dissolved nucleohistone surround the swollen nuclei. Small amounts of salt, and especially the addition of divalent cations, reverse these effects. The amount of salt required varies with the pH of the medium. The pH and ionic strength of the medium also influence the internal morphology of the nucleus; a transition from optical homogeneity to intense granularity can be observed when the pH is lowered, or when the ionic strength is raised to 0.2.

On the assumption that nuclear morphology constitutes a satisfactory guide in selecting the isolation medium, Philpot and Stanier (1956) recommended a mixture of sucrose-potassium glycerophosphate-magnesium chloride, and glycerol. This medium provides optically homogeneous nuclei of a far higher standard of purity than is usually attained using 0.25 M sucrose alone. Unfortunately, it has since been found that such nuclei have lost their capacity for protein and ribonucleic acid synthesis (Allfrey *et al.*, 1956). It is a curious fact that when calf thymocyte nuclei are isolated in 0.25 M sucrose-0.003 M CaCl₂, they are granular in appearance, but active in many synthetic systems; if they are isolated in a hypertonic medium which preserves optical homogeneity they lose their synthetic capacity. This is discussed further in Section V, C, Standardization).

The foregoing discussion should make clear the complexity and difficulty which underlie the choice of aqueous isolation media. Problems of adsorption and transfer of soluble components, of autolysis, of osmotic balance, breakage characteristics, pH changes, and many other variables all enter the picture. The solution and the choice can only be made empirically, on the basis of experiments which test the isolated sub-cellular component for retention of function, and by comparing its composition, when possible, with that of standards isolated in other ways.

In many respects the choice of media for nonaqueous isolation procedures seems simple in comparison. Because the tissue is rapidly frozen and lyophilized at the outset, transfer and adsorption of water-soluble components is usually not a problem. Autolysis, in the absence of water, is negligible. Clumping and agglutination are not as common, and pH is no longer a variable.

Against these very considerable advantages there must be weighed several considerable disadvantages. The most serious of these is enzyme inactivation. In the original Behrens technique nuclei were separated in benzene-carbon tetrachloride or ether-chloroform mixtures (Behrens, 1932). Enzyme studies (Allfrey *et al.*, 1952) indicated that benzene

inactivates some enzymes (e.g., adenosine deaminase) to a far greater extent than does cyclohexane, and the latter solvent was substituted for it. An attempt was also made to find a heavy solvent more suitable than carbon tetrachloride. However, the number of pure, commercially available solvents of comparable density is limited. Substitution of the heavy solvent, dibromocyclohexane, for carbon tetrachloride led to complete inactivation of several enzymes considered. The possibility remains, however, that the applicability of the method to enzyme studies of isolated cell components can be broadened by the substitution of other organic solvents, silicones, or fluorocarbons, for the cyclohexane- CCl_4 mixtures used. The nonaqueous preparations described below (Section III, B; III, D, 4; and IV, A, 3) are comparable to the acetone-extracted powders commonly used in the investigation of many enzyme systems. The broad literature on acetone powders can be used as a guide in the selection of enzyme systems which are likely to resist inactivation during the non-aqueous isolation procedures. In careful comparative studies of nuclei isolated in sucrose solutions and in nonaqueous media (Stern and Mirsky, 1953) many enzymes were shown to retain their activities despite lyophilization and prolonged exposure to petroleum ether, cyclohexane, and carbon tetrachloride. Such comparisons are further discussed in connection with standardization procedures (Section V, A). They are of great value in extending the range and significance of both aqueous and nonaqueous isolation procedures.

A second disadvantage of the nonaqueous methods is the damage caused to integrated function and morphology, due, in part, to the extraction and removal of essential lipids. Thus thymus nuclei prepared by the Behrens procedure do not incorporate amino acids; those prepared in 0.25 M sucrose solutions do. Because isolations in nonaqueous media have been largely directed to the study of nuclear composition, similar comparisons of cytoplasmic particulates prepared in the different systems are not available, but the high lipid content of both mitochondrial and microsome fractions (prepared in sucrose) suggests that similar fractions prepared in nonaqueous media would be seriously modified in both form and function. However, the possibility should be mentioned that function may be restored by suitable extracts containing lipid cofactors.

The most important variable in the nonaqueous isolation procedures is the density of the medium. In almost all cases, the nuclei have a characteristic and relatively high density which permits their separation from the blocks of free cytoplasm, the partly ground cells, and assorted granules which form a large part of the ground tissue suspension. If the suspension medium (a blend of light and heavy solvents) is adjusted to a specific gravity slightly lower than that of the nuclei, centrifugation

will sediment the nuclei and float the lighter (cytoplasmic) components. And in organic media of higher specific gravities, the nuclei can be floated away from heavier cellular debris. In practice, the number of successive sedimentations and flotations required to purify the nuclei depends mainly on the difference in density between nucleus and cytoplasm, and, secondly, upon the nuclear fraction of the ground cell suspension.

Both the density and the nuclear fraction vary from tissue to tissue. These variations form the basis for selecting the particular solvent mixtures used in a given isolation procedure. The choice is made empirically, usually by increasing the density of the medium in a step-wise fashion and observing the distribution of the nuclei after centrifugation. For the final steps in more difficult fractionations, centrifugation may be carried out in a density gradient. Further descriptions of these experimental procedures are given below in Sections III, D, 4; III, E, 2, and IV, A, 3.

In some instances in which the nuclear density coincides or nearly coincides with that of a cytoplasmic component, the latter may be removed by techniques which exploit differences in size. Small particles, for example, sediment more slowly than larger particles of the same specific gravity. A nuclear fraction contaminated by small granules can often be further purified by sedimentation in a gravitational or centrifugal field, selecting the time and other conditions so that nuclei sediment but smaller particles remain in suspension. On the other hand, when the cytoplasmic contaminant is relatively large, it can be removed by filtration through fine wire screens or bolting cloth. This device is used in many nuclear preparations and is particularly effective in the removal of contaminating fiber from heart muscle nuclei.

In many tissues the isolation is complicated by the fact that the nuclei form only a small fraction of the ground cell suspension. Under these conditions a straightforward separation of nucleus and cytoplasm cannot be achieved by sedimentation at specific gravities immediately below some fixed value characteristic of the nucleus. Instead the nuclei are trapped and carried along by the other components of the suspension. For example, if a ground erythrocyte suspension is adjusted to a specific gravity of 1.32 and centrifuged, all cellular components are brought to the surface. Yet an appreciable fraction of chicken erythrocyte nuclei, once isolated, will sediment at specific gravities above 1.34. Obviously, the extent to which nuclei are entrapped and carried along by other elements of the ground cell suspension is a function of concentration. The entrapment can be minimized in two ways: (1) by working with very dilute suspensions, or (2) by a stepwise removal of the cytoplasm, beginning with the lighter, fatty fractions and progressing to specific gravities slightly below those of the nuclei. In practice,

the second of these alternatives is the more useful, since it permits one to follow the fractionation, taking note of the nuclear losses, and at the same time, it allows an estimate of nuclear density, needed for further steps in the isolation. Some of the methods employed in this stepwise removal of cytoplasm (the adjustment of specific gravity, construction of density gradients, removal of fractions, useful staining procedures, etc.) are presented in detail in Sections III, D and E and V, A, 1.

In a number of tissues the nuclear densities fall in a range which coincides with that of elements of the cytoplasm, and separations based on specific gravity differences alone will not yield pure nuclei. Behrens (1938) encountered this difficulty in preparing nuclei from the liver of the guinea pig. He observed that a prior fasting (to deplete the liver cells of glycogen) led to a drop in cytoplasmic density which permitted the separation of the nuclei. Similar observations were made on rat liver (Allfrey *et al.*, 1952). In other issues (e.g., fowl reticulocytes; turtle erythrocytes; sheep, pig, and chicken liver; calf bone marrow) nuclear and cytoplasmic densities coincide closely and pure nuclear preparations could not be obtained.

The foregoing discussion illustrates some of the considerations which determine the outcome of separations in nonaqueous media. The nonaqueous methods offer great advantages in permitting the isolation of nuclei in which a loss or adsorption of water-soluble substances has not occurred. The value of such nuclei as "standards of comparison" has already been mentioned. Their use in studying the enzymatic constitution of the nucleus will be referred to subsequently (Section V, A, 4 and V, B).

Other applications of the nonaqueous fractionation procedures include the separation of leucocytes (Behrens and Taubert, 1952a, b), the isolation of hemosiderin granules from the spleen, and the preparation of a colloid from the thyroid gland (Behrens and Ascher, 1933).

D. The Separation of Particles in Suspension

In both the aqueous and the nonaqueous systems, grinding of the tissue produces a mixed suspension of particles which differ in properties such as size, shape, density, surface charge, degree of solvation, etc. These differences in properties (especially in size, shape, and density) form the basis for the physical methods which are employed to fractionate the mixture into its separate components. These methods are discussed in some detail in Sections III, C; III, E; and IV, I.

Centrifugation techniques form an essential core in nearly all isolation procedures. Their success rests in the fact that particles which differ in size and in density will sediment at different rates in a centrifugal field. In differential centrifugation, the field strength and/or the time

of centrifugation are increased in a stepwise fashion, and fractions are collected successively at the bottom of the centrifuge tube. Thus, in the typical "four-step" fractionation of sucrose homogenates, very low fields are employed to sediment the nuclei and whole cells; higher speeds bring down the mitochondria, and prolonged centrifugation at very high speeds yields the microsome fraction. Cell fractions obtained in this way are usually mixtures of particles, and some cross contamination of one fraction by another usually occurs. These problems are considered below in connection with centrifugation (Section III, E) and standardization procedures (Section V).

By modifying the medium through which the particles sediment, e.g., by employing density gradients or "layering" techniques, one can often achieve a more precise fractionation of different subcellular components and effect a considerable purification of fractions which could not be further improved by differential centrifugation alone. The use of density gradients in cell fractionation was first due to Behrens (1938), but the procedure is now a valuable adjunct in both aqueous and non-aqueous isolation methods. Some of the techniques employed in constructing gradients and removing layered fractions are given in Section III, E.

It has already been pointed out that in the nonaqueous methods the density of the medium is deliberately varied to float some particles and sediment others. A major role of centrifugation in such systems is to speed up the redistribution of the particles on this specific gravity basis. The separation is often complicated because small, dense particles may sediment at the same rate as larger, lighter components of the suspension, but by varying the time of centrifugation together with the density of the medium, good fractionations can usually be achieved.

There are a number of other techniques employed in separating cell components in suspension. These include methods such as screening and filtration (which exploit differences in shape or size) and chromatography, adsorption and electrophoresis (which depend on surface charge). Some applications of these methods will be described subsequently (Sections III, C; IV, I).

III. THE TECHNIQUES OF CELL FRACTIONATION

A. *Methods of Breaking Cells*

In cell fractionations few steps are more important than the initial process of breaking the cell. The ideal procedure would break all the cells in a given sample of tissue and release their contents, intact and unaltered, into a suitable medium. Though a wide variety of techniques

for cell breakage exist, all fall short of this ideal, and the method of choice for particular purposes is best determined empirically. Because the way in which cells are broken has such a decisive effect on the course and outcome of cell fractionations, a brief discussion of the techniques will now be given.

Most of the common procedures for the isolation of subcellular components break cells mechanically, usually by the application of shearing forces when isolations are carried out in aqueous media, and by grinding and maceration when cells are fractionated in nonaqueous media. Other mechanical methods for breaking cells include freezing and thawing, osmotic shock, sonic and ultrasonic vibration, and high-speed centrifugation under conditions which tear cells in suspension apart (Section III, A, 4).

1. *Coaxial Homogenizers*

There are many devices which permit shearing in a liquid velocity gradient sufficiently sharp to rupture the cell membrane without excessive damage to nuclei, mitochondria, or other cell particulates. The most commonly used instrument is the coaxial homogenizer, in which a pestle is rotated or moved up and down in a tube of slightly larger diameter. Several of the more familiar tube and pestle (or piston) designs are illustrated in Fig. 1. The rate of shear in such instruments depends on the radii of the pestle and the tube and on the rate of rotation of the pestle (the tube being held fixed). It is obvious that the rate of shear will be high when the speed of rotation is high and the liquid is rapidly forced through the annular space between the pestle and tube. Since the tube is fixed while the pestle rotates, the liquid velocity ranges from a minimum at the wall of the tube to a maximum at the surface of the pestle, and the gradient will be sharper, the smaller the radial distance between these surfaces. Closeness of fit therefore, has much to do with the efficiency of a given type of homogenizer; but other factors are equally important. In practice, the use of coaxial homogenizers almost always entails an up-and-down movement of the pestle, forcing the entire brei volume through the annular space between the pestle and the tube: if this is done rapidly, high shearing forces are obtained, and the efficiency of homogenization is increased correspondingly. Rapid up-and-down homogenization by hand is often more effective than homogenization using a motor-driven pestle with only slow up-and-down movement.

For this reason, several recent designs of homogenizers (e.g., Dounce *et al.*, 1955; Stern and Mirsky, 1953) utilize short pestles of round or half-round design (see Fig. 1, D and G). When these are operated in

an up-and-down manner, the entire brei volume can be forced through the small, circular shearing volume in very short time (without the jamming or binding which might occur using tight-fitting pistons); high and relatively efficient shearing fields can be obtained. Local heating due to friction and the maceration of cells between pestle and wall surfaces (both of which always occur to some extent in coaxial homogenizers unless they are carefully centered) are also minimized in these designs.

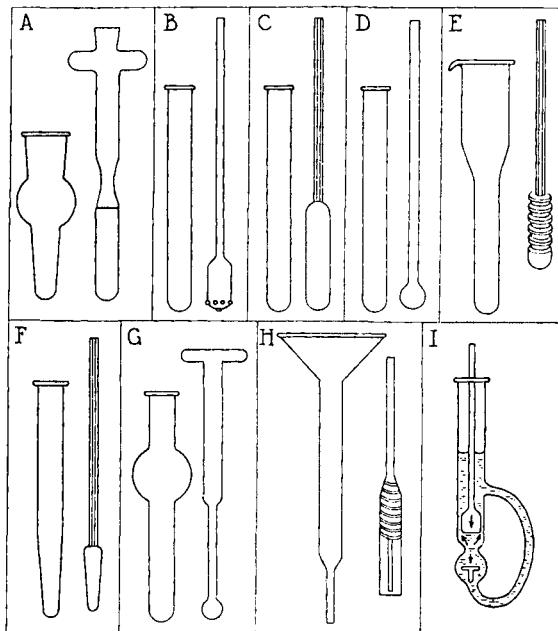


FIG. 1. Nine types of tissue homogenizer: (A) All-glass instrument of Ten-Broeck (1931); (B) glass homogenizer designed by Potter and Elvehjem (1936); (C) Teflon pestle mounted on stainless steel shaft, described by Pierce *et al.* (1953); (D) all-glass homogenizer with small spherical pestle used by Stern *et al.* (1952); (E) Lucite pestle with machined spiral and matching tube designed by Brendler (1951); (F) plastic pestle polymerized in conical centrifuge tube for close fit (Schlegel and Rawlins (1954); (G) glass plunger-type homogenizer of Dounce *et al.* (1955); (H) continuous-feed homogenizer; plastic pestle carries machined spiral; Harris and Mehl (1954); (I) glass plunger-type homogenizer of Lang and Siebert (1952).

The preparation of homogenates or broken cell suspensions with instruments of this sort is a small-scale operation (usually involving about 10-50 ml. of suspension), and the step must be repeated again and again when larger volumes are required. This is time-consuming

and increases the risks of extraction, adsorption, and autolytic changes. A coaxial homogenizer capable of handling larger amounts of suspension has been described by Harris and Mehl (1954) (Fig. 1, H). This instrument is open at the bottom and allows a continuous feed of tissue suspension at the top. (A prior homogenization of the whole tissue in the Waring Blender is required to produce a free-flowing suspension.) The pestle in this homogenizer is made of Lucite, following the lead of Brendler (1951) (Fig. 1, E). The use of Lucite or other plastic pestles has several advantages; they wear well, in contrast to glass pestles, which abrade very rapidly and contaminate the homogenate with glass fragments. Abrasion in the all-glass homogenizers also leads to changes in the dimensions of the shearing field and loss of reproducibility in successive homogenizations. Plastic pestles have less tendency to jam or bind during homogenization: they may be machined with spirals to direct the flow of suspension toward the shearing area. Also, by polymerizing the plastic in the homogenizing tube, pestles can be obtained which closely match the dimensions of the tube (Schlegel and Rawlins, 1954) (Fig. 1, F). Similarly, polyethylene pestles can be closely fitted to matching tubes by warming (Kamphausen and Morton, 1956).

An important feature of coaxial homogenizers is the relative gentleness of their action, which leaves mitochondria essentially undamaged. This accounts for their extensive use in mitochondrial isolations in preference to other instruments such as the Waring Blender, which break cells more efficiently but which may also damage the mitochondria (Berthet and de Duve, 1951). The gentle action of many coaxial homogenizers is sometimes a serious disadvantage. This is especially true in nuclear isolations where such instruments often fail to break a sufficiently high proportion of the cells. These residual whole cells will contaminate the nuclear fraction obtained in the customary fractionation procedures in isotonic sucrose, and further purification of the nuclei is usually required (see Section III, D, 2). Dounce (1955) has reported that the relatively tight fit between the ball and barrel of the plunger-type homogenizer minimizes this problem by making it possible to break a very high percentage of the cells without appreciable damage to nuclei (or mitochondria).

The efficiency of coaxial homogenizers can vary considerably when connective tissue is present. Large amounts of connective tissue fiber introduce mechanical problems such as sticking and binding of the pestle and lower the efficiency of cell breakage. When fractions are to be isolated from a relatively nonfibrous tissue (e.g., rat or mouse liver), the problem is usually solved by a short homogenization, filtration through cloth (see Section III, C) to remove the fiber, and rehomogen-

ization of the filtrate. If the tissue to be fractionated contains a large amount of fiber it is preferable to remove it at the outset. One way in which this can be done is to force the tissue through a perforated stainless steel plate, leaving most of the fiber as a mat on the upper side of the plate. Devices for removing fiber in this way have been described by Hogeboom and Schneider (1952b) and by Dounce (1955) (see Fig. 2). It should be mentioned that Hogeboom and Schneider consider that the use of such presses to remove fiber may lead to some damage to the nuclei.

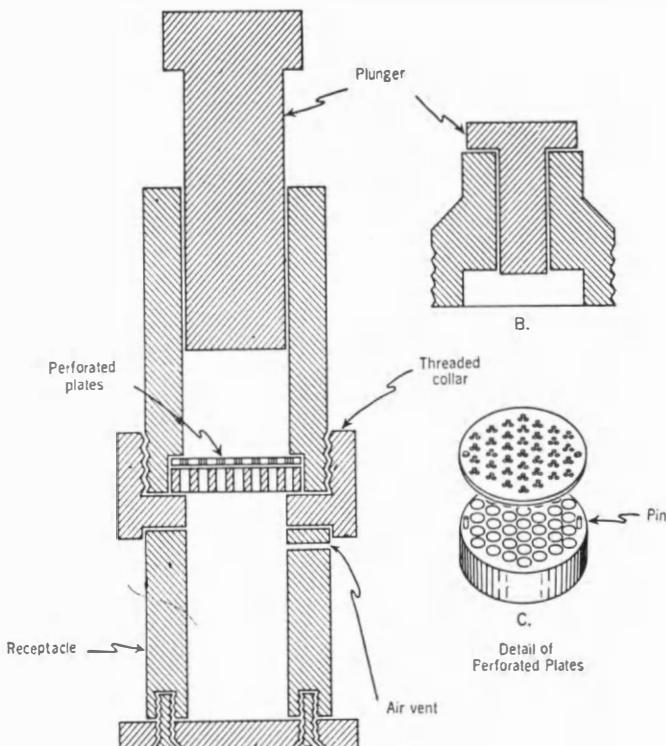


FIG. 2. Tissue press for the removal of connective tissue; from Dounce (1955).

A number of instruments are available which supply high shearing forces and are also capable of dealing with large amounts of cell suspensions; these will now be discussed.

2. Colloid Mills

One of the most adaptable of shearing devices is the colloid mill. This instrument consists of a truncated cone (with its axis vertical and

base down) which can be rotated at very high speed inside an outer stationary cone (see Fig. 3, B). These cones are machined to fit each other very exactly and are usually constructed of stainless steel, or steel covered with an abrasive. The spacing between the cones is adjustable over a wide range, but very small clearances (of the order of 0.03 mm. or less) are generally used. Because of this close spacing and the high velocity of rotation, the shearing forces obtainable in such mills are considerable. The material to be homogenized is fed into the mill through a funnel which leads to the space between the cones. The rotation of the inner cone gives rise to centrifugal force which throws the liquid outward at the base of the cones, where it is passed through a spout and collected.

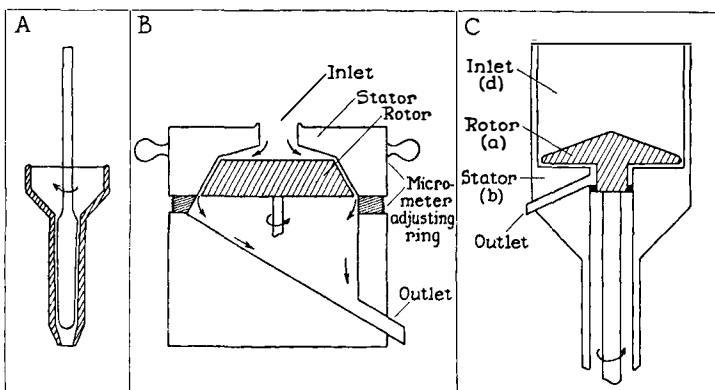


FIG. 3. Mills used in tissue fractionation: (A) stainless steel "nuclear mill" of Lang and Siebert (1952); (B) colloid mill allowing adjustable spacing of grinding surfaces (this type of mill is made by the Premier Mill Corp., Geneva, New York); (C) tissue mill described by Poort (1957). For further details, see text.

A great advantage of the colloid mill is its capacity. Large volumes of homogenate may be processed in a relatively short time (often in a few minutes) and the material may be readily fed back into the mill until cell breakage is complete.

The shearing forces produced in colloid mills are of course, a function of the speed of rotation, and the adaptability and range of the instrument can be increased greatly by operating the motor on the output of a variable transformer. At high speeds the colloid mill will disrupt both nuclei and mitochondria. Its use in cell fractionation is due to Mirsky, who first applied it to the preparation of isolated chromosomes (Mirsky and Ris, 1951). Temperature control during milling (as in all types of homogenization) is essential. The mill design may include

provision for a cooling jacket; failing this, operation in the cold room, or precooling (with crushed ice, etc.) is recommended.

A disadvantage of the colloid mill is the requirement for some form of preliminary homogenization of the tissue. This can be carried out by a short run in the Waring Blender at low speed followed, where necessary, by filtration through cloth to remove fiber.

Lang and Siebert (1952) have described a nuclear mill which has much in common with both colloid mills and coaxial homogenizers. A cross section of this instrument is shown in Fig. 3, A. It consists of a motor-driven stainless steel pestle which is tapered to closely fit a matching outer cylinder. The pestle is precisely centered, leaving a radial distance of about 0.1 mm. between pestle and cylinder. The milling is carried out at comparatively low speed (about 1400 r.p.m.) and the cylinder is surrounded by a cooling mantle so that local heating effects are avoided. Operation of this mill also requires a prior homogenization of the tissue (using the piston-type homogenizer shown in Fig. 1, I), followed by filtration through cloth to remove fiber. The filtrate is then fed into the funnel above the milling cylinder and is drawn by gravity into the narrow annular slit between the two conical surfaces. The broken-cell suspension flows downward and is collected as it leaves the aperture at the base of the mill. Rates of flow vary with the setting of the pestle and the type of tissue homogenized.

A more recent design embodying similar principles of continuous feed and permitting a close, but adjustable, spacing of milling surfaces has been described by Poort (1957). In this case (Fig. 3, C) the tissue suspension is poured into the upper cylindrical chamber (*d*) and is drawn by suction between the rotating disk (*a*) and the plane upper surface of (*b*). This high-capacity mill has been used to prepare nuclei in 70% glycerol from large quantities of beef pancreas tissue.

3. Waring Blendors and Similar High-Speed Mixers

One of the most versatile of the instruments used for fragmenting tissues and breaking cells is the Waring Blender. This device consists essentially of a high-speed stirrer mounted in a cylindrical vessel, the walls of which are indented from top to bottom to form a clover-leaf cross section. The number and design of the stirring blades varies in different instruments; they are usually sharpened, at right, or near right, angles to each other, and inclined at different angles to permit efficient mixing of vessel contents. The combination of complex blade design and the irregular outline of the vessel gives rise to very complex shearing fields, characterized at high speeds by considerable turbulence and cavitation.

In most of the commercially available blenders the blades are mounted on a central bearing in the base of the cylinder. The vessel is

placed over the motor and the bearing is driven from below. This design, although usually satisfactory, introduces problems of leakage through and around the bearing, especially when working with organic solvents. More recent designs make use of overhead drives, in which the blades are mounted on a vertical shaft, and the vessel is placed beneath the motor (see Fig. 4). This eliminates any problem of leakage around bear-

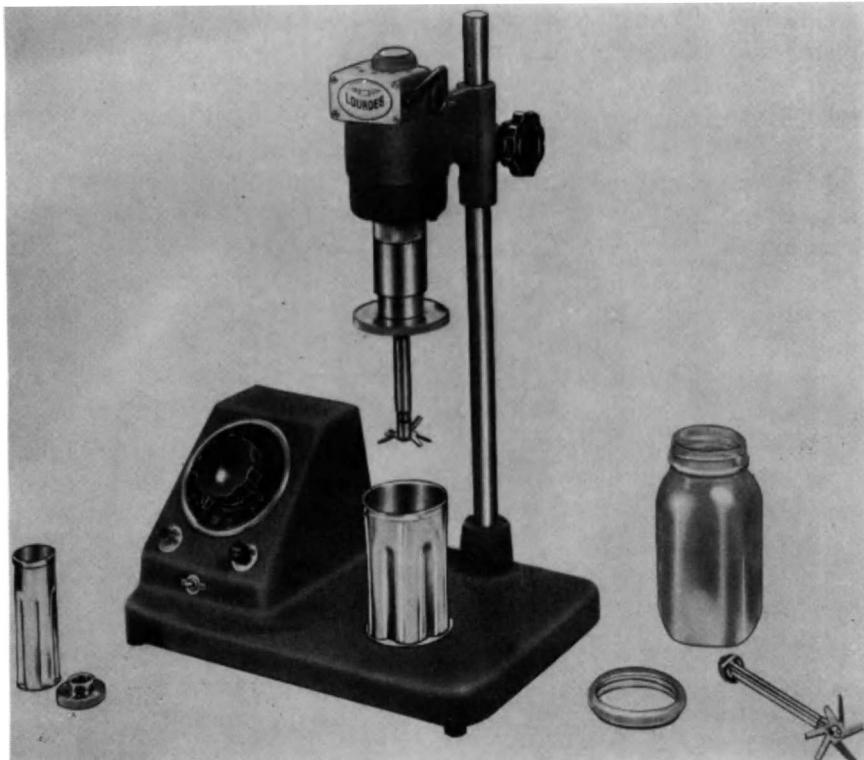


FIG. 4. A variable-speed, overhead-drive mixer. This design permits easy cooling of the blending vessel and also allows a great deal of flexibility in interchanging various-sized vessels without the need for many blade assemblies. (Photo: courtesy of the Lourdes Instrument Co., Brooklyn 32, New York.)

ings and also permits a great deal of flexibility in interchanging various-sized vessels without the requirement of additional stirrers.*

Blenders offer many advantages in cell fractionations. The combina-

* There are a number of commercially available mixers which feature overhead drives, variable speed control, and interchangeability of mixing vessels. Among these are the "Multi-Mixer" manufactured by the Lourdes Instrument Co., Brooklyn 32, New York (shown in Fig. 4); the "Omni-Mixer" of Ivan Sorvall, Inc., Norwalk, Connecticut; the "Homogeniser" of the Measuring and Scientific Equipment, Limited, Spenser Street, London.

tion of cutting action and shearing force makes possible a direct homogenization of many tissues which could not be handled as simply in the other devices mentioned above. A wide choice of vessel sizes makes it possible to vary volumes and proportions in many ways. Additional flexibility results when the blender speed is controlled by a variable transformer. For certain isolations, e.g., the preparation of thymocyte nuclei (Allfrey *et al.*, 1957a), such control is essential.

Although blenders have several disadvantages, most of the criticisms directed against their use are based on their performance at very high speeds. At such speeds the blender will disrupt mitochondria (Still and Kaplan, 1950; Harman, 1950; Berthet and de Duve, 1951), inactivate enzymes (Stern and Bird, 1949), denature proteins (Naimark and Mosher, 1951), and introduce problems of foaming and overheating. The latter problem can be solved in overhead-drive blenders simply by immersing the stainless steel cylinder in an appropriate cooling bath; for the other models, special cooling mantles (Mirsky and Ris, 1947), the addition of crushed ice to the homogenate, or intermittent operation in the cold have been used to avoid damage due to overheating.

The shearing forces obtained by high-speed blending are sufficient to break nuclei (although this becomes progressively more difficult as the pH of the medium is lowered), and this technique has been used in preparing isolated chromosomes (Mirsky and Ris, 1947, 1951). Low-speed blending, on the other hand, has made it possible to fragment thymocyte and lymphoma cells without excessive damage to their nuclei (Allfrey, 1954). This versatility of the blender accounts for its frequent utilization in cell fractionation techniques. Some of these applications are described in detail below (Section IV, A).

4. Other Mechanical Methods of Breaking Cells

Emanuel and Chaikoff (1957a) have described the design and operation of a homogenizer which utilizes the shearing forces obtained when a tissue suspension is forced through a narrow orifice at high velocity (see Milner *et al.*, 1950). This device consists of a cylinder with a tapered outlet tube centered in its base; rods of different diameters can be inserted through this tube to form a circular orifice of varying size at its base (Fig. 5). The homogenization procedure consists of adding the minced tissue and its suspension medium to the cylinder; a piston is inserted into the top of the cylinder and the entire assembly placed in a Carver laboratory press. The application of pressure forces the tissue through the orifice at high speed, thus breaking the cells. (To remove heat formed in the cylinder as a result of compression, the outside of the cylinder is surrounded by a cooling jacket through which cold brine is circulated.) The type of breakage achieved depends on the dimensions of the orifice (which can be closely controlled by varying

the diameter of the center rod) and on the velocity of the jet. This instrument has been used in separating nuclei from a variety of mammalian and invertebrate tissues.

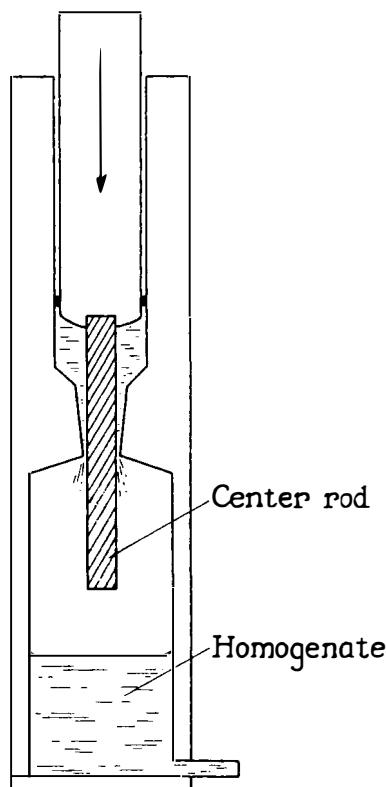


FIG. 5. Piston-press homogenizer of Emanuel and Chaikoff (1957a). The minced tissue, suspended in a suitable medium, is forced by the downward movement of the piston through the very narrow annular orifice between the center rod and the tapered section of the cylinder. The dimensions of the orifice are varied by changing rods. (This instrument is made by the Micro-Chemical Specialties Co., 1834 University Ave., Berkeley 3, California.)

The shearing forces obtained in high-speed jets are sufficient to break bacterial cell walls and rupture chloroplasts. Their use for this purpose has been described by Milner *et al.* (1950).

Other mechanical devices for breaking cells in aqueous media will not be discussed in detail here. A number of references are included in the brief summary below which supply representative applications of these other techniques.

Cells can be broken by freezing and thawing. Warburg (1910) and Miyake (1933) applied this technique for breaking nucleated erythro-

cytes. For many purposes this causes excessive damage to cell components due to their penetration and rupture by ice crystals and to drastic changes in osmotic pressure.

Cells can be broken by grinding in a mortar and pestle either with or without added abrasives (Bensley and Hoerr, 1934; Claude, 1946; Claude and Potter, 1943). Drastic grinding is often required for the preparation of subcellular components from yeast or bacterial cells and in the isolation of chloroplasts from plant cells (e.g., Kalnitsky *et al.*, 1945). Bacterial cells can be broken by agitation with glass beads (Curran and Evans, 1942; Mickle, 1948). This can be carried out with high efficiency by homogenizing the bacterial suspension in the Waring Blender together with small glass beads (0.2 mm. diameter) (LaManna and Mallette, 1954). An excellent summary of the special techniques used in breaking bacterial cells has been prepared by Hugo (1954).

In recent years increasing use has been made of ultrasonic or sonic vibrations as a means of breaking cells or disrupting cell components. Such vibrations may be transmitted by magnetostriction or electrostriction devices to vessels containing the cell suspension. Sound vibrations have been used in lysing erythrocytes (Wood and Loomis, 1927), in preparing bacterial extracts and particulates (e.g., Gale and Folkes, 1954; Gunsalus *et al.*, 1953), in solubilizing mitochondrial enzymes (Hogeboom and Schneider, 1950, 1952b), and in disrupting nuclei (Billingham *et al.*, 1956) (review by Naimark *et al.*, 1951).

Other methods include bursting due to osmotic shock, which is widely used in lysing bacteriophage (Anderson, 1949), and sudden decompression (Fraser, 1951).

A special case of some interest is a technique for breaking oocytes by centrifuging a cell suspension in sea water which overlays a 0.95 M sucrose solution. When the cells rupture, the lighter nucleated halves go to the top of the saline solution and the red pigmented halves go to the bottom of the centrifuge tube (Harvey, 1931, 1938; Shapiro, 1935).

B. Breaking Cells in Nonaqueous Media

In the nonaqueous isolation procedures the methods used for breaking the cell differ markedly from those discussed in Section III, A above. The most significant difference is the fact that the cells are fragmented either in the dry state or suspended in inert organic solvents.

1. Lyophilizing Techniques

Because freezing and drying constitute the first steps in all but a few modifications of the nonaqueous isolation procedure, a brief discussion of freeze-drying techniques is included as a preface to the description of the grinding procedures themselves.

In the original Behrens procedure (1932), organs were frozen in CO₂, wrapped in cloth, and placed in a vacuum dessicator over concentrated sulfuric acid. The removal of water by this slow procedure took several days, and the method has since been supplanted by more rapid and efficient techniques, some of which will now be described.

Since the aim of nonaqueous isolation procedures is to minimize the transport of soluble cell components from cytoplasm to nucleus, or vice versa, emphasis must be placed on the need for rapid freezing and for the removal of water without thawing of the tissue. Freezing can take place in a matter of seconds when small segments of tissue are dropped into liquid air or liquid nitrogen, or into isopentane or propane cooled by liquid nitrogen. Liquid-air freezing was employed in the preparation of nuclei from beef heart, calf liver and thymus, and chicken kidney (Allfrey *et al.*, 1952). Another technique for rapid freezing consists of grinding the tissue in a Waring Blender or similar high-speed mixer together with dry-ice (CO₂) powder (Behrens, 1952). Pulverizing in CO₂, however, introduces the possibility of chemical changes due to a lowering of the pH of the tissue. Rapid freezing of suspensions of free cells (e.g., erythrocytes) is a comparatively simple matter. The suspension can be placed in a lyophilizing flask, and the flask immersed in a dry-ice and acetone mixture; by rapid rotation, the cell suspension is frozen as a thin film on the walls of the flask.

The slower freezing methods (e.g., placing the tissue in a freezing chest at -26° C.) are probably less satisfactory than instantaneous freezing in liquid air. Nevertheless, a comparison of enzyme and soluble protein distribution in nuclei prepared after freezing in these ways showed no significant differences (Allfrey *et al.*, 1952).

When solid tissues are frozen, they can be fragmented quickly by a blow with a hammer, or sliced into small chips. The small, frozen segments are quickly transferred to lyophilizing vessels immersed in dry ice-acetone, in readiness for drying.

The first requirement of the drying procedure is that water be removed under conditions which preclude thawing of the tissue. This aim can be achieved or closely approximated by a variety of lyophilization techniques, but it should be emphasized that working with large amounts of tissue prevents the utilization of many elegant devices employed in the freeze-drying of tissue sections (see Bell, 1952; Moberger *et al.*, 1955). The drying techniques have in common the use of very low pressures to hasten evaporation, and the removal of water vapor in freezing traps maintained at very low temperatures. The high vacuum is usually maintained with an oil pump; the low temperature is commonly achieved by immersing the freezing trap in a dry-ice-acetone or similar mixture (-60° to -70° C.). A great deal depends on the design

of the lyophilizer and the capacity of the pump. Although space does not permit a detailed consideration of these design factors, the essentials are quite simply stated: (1) All tubes leading from the lyophilizing vessel to the freezing trap must be of as wide a bore as possible; any narrow constrictions must be avoided, and the shorter the vapor path, the better. (2) The freezing trap should have a large surface in contact with the freezing mixture in which it is immersed, and its dimensions should be large enough to permit the removal of all the water in the tissue load without setting up an ice-crystal barrier on the way to the pump. For convenience in cleaning, it should allow easy draining of accumulated water. (3) The temperature of the trap should be kept low to minimize evaporation of its contents; this keeps the pressure of the system low and reduces evaporation and transport of water into the vacuum pump. (4) Most lyophilizing units make use of the low pressures obtainable using the common laboratory vacuum pumps (0.0001 mm. Hg); it is obvious that the condition of the pump will determine the efficiency of the drying process. Some provision for venting air back into the system after lyophilization is necessary; a needle valve or capillary-feed stop-cock is convenient for this purpose.

If the flask containing the tissue fragments is immersed in a freezing bath throughout the process, thawing cannot occur and soluble cell components must remain fixed as frozen. The time required for lyophilization is, of course, extended when the tissue is maintained at very low temperatures, and the rate of evaporation is correspondingly diminished. In efficient systems, even if the flasks are not kept in a freezing mixture, one usually observes that low temperatures are maintained: flasks exposed to the moisture of the air gradually accumulate a thick jacket of ice, which is retained throughout most of the drying period. This is an indication of the appreciable cooling effect due to evaporation, an effect which helps to maintain the frozen state of the tissue even without supplementary cooling. However, it should be mentioned that the stability of certain enzymes (catalase, for example) is quite low during lyophilization, and it has been shown that maintaining the flask at low temperatures throughout the drying period allows a greater recovery of enzyme activity (Dounce and Schwalenberg, 1950).

In drying solid tissues it is important that the tissue segments permit the easy escape of water vapor from the interior. For this reason thin slices are preferable to thick pieces or chunks; and tissue powders which are spread as a thin film dry very rapidly indeed.

Following the lyophilization, a convenient next step is to prepare the dry tissue for grinding by pulverizing or shredding it in the Waring Blender. It is then stored at subfreezing temperatures in readiness for grinding.

In a recent method Behrens and Taubert (1952b) have described a combining freezing and drying technique in which the tissue is homogenized in cold, dry acetone prior to grinding and separation of the nuclei (see Mayer and Gulick, 1942). Whether this procedure adequately protects against extraction and adsorption of water-soluble components has not yet been tested. It may be that considerable translocation of material, especially of low molecular weight, might take place before the acetone concentration becomes high enough to prevent it.

2. Grinding and Milling

Most of the nonaqueous isolation procedures begin with a milling or grinding procedure which breaks cells as a result of impact, tearing, and maceration between hard grinding surfaces. In the original Behrens procedure, this was carried out by suspending the dry tissue powder in organic solvents and grinding between two closely fitting Carborundum plates in a mill especially designed for that purpose, and by using a ball mill (Behrens, 1938). In subsequent methods (Dounce *et al.*, 1950; Allfrey *et al.*, 1952), grinding in the ball mill has been found satisfactory for breaking a wide variety of cell types, and the nuclear isolation procedures described below (Section IV, A, 3) make use of this instrument. For fragmenting small amounts of tissue a coaxial homogenizer fitted with a plastic piston may be used (Kay *et al.*, 1956).

A common form of ball mill consists simply of a porcelain jar with a tight-fitting, rubber-gasketed cover which locks into position. The porcelain jars come in a variety of sizes ranging from 950 ml. to a capacity of several liters. (Smaller volumes can be handled in stainless steel beakers fitted with tight cork covers.) The grinding vessel is loaded with stones (flint pebbles) or with porcelain or steel balls to supply grinding surfaces which are in constant action during the milling process. The tissue and some suitable suspension medium (such as petroleum ether, b.p. 60–70° C.) are then added to complete the load. For increased milling efficiency the jar is not filled to capacity but is operated with about one-third of its volume free. When it is loaded and sealed, the jar can be rolled, shaken, or agitated in a variety of commercially available mills. For many nuclear isolations a satisfactory procedure is to mount the jar on its side on motor-driven rollers and rotate at about 110 r.p.m. for 44–48 hours (see Fig. 6). Under these conditions the free space in the jar permits a continuous tumbling and falling of stones during rotation, and the grinding action is supplemented by impact. The time required for grinding and the results obtained vary with the particular mill, the size of the jar, the number and size of the stones, and with the amount, nature, and concentration of the tissue suspension.

The purpose of grinding is to convert a tissue suspension which, at

the outset, is completely cellular, to a mixture containing a high proportion of free nuclei. Its success depends on the relatively high resistance of the nuclei to the milling conditions. But because the nucleus is not entirely refractory to grinding, the process must be stopped before

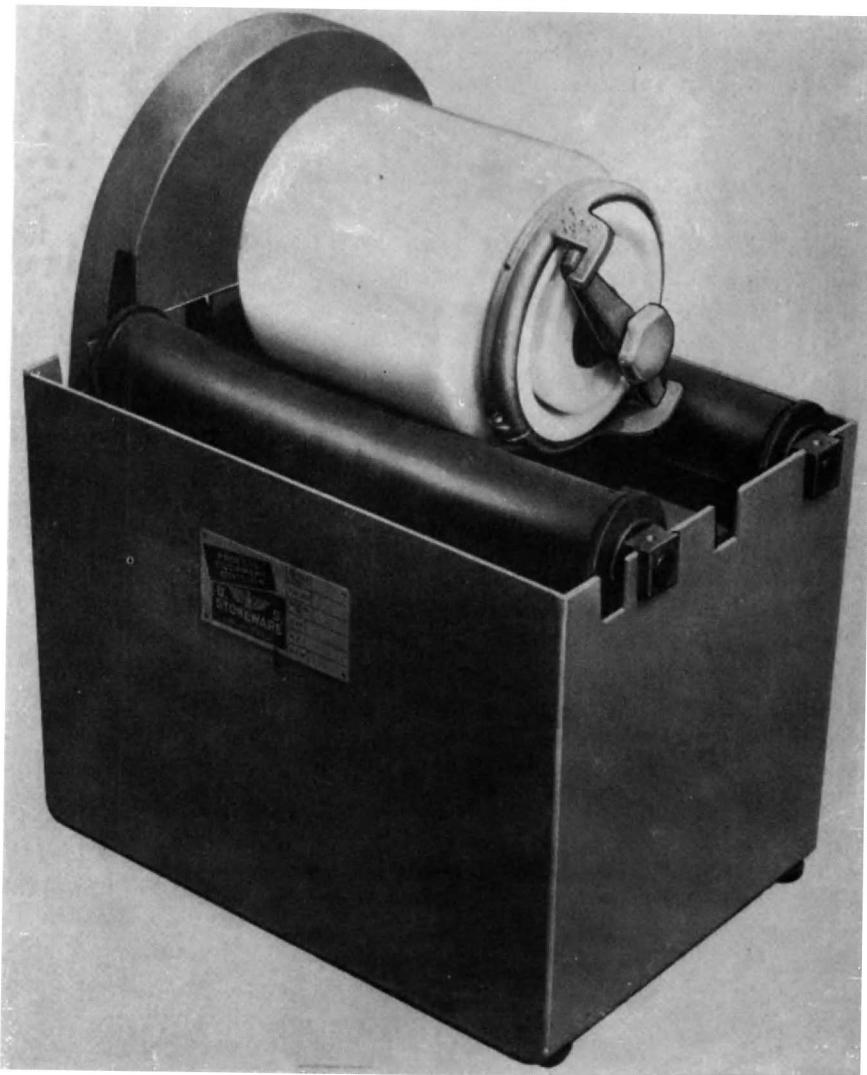


FIG. 6. A roller-type ball mill and porcelain milling jar. The roller mill will accommodate any of a large number of different grinding jars. (This equipment is available commercially from the Cole-Parmer Instrument Co., 224 W. Illinois St., Chicago, Illinois.)

any extensive nuclear breakdown occurs. In practice, this end point corresponds to a ground-cell suspension in which only a fraction of the nuclei are completely devoid of cytoplasm. The rest of the suspension comprises blocks of free cytoplasm, intact and partly ground cells, nuclei with cytoplasmic tabs, pigment granules, fiber, etc. If the grinding is not carried to excess, the isolation of nuclei from this mixture is not complicated by the presence of ground nuclear debris.

The possibility that cytoplasmic proteins are ground into the nucleus during this type of milling procedure has been tested and found untenable. Calf thymus nuclei prepared from a mixed suspension of thymus tissue and avian erythrocyte debris were not tinged with red, and their normal iron content (0.0066%) indicated that no contamination by extraneous hemoglobin had occurred (Allfrey *et al.*, 1952). Further evidence for the absence of cytoplasmic contamination in nuclei prepared in nonaqueous media is presented below in connection with standardization procedures (Section V, A).

C. Separations Based on Size Differences

Both aqueous and nonaqueous isolation procedures make use of the fact that a number of contaminants, and fiber in particular, can be removed by techniques which exploit differences in size. The more common of these techniques involve filtration through gauze or cloth, or through wire sieves of varying porosity. Other methods exploit the differences in sedimentation rate which are due to differences in the size and shape of suspended particles. Nearly all of the isolation procedures in common use make some application of sieves or filters; the use of sedimentation chambers to remove rapidly sedimenting impurities has been described by Behrens (1938).

It has previously been pointed out, in the discussion of grinding techniques, that tissues containing excessive amounts of fiber may be forced through a perforated plate, leaving most of the fiber as a mat on the upper side of the plate (see Fig. 2). This procedure, which is useful only for small amounts of tissue, is carried out before homogenization, and it can be omitted altogether in fractionating relatively nonfibrous tissues (e.g., mouse liver). For isolations on a larger scale, the removal of fiber is accomplished after the tissue is homogenized in the blender or preground in other ways. In most cases the preliminary steps involve filtration through gauze, bandage cloth, or 10-XX silk screening. Different isolation procedures differ in the details of this filtration: the variables, apart from the fiber content of the homogenate, include the pore size of the cloth, whether it is napped or not, and the number of thicknesses used. Where detailed methods are not available as a guide, it is

often satisfactory to proceed by passing the homogenate through progressively finer grades of cheesecloth or gauze until it passes 60- or 90-mesh. Remaining fiber and clumps of cells can then be removed by subsequent filtration through flannel, silk screening, bolting cloth, or fine stainless steel wire meshes. These materials and a wide variety of sieves are commercially available in graded pore sizes (Table I). Wire meshes

TABLE I
MESH APERTURES OF STANDARD WIRE SIEVES AND XX QUALITY SILK SCREEN
SIEVE SERIES

Meshes/ inch	U. S. sieve no.	XX SILK					
		Aperture width Mi- crons	Aperture width Milli- meters	Threads/ inch	Cloth no.	Aperture width Mi- crons	Aperture width Milli- meters
27.62	30	590	0.590	23	000	910	0.910
32.15	35	500	0.500	29	00	680	0.680
38.02	40	420	0.420	38	0	500	0.500
44.44	45	350	0.350	48	1	370	0.370
52.36	50	297	0.297	54	2	320	0.320
61.93	60	250	0.250	58	3	300	0.300
72.46	70	210	0.210	62	4	280	0.280
85.47	80	177	0.177	66	5	250	0.250
101.01	100	149	0.149	74	6	220	0.220
120.48	120	125	0.125	86	8	180	0.180
142.86	140	105	0.105	97	9	150	0.150
166.67	170	88	0.088	116	11	120	0.120
200.00	200	74	0.074	129	13	100	0.100
238.10	240	62	0.062	150	15	80	0.080
270.26	270	53	0.053	163	17	70	0.070
323.00	325	44	0.044				

are especially useful in purifying nuclear fractions obtained by the non-aqueous isolation methods (Allfrey *et al.*, 1952). An alternative procedure to the use of fine screening is filtration through napped cloth (flannel or flannelette). The fine nap in flannelette serves as an efficient trap for fiber and clumps of cells in the preparation of lymphocyte and thymus nuclei in sucrose solutions (Schneider and Peterman, 1950; Allfrey *et al.*, 1957a). Other procedures for removing fiber and large contaminants include filtration through fiber glass cloth, or through filter pads of absorbent cotton (Behrens, 1938; Cutter *et al.*, 1952).

Sedimentation columns were used by Behrens, who allowed cell suspensions to stand long enough for larger fibers to settle; smaller fiber was then removed by filtration through cotton. Particles of equal density can also be separated according to size using the counterstreaming centrifuge developed by Lindahl (see Section III, E, 5).

In preparing nuclei in concentrated sucrose solutions, fibrous contaminants are removed on the basis of their density, and filtration through flannel or bolting cloth is not always required (e.g., see Chauveau *et al.*, 1956). Similar results are observed in many fractionations in nonaqueous media, but where large amounts of fiber are involved, nuclear isolations are considerably improved by filtration through fine screening.

D. Isolation Media

The importance of the isolation medium in determining the outcome of cell fractionations has already been stressed (Section II, C). In aqueous isolation procedures the composition, ionic strength, pH, and osmotic pressure of the medium all play an important part in deciding the final appearance and function of isolated subcellular components. In the nonaqueous fractionation procedures the important variables are the composition and density of a binary organic solvent mixture. The way in which some of these variables are controlled and some factors which influence the choice of an isolation medium will now be discussed.

1. Citric Acid Solutions

Isolations in citric acid solutions have been widely used as a source of cell nuclei. The course of such separations depends considerably on the pH of the suspension. Where the aims of the fractionation permit the use of low pH's (as in some studies of DNA and lipid composition), strong citric acid solutions facilitate the isolation of very clean nuclei from a wide variety of tissues. Citric acid methods have been described by many workers (Stoneburg, 1939; Marshak, 1941; Haven and Levy, 1942; Mirsky and Pollister, 1946; Barnum *et al.*, 1950; Vendrely, 1952; Frazer and Davidson, 1953). A typical isolation procedure using 1% and 0.2% citric acid is described in detail in Section IV, A, I. Dounce and co-workers have described nuclear isolations carried out with very dilute citric acid solutions at pH 5.9–6.0; these methods are applicable to liver, kidney, and pancreas tissue, but not to the thymus, of young animals. Tissues of older animals were found to yield a high proportion of unbroken cells (Dounce, 1950a). The isolation of nuclei at pH 6.0 can be facilitated by the use of gum arabic solutions to minimize the adsorption of cytoplasmic particulates (Dounce and Litt, 1952).

The disadvantages of citric acid isolations have already been mentioned. Apart from the damage to enzyme function and nuclear organization brought about by low pH's, there is a serious extraction problem. As much as 55% of the protein of liver nuclei may be lost during citric acid isolations (Allfrey *et al.*, 1952) and some nuclear ribonucleic acid may be lost as well (Smellie *et al.*, 1955). Despite these disadvantages

the citric acid methods are often extremely useful. They permit the rapid isolation of very clean nuclei in high yield from many tissues. Such nuclei have been used in studies of lipid composition (Stoneburg, 1939) and DNA and histone content (Mirsky and Ris, 1949; Daly *et al.*, 1951).

2. Sucrose Solutions

Procedures for the isolation of cell nuclei in sucrose solutions have been described by many workers (Hogeboom *et al.*, 1948, 1952; Arnesen *et al.*, 1949; Schneider and Peterman, 1950; Wilbur and Anderson, 1951; Dounce, 1952; Stern and Mirsky, 1953; Allfrey *et al.*, 1957a; Allfrey, 1954; Chauveau *et al.*, 1956; Philpot and Stanier, 1956). The methods differ in many ways, the most important of the variables being the concentration of the sucrose solution, the pH of the suspension, the presence or absence of divalent ions, and the use of other agents (e.g., glycerol or gum arabic) to facilitate the isolation.

One of the main applications of sucrose media is in the preparation of cytoplasmic particulates, notably the mitochondria and microsome fractions. The first sucrose isolation procedures employed neutral, hypertonic (0.88 M) and fairly viscous solutions (Hogeboom *et al.*, 1948). In several subsequent methods the sucrose concentration was lowered to isotonic (0.25 M) (Hogeboom *et al.*, 1952), and the pH was sometimes lowered to 6 (Dounce, 1952). Rather than present a detailed history of the development of isolations in sucrose, a brief summary of the present state of such isolations will be given.

There is now good reason to believe that the most desirable sucrose concentration is the isotonic level (in mammalian tissues, about 0.25 M). This conclusion is based on studies of protein and ribonucleic acid synthesis in isolated thymocyte nuclei, where there is a clear-cut functional optimum in the isotonic range. Nuclei prepared in more concentrated sucrose solutions (0.4 – 1.0 M) fail to incorporate amino acids into protein or pyrimidine precursors into RNA (Allfrey *et al.*, 1957a).

Similar functional optima are observed in isolated cytoplasmic particulates. Sarcosomes prepared from insect thoracic muscle, for example, show a maximum P:O ratio in 0.36 M sucrose solution, a concentration isotonic with the insect hemolymph (Lewis and Slater, 1954). There is good evidence that animal mitochondria behave similarly and that an osmotic balance with the medium is essential in maintaining the structures involved in coupled oxidative reactions (Harman and Feigelson, 1952).

There are many modifications of sucrose isolation media which were devised for special purposes. The addition of calcium ions to 0.25 M

sucrose permits the isolation of thymus nuclei without the clumping, agglutination, and gel formation one obtains in sucrose alone (Allfrey *et al.*, 1957a; Schneider and Peterman, 1950). Chelating agents (e.g., ethylenediaminetetraacetic acid) have been used to stabilize the α -ketoglutaric acid oxidase system in rat heart and insect sarcosomes (Slater and Cleland, 1953; Lewis and Slater, 1954). Polyvinylpyrrolidinone (PVP) added to isotonic sucrose preserves mitochondrial form (without loss of function) and avoids the swelling and distortion obtained in 0.25 M sucrose alone (Novikoff, 1957).

Most sucrose isolations are carried out at pH values near neutrality. Buffers are sometimes added to maintain the desired pH, as in the preparation of mitochondria from plant cells (e.g., avocado mesocarp) where a 0.4 M sucrose medium is buffered at pH 7.3. A buffered sucrose medium was also employed in the fractionation of cytoplasmic particulates from beet petioles, wheat roots, and other nonphotosynthetic plant tissues (Stumpf and Barber, 1957; Martin and Morton, 1957). Isolations from mammalian tissues usually do not involve special precautions for maintaining a fixed hydrogen ion concentration in the tissue suspension. In homogenates of rat liver and kidney, for example, the pH remains very close to 7 even after 1 hour at 5° C. (Wilbur and Anderson, 1951). In other tissues, however, a rapid autolysis may cause a fall in the pH of the suspension; this has been observed in rabbit kidney breis prepared in isotonic KCl (Green *et al.*, 1948). In such cases buffering is probably desirable, but it should be pointed out that changes in ionic strength and tonicity of the medium will probably alter the course and outcome of the fractionation.

Sucrose media have frequently been modified with a view to improving the yield, purity, or morphology of the isolated component. The use of PVP in isolating rod-shaped mitochondria has already been mentioned. Some effects of the medium on the morphology of liver nuclei have been summarized by Anderson (1956), and other studies of nuclear morphology in different suspension media have been made by Philpot and Stanier (1956). Nuclear fractions prepared in 0.25 M sucrose are usually grossly contaminated by whole cells, cell fragments, and adhering cytoplasmic debris. The problem of removing such contaminants from liver nuclei led to several modifications of the sucrose isolation procedure. These include "layering" techniques, in which the homogenate is layered over a denser medium in the centrifuge tube; this acts as a barrier to the sedimentation of whole cells and other contaminants of lower density than the nuclei (Wilbur and Anderson, 1951; Maver *et al.*, 1952; Hogeboom *et al.*, 1952). Other procedures employ hypertonic isolation media whose high density and viscosity

facilitate the separation of the nuclei from lighter and slower sedimenting impurities (Krakauer *et al.*, 1952; Lang and Siebert, 1952; Philpot and Stanier, 1956; Chauveau *et al.*, 1956). Unfortunately, isolation in hypertonic media leads to a loss of synthetic capacity (see Section V, C), and such nuclear fractions lose much of their value for studies of nuclear function *in vitro*.

3. Other Aqueous Isolation Media

a. Salt solutions. Although many of the early cell fractionation procedures were carried out in saline or mixed salt solutions (Bensley and Hoerr, 1934; Claude, 1943; Chantrenne, 1947), the use of salts as suspension media is no longer common practice in fractionating mammalian cells. The change to sucrose isolation media is largely due to the effects of salt solutions in causing the aggregation of cytoplasmic particulates (Hogeboom *et al.*, 1948; Dalton *et al.*, 1949) and in modifying the structure and function of the mitochondrion (Berthet *et al.*, 1951; Cleland, 1952) and the nucleus (Allfrey *et al.*, 1952, 1955).

Nevertheless, there are examples which illustrate that in some systems the functional activity of the isolated particle is retained in salt solutions without the use of a sucrose suspension medium. Thus, salt solutions have been used for the preparation of chloroplasts which are capable of photosynthetic phosphorylation (Arnon *et al.*, 1956). Bacterial pigment granules suspended in tris buffers also synthesize ATP (Newton and Kamen, 1957). Protoplasts of *Bacillus megatherium* are capable of division in a 0.5 M phosphate buffer (McQuillen, 1956), and cell fragments of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) can incorporate radioactive amino acids when they are suspended in buffered saline (Gale and Folkes, 1955).

Salts are frequently added in functional tests of cell components isolated in sucrose solutions; this is discussed further in Section V, C.

b. Solutions of glycerol, ethylene glycol, and sugars other than sucrose. Other aqueous isolation media include solutions of glycerol, ethylene glycol, and the Carbowaxes (polymers of ethylene glycol). These are usually dissolved in concentrations which yield viscous or syrupy media; the high viscosity often aids in the separation of particles by modifying their sedimentation rates (see Section III, E).

The isolation of nuclei in ethylene glycol was briefly described by Mazia (1952) and subsequently tested by Dounce (1955), who prepared liver nuclei in 70% ethylene glycol solution and found them to be swollen and of a reddish color due to the presence of adsorbed hemoglobin. The yields were low due to difficulty in breaking the cells, and whole cell contamination was evident.

Some of the glycerol methods which have been employed use a buffered 70% glycerine solution which seems to preserve the nuclear morphology and minimize the loss of protein into the medium (Schneider, 1955). Beef pancreas nuclei have been prepared in this way (Poort, 1957). Liver nuclei have been isolated in 95% glycerol (Dallam and Thomas, 1953), in 70% glycerol (Dounce, 1955), and in 40% glycerol plus potassium glycerophosphate, sucrose, and magnesium chloride (Philpot and Stanier, 1956). These are all viscous, hypertonic media which would have a damaging effect on the isolated thymus nucleus (see Section V, C). Neither glycerol nor ethylene glycol in isotonic concentrations can preserve the synthetic capacity of the thymus nucleus (Allfrey *et al.*, 1957b).

The polyethylene glycols or Carbowaxes have been used for the preparation of plastids from plant cells (McClendon and Blinks, 1952). It has since been shown that polyethylene glycol will coprecipitate soluble proteins (e.g., amylophosphorylase) which would contaminate the isolated particles (Stocking, 1956).

Sugars other than sucrose have been used in preparing chloroplasts (Granick, 1938) and in isolating nuclei from calf thymus (Allfrey *et al.*, 1957b). It was found that the nature of the sugar made little difference to the synthetic capacity of the isolated nucleus as long as the isolation was carried out under isotonic conditions (see Section V, C).

Other isolation media include solutions of gum arabic (Dounce and Litt, 1952), pectin (Brown, 1951), polyvinylpyrrolidinone (Woods, 1954), and albumin (Lewis and Slater, 1954). The reader is referred to the original papers for further details.

4. Nonaqueous Isolation Media

In the nonaqueous isolation methods the suspension medium is a blend of a light organic solvent with a heavier one, and the density of the mixture depends on the relative amounts of these two liquids. The choice of organic solvents, although potentially a broad one, has been limited in practice to very few binary mixtures, such as ether-chloroform, benzene-carbon tetrachloride, cyclohexane-carbon tetrachloride, and petroleum ether- CCl_4 . The ether-chloroform mixtures are extremely volatile, and this is a disadvantage because it introduces the problem of uncontrolled density and volume changes. Behrens, who introduced this mixture, also recommended benzene-carbon tetrachloride as being more generally useful, and the latter blend was found to be satisfactory for nuclear isolations from many types of tissue (Behrens, 1938; Dounce *et al.*, 1950; Allfrey *et al.*, 1952).

Cyclohexane was substituted for benzene when it was found that

benzene inactivates some enzymes (e.g., adenosine deaminase) to a greater extent than does cyclohexane. Attempts were also made to find other heavy solvents which could substitute for carbon tetrachloride, but the number of pure, inert, commercially available solvents of comparable density is limited. Substitution of dibromocyclohexane for CCl_4 led to complete inactivation of several nuclear enzymes (Allfrey *et al.*, 1952). However, the possibility remains that the use of other organic solvents, silicones, or fluorocarbons may broaden the range and utility of the nonaqueous methods.

The rationale of cell fractionation in nonaqueous media has already been discussed (Section II, C). In preparing cell nuclei from mammalian tissues one begins by suspending the ground tissue in a solvent mixture of relatively low specific gravity (e.g., 1:1 benzene- CCl_4 or 1:1 cyclohexane- CCl_4). Centrifugation in these mixtures will sediment the nuclei (900 g for 20 minutes), and some cytoplasm may be removed in the supernate. In subsequent stages of the isolation the proportion of the heavy solvent in the mixture is increased stepwise. This brings the specific gravity closer and closer to the nuclear range, and each centrifugation removes more and more of the cytoplasm as the supernates are discarded. In the final stages the medium is adjusted to specific gravities immediately below and above the range for free nuclei. Under these conditions the nuclei are sedimented or floated away from lighter and heavier contaminants in the suspension. This simple procedure often yields nuclear preparations of great purity (see Section V, A).

In making up the solvent mixtures for nonaqueous fractionations a method for the accurate determination of specific gravity is essential. The usual procedure is to use a hydrometer which reads to the third decimal place. A set of five hydrometers can be obtained which cover the range 1.180 to 1.490 in steps of 0.001 unit; this includes the effective fractionation range for mammalian tissues. The use of such hydrometers requires volumes of 40 ml. or more, and this may preclude their use in very small-scale fractionations. An alternative method is to use a specific gravity balance, or one can weigh a known volume of the solvent mixture (in a closed tube, to minimize evaporation) and calculate its density.

Specific gravity determinations can be carried out on very small amounts of fluid using a capillary buret and a vacuum system (Behrens, 1938). The capillary is inserted into the medium and a negative pressure applied to the upper end; this causes the liquid to rise (see Fig. 7). The negative pressure required for a given rise is proportional to the specific gravity of the medium. In Behrens' device this pressure is read directly on a parallel manometer which contains a fluid of known specific gravity.

Since the specific gravity of the medium changes appreciably with changes in temperature, it is important that all stages in the isolation (especially the adjustment of specific gravities and centrifugations) be carried out at constant temperature. Although autolysis is not a serious problem in the nonaqueous isolation methods, it is preferable to work in the cold.

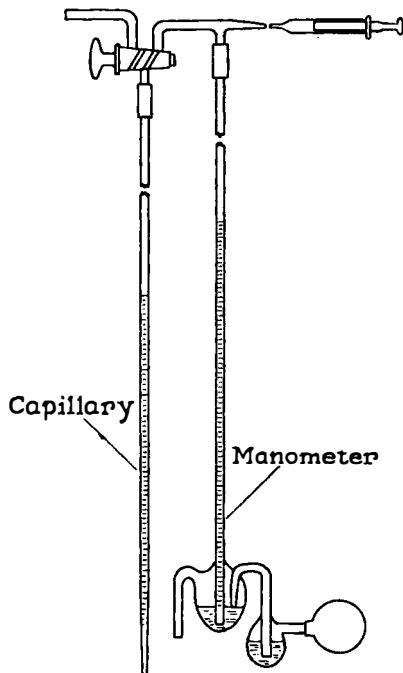


FIG. 7. Capillary densitometer of Behrens (1938). The tip of the capillary is inserted into the solution to be measured and a negative pressure is applied. The suction causes the solution to rise and also raises the level of the liquid in the parallel manometer tube. Since the density of the manometer fluid is known, and both liquid column heights are measured, the specific gravity of the test fluid is easily calculated.

It has already been pointed out (Section II, C) that working with dilute suspensions reduces the possibility of entrapment of the nuclei by other components of the suspension. Clumping and agglutination are usually not serious problems in organic solvents. When nuclei do agglutinate it may be of value to add small amounts of lecithin to the suspension medium (Behrens and Taubert, 1952b; Behrens and Marti, 1954).

Centrifugation is usually carried out in glass tubes because the solvents may attack plastic or celluloid tubes. Specific gravity separations are carried out in centrifugal fields ranging from 2000 to 3000g for

periods of one-half to two hours, depending on the size of the centrifuge tubes and the separation desired.

Nuclear fractions obtained by the procedures described above may require further purification. This can sometimes be accomplished by centrifugation in a specific gravity gradient. This technique is discussed below in connection with centrifugation procedures (Section III, E, 2).

It should be emphasized that the "nuclear specific gravity" in any given tissue is not a single, sharply defined value. Instead it covers a range of 0.3 to 0.5 unit. (Calf thymus nuclei, for example, have specific gravities between 1.37 and 1.41.) It follows, therefore, that in the lower part of the range, light nuclei will sediment together with heavier nucleoli bearing cytoplasmic tabs. In practice, this mixture is discarded. Similarly, nuclei at the upper end of the specific gravity range are often discarded with heavy fiber and dense cytoplasmic debris. Thus, depending on the tissue, an element of selection is necessarily introduced which leads to nuclear preparations in which all nuclear densities are not represented. There does not appear to be a corresponding fractionation according to shape or size; nor do the heavier components of a nuclear preparation differ appreciably from the lighter in enzyme content.

The nuclear specific gravity range is a fairly reproducible factor in different preparations from the same tissue, but it may vary with changes in the physiological state of the tissue. Prolonged fasting, for example, leads to a decrease in the specific gravity of liver nuclei. Failure to remove all the water during lyophilization of the tissue will also affect the densities at later stages in the isolation.

E. Centrifugation

1. Some General Considerations

Centrifugation techniques lie at the core of all the isolation procedures described in this review. A brief discussion of aims and principles may be of value in indicating the rationale of the fractionations described, and may serve as a guide in modifying these procedures to fit other tissues, other media, or other purposes.

Fractionation by means of centrifugation is possible because particles which differ in density or in size (or shape) will sediment at different rates in a field of centrifugal force. The rate of sedimentation is directly proportional to this field, which in a centrifuge is directed outward, and radially, and increases with the square of the angular velocity, ω , and with the radial distance, r , from the axis of rotation:

$$G = \omega^2 r$$

The angular velocity, ω (in radians per second), can be readily expressed in terms of rotor speed (revolutions per minute) as:

$$\omega = \frac{2\pi \text{ r.p.m.}}{60}$$

and the field, in terms of r.p.m. is then:

$$G = \frac{4\pi^2 \text{ r.p.m.}^2}{3600} \cdot r$$

Its dimensions are cm. per second².

It is clear from the above that descriptions of centrifugal separations which report only the time and rotor speed in r.p.m. are of little value unless radial dimensions are included. However, it is far more convenient to express the centrifugal force as a multiple of the ordinary gravitational force acting on the particle (i.e., the ratio of the weight of a mass in the centrifugal field to the weight of the same mass when acted on by gravity alone). This relative centrifugal force is proportional both to the absolute force acting on the suspended particle and its speed of free sedimentation.

A very useful nomograph permitting a ready conversion of r.p.m. to G has been prepared by Dole and Cotzias (1951), and is reproduced in Fig. 8. A thread or straightedge stretched across the chart so as to intercept the known radius and r.p.m. will also intercept the equivalent relative centrifugal force, G. The chart can be used for calculating values outside the indicated ranges by shifting the decimal points. A change in radius by a factor of 10 alters G to the same extent; a change in r.p.m. by a factor of 10 changes G by a factor of 100. The use of the nomograph requires a measurement of the radial distance. Since G increases with radial distance it is obvious that the field increases down the length of the centrifuge tube and is greater at the bottom than elsewhere. A satisfactory convention is to select the distance from the axis of rotation to the bottom of the tube, since this gives a maximal value, G_{\max} , which is independent of the fluid level in the centrifuge tube. Many workers, however, prefer to use the distance to the middle of the tube and to describe separations in terms of this average G.

The rate of sedimentation of a particle in a centrifugal field is proportional to the strength of the field, as mentioned. The rate also varies with the density of the particle and with the density and viscosity of the medium. The relationship between particle size and density, and the density and viscosity of the medium can be illustrated by Stokes' law for sedimentation of a spherical particle:

$$\frac{dR}{dt} = \frac{2}{9} \cdot \frac{r^2 (d_p - d_m)}{\eta} \cdot g$$

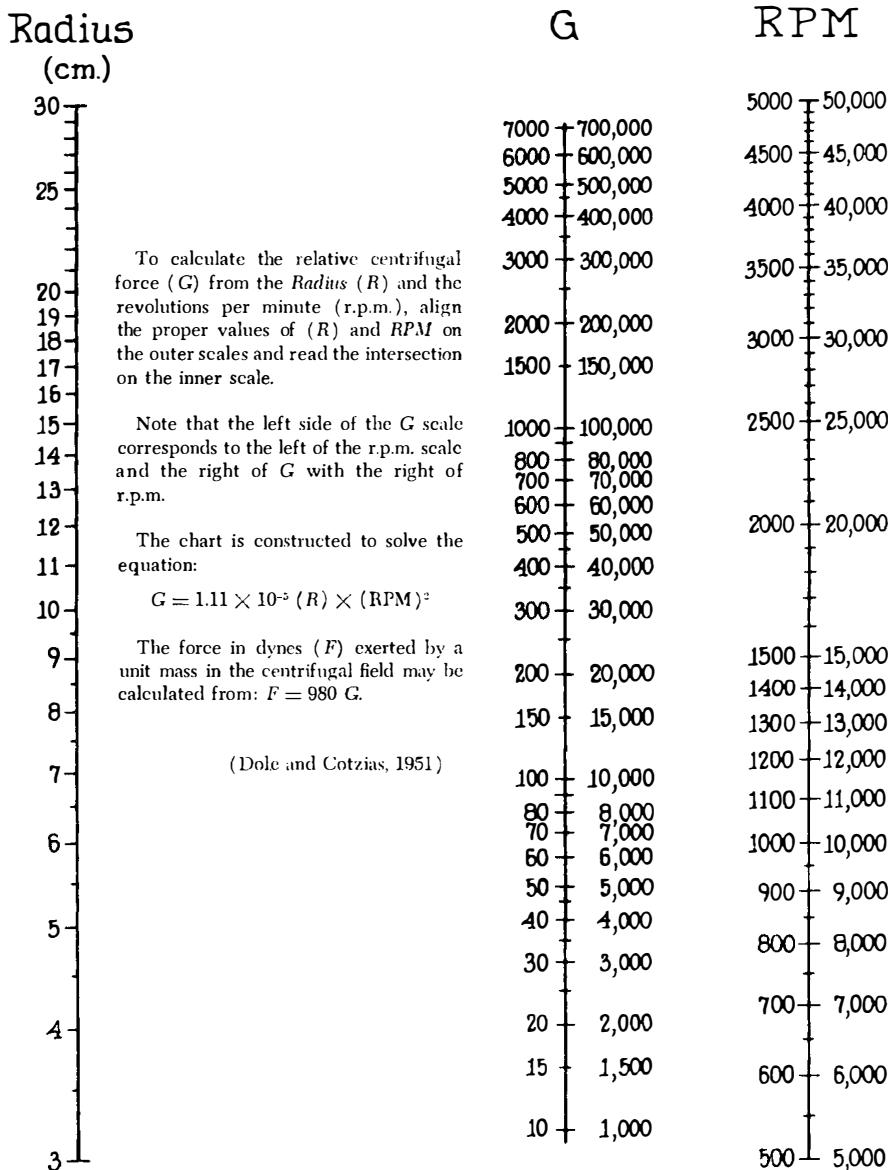


FIG. 8. Nomograph relating relative centrifugal force (G) to rotor speed and radius of the centrifuge head. A thread or straightedge stretched across the chart so as to intercept the known radius and r.p.m. will also intercept the equivalent relative centrifugal force, G .

where dR/dt is the distance traveled in the direction of the field in unit time (cm./sec.), d_p is the density of the particle and d_m the density of the medium (both in grams per cubic centimeter), r is the radius of the particle (in centimeters), η is the viscosity of the medium (in poises), and g is the gravitational field (980 cm./sec.²). The constant 2/9 is the shape factor for a sphere.

The equation illustrates three main points: (1) Particles in suspension will sediment at rates proportional to the square of their radii; (2) sedimentation rate is directly proportional to the difference in density between the particle and the medium; and (3) the more viscous the medium, the slower the sedimentation. It should be mentioned that the relationship between the sedimentation rate and the size of the particle (1) will be modified somewhat when nonspherical particles are considered, and pronounced asymmetry will vary the rate considerably. The density of the medium is one of the important variables in cell fractionation, and it is discussed separately below. Viscosity, the third variable, is especially significant in isolations utilizing concentrated sucrose or glycerol solutions, and either the field or the time of centrifugation must be increased correspondingly.

The integration of Stokes' law (see Svedberg and Nichols, 1923) gives the sedimentation time in a centrifugal field as a function of these variables and also in relation to the distance of travel of the particle in the centrifuge tube:

$$t = \frac{9}{2} \frac{\eta}{\omega^2 r^2 (d_p - d_m)} \cdot \ln \frac{R_{\max}}{R_{\min}}$$

Here R_{\max} is the radial distance to the end point of travel (in most separations this is the distance to the bottom of the centrifuge tube), and R_{\min} is the distance to the meniscus. The equation then gives the time required for a particle at the top of the tube to reach the bottom.

It is clear that this time increases with the viscosity of the medium, and decreases with the strength of the field, with the square of the particle size, and with the difference in density between particle and medium. (The equation does not take into account the changes in field or accelerating and decelerating the centrifuge.)

It is not the purpose of this discussion to treat the subject further in mathematical terms. The above equations were selected because they show, in concise form, the relationship between some of the important variables involved in centrifugal separations. For further mathematical expressions the reader is referred to Svedberg and Pederson (1940), de Duve and Berthet (1953, 1954), Anderson (1956), Pickels (1943), and Chauveau (1954). The latter papers are of special interest be-

cause they treat sedimentation in conical-head centrifuges, in which the tubes are inclined with respect to the horizontal plane. In such conical rotors the sedimentation begins horizontally but later proceeds along the outer wall of the tube. This is discussed further below, but for most practical purposes in cell fractionation (and in the use of the nomogram relating r.p.m. to G) it is usually satisfactory to treat the inclined tube as a horizontal tube with R_{\max} equal to the radial distance from the axis of rotation to the bottom of the tube.

The equations for sedimentation in centrifugal fields can be used in other ways. Thus it has been pointed out by de Duve and Berthet (1954) that the efficiency in separating two classes of particles by a single sedimentation is usually low. In the time required for complete sedimentation of the heavier particles some of the lighter particles will also have reached the bottom and will contaminate the sediment. The extent of such contamination is appreciable; for particles having a value of $r^2 (d_p - d_m)$ equal to one-third that of the heavier particles, complete sedimentation would give a heavier fraction with at least 40% contamination (taking $R_{\max} = 10$; $R_{\min} = 5$). This example illustrates the need for careful washing by resedimentation of the first fractions isolated, and the importance of frequent checks (microscopic or chemical) on the purity of cell components prepared by differential centrifugation.

Considerable discrepancies exist between the theory of centrifugation as outlined above and the practice of centrifugation as usually carried out. Some of these discrepancies will now be discussed.

The sedimentation properties of mixed classes of particles depend in a very complex way on variables not mentioned in the equations, such as the concentration of the suspension, the nature of the medium, and the particular design and handling of the centrifuge. In differential centrifugation, as ordinarily carried out, fractions are collected consecutively at the bottom of the centrifuge tube. In practice, the species purity of each sediment is usually lower than predicted, and larger particles often remain in suspension while lighter, smaller particles are brought to the bottom. Some of this difficulty is due to the fact that particles in a centrifugal field do not separate in simple parallel lines, but rather fan out from a center at the axis of rotation. Particles of all sizes are thus brought to the walls of the conventional cylindrical centrifuge tube, where they adhere, or agglutinate, and set up convection currents as they travel down the wall. In this way many small particles, which would otherwise not have been sedimented, are entrapped and carried to the bottom of the tube. The result of this compounding of agglutination and convection is a loss of resolution in fractionating particles on a size basis. In angle-head centrifuges the production of convection

currents by wall effects can make an appreciable contribution to the sedimentation rate; this is discussed by Pickels (1943). Similar convection or wall effects also occur in the cylindrical tubes used in horizontal swinging-tube rotors. These effects can be eliminated or minimized in such horizontal systems by the use of sector-shaped centrifuge tubes, as is done in the ultracentrifuge (see Svedberg and Pederson, 1940). The design and operational characteristics of sector-shaped tubes of large capacity have been described by Anderson (1955b, c).

A related difficulty is the setting up of thermal convection currents during the operation of the centrifuge, especially in long runs at high speed. These effects are largely eliminated in modern high-speed centrifuges (such as the Spinco Preparative Model E) in which the head is refrigerated and rotates in a vacuum. When a centrifuge operates in air, however, the heating effects due to friction may be considerable; for careful separations refrigeration is necessary. (Low temperatures are also desirable because they reduce autolytic changes in tissue homogenates.) Variations in temperature along the length of the tube can affect sedimentation rates appreciably because the concomitant density changes in the medium are greatly magnified at high centrifugal fields.

One of the most important variables in centrifugation is the way in which the rotor is started and stopped. Acceleration and deceleration both produce mechanical disturbances which may lead to swirling and mixing of the tube contents. This is not a serious problem in ordinary differential centrifugation technique as long as abrupt starts or stops (especially the latter) are avoided. But with layered systems and with density gradients (both discussed below) gradual acceleration and deceleration is essential. The transition from a vertical tube position to a horizontal one in the swinging-tube centrifuge, and the corresponding swing of the meniscus in an angle-head centrifuge must be achieved slowly if swirling and mixing of layers is to be avoided. Detailed procedures for gradual acceleration and deceleration in the International Model PR-2 centrifuge have been given by Anderson (1956).

It has already been pointed out that the isolation medium can influence the course and determine the success of a fractionation. Media which clump or agglutinate particles will cause obvious difficulties in differential centrifugation. Media which slowly alter the composition of particles and so alter their density also introduce complications. This sometimes occurs in the nonaqueous isolation procedures, in which a slow removal of lipid from the nucleus causes a corresponding increase in its density. Related to this is the problem of cell particulates imbibing the medium and changing their sedimentation properties. The behavior of particulates in the centrifuge also depends to a very large extent on

the concentration of the suspension. In heavy suspensions a certain amount of agglutination and entrapment is inevitable, its extent depending on the shape and surface properties of the particles in the particular medium. This can be especially troublesome when microfibrils are present. It is, of course, essential that the particles be well dispersed before centrifuging is started; this dispersion of sediments and suspensions is usually accomplished using either efficient stirrers or piston-type homogenizers which fit the centrifuge tube. Low shearing forces are generally adequate for breaking up clumps and dispersing particles.

2. "Layering" and Density Gradient Techniques

These two procedures are often employed in cell fractionations for the purification of fractions which cannot be further resolved by differential centrifugation alone. In "layering" techniques the suspension is carefully layered over a medium of higher density which acts as a barrier to particles of low specific gravity and permits other denser elements of the suspension (e.g., nuclei) to pass to the bottom of the tube. In carrying out this technique one sometimes observes streaming anomalies; masses of the material to be fractionated pass in streams through the denser layer and form a mixed sediment. This may occur as the layers are being formed, or on standing, or during centrifugation. Particles of all sizes, together with the liquid between them, move as a unit. This streaming effect can be minimized by working with dilute suspensions and by increasing the density of the lower layer. A related anomaly is the "turnover" effect, sometimes seen when particles are sedimented through an interface and become concentrated at the top of the lower, denser layer. The packing of the particles may then make this zone denser than the medium below it, and it then "turns over," moving as a body to the bottom of the centrifuge tube (Anderson, 1955b).

"Layering" techniques, despite the difficulties mentioned, make possible some very useful modifications of both aqueous and nonaqueous fractionation procedures. Their use in cleaning up nuclear fractions obtained in 0.25 M sucrose has already been mentioned.

A mixture of particles can often be separated into several fractions which differ in specific gravity by centrifuging them through a density gradient until each species finds its own density level. The use of such gradients by Behrens (1938) is a model of the experimental design; in his experiments a liver homogenate was separated into red cells, cytoplasm, connective tissue and nuclei, in a single centrifugation. Behrens employed mixtures of organic solvents, but density gradients in aqueous media have also been employed for many fractionations. Brakke, for

example, separated potato dwarf yellow virus by centrifugation in sucrose density gradients (Brakke, 1951, 1953). A clear resolution of the layers in such gradient systems requires a slow acceleration and deceleration of the centrifuge in order to minimize swirling effects.

There are a number of ways in which specific gravity gradients can be produced in a centrifuge tube. The most direct way is to make up mixtures of known specific gravity and layer them carefully over each other in order of decreasing density. A modification of this technique employs an inverted layering sequence which causes a slight mixing of the boundaries and so produces a smooth, continuous density gradient rather than a series of discontinuous steps (Behrens and Marti, 1954). A simpler but less precise technique produces a density gradient by layering a light solvent over a heavy one; the boundary is then spread mechanically by tipping the tube or stirring with a fine wire. In another simple procedure the centrifuge tube is partly filled with the heavy solvent; a thin-walled glass tube (about one-third the diameter of the centrifuge tube) is then inserted and filled with the lighter solvent. When this inner tube is withdrawn the lighter solvent flows out and blends with the heavier fluid to produce a density gradient (Behrens, 1938).

Instruments for the production of continuous specific gravity gradients have been described by Behrens (1938) and by Anderson (1955d). In both cases the gradient is produced by a gradual, precisely controlled addition of a lighter solvent to a heavier one. In the Behrens method the blend is achieved by mixing the outflow of two parallel burets, one containing the lighter solvent; the other containing the heavier solvent. The buret containing the lighter solvent is fitted with an internal wedge which runs its length, leaving a corresponding wedge-shaped column of liquid (see Fig. 9). This simple but elegant device permits more and more of the lighter solvent to blend with a fixed amount of the heavier, thus making possible a variety of gradients, depending on the choice of solvents and the rates of flow.

A wide range and a more precise control of gradients are possible when a gradient engine is used. In the instrument described by Anderson (1955d), two differentially driven syringes are filled with a light and heavy solvent and their outflows are blended to produce the gradient. Both the shape of the concentration vs. delivery curve, and the amount of fluid in the gradient can be varied by changing the syringe size and the rate of advance of the piston. Gradients made from 7.4 and 60% sucrose solutions have been used to fractionate a rat liver homogenate into "soluble," "microsomal," "mitochondrial," and "nuclei + whole cell" fractions. No cross contamination of particles was observed

in these experiments; wall and streaming effects were minimized both by the density gradient, and by the use of sector shaped tubes (Anderson, 1956). Other devices for the production of gradients have been described by Ling and Bock (1954), Drake (1955), and Wetlaufer (1957). These instruments, like others which were designed for the production of solution concentration gradients (for use in chromatographic elutions) can also be applied to the production of density gradients.

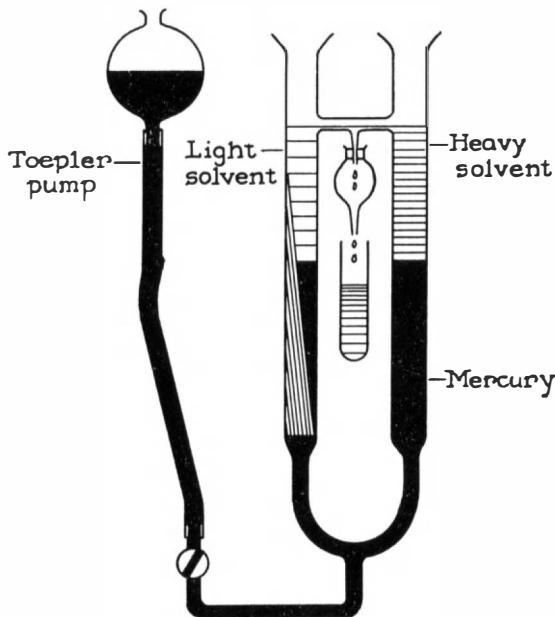


FIG. 9. Instrument for the production of density gradients (from Behrens, 1938). The gradient is produced by mixing the outflow of the two parallel burets, one containing the heavy solvent and the other, fitted with the wedge, containing the light solvent. As the fluids are displaced by the column of mercury rising from below, a gradually increasing amount of the lighter solvent is added to a fixed amount of the heavier solvent. In this way a variety of density gradients can be produced, depending upon the choice of solvents and the dimensions of the columns.

3. Collecting Isolated Fractions

The collection of sediments after differential centrifugation usually poses no special problems. Where the sediment is hard-packed, simple decantation of the supernate is usually employed, supplemented if necessary by wiping the walls of the tube. In centrifugations which yield a loosely packed sediment, the supernate can be removed using a syringe fitted with a long stainless steel needle, a pipet fitted with a rubber

suction bulb to control the intake, or a length of narrow-bore glass tubing connected to a vacuum line and receiving trap.

Where layering or gradient techniques have been employed and the desired fractions lie in loose parallel zones across the tube, special devices for fraction-collecting can be used, although, if the zones remain fairly fluid, collection using the methods described above is still possible. If a syringe is used to remove the zones, a convenient system is to clamp the centrifuge tube in any holder which permits good visibility and to mount the syringe on a rack-and-pinion stand; this simplifies lowering the tip of the needle into position for withdrawing samples. The stainless steel syringe needle may be straight, or bent back on itself to form a small J. Fractions are withdrawn, usually beginning at the top of the tube, at rates slow enough to preclude mixing of the zones. By using appropriate valve systems, the syringe may be used only as a pump, and the fractions transferred directly to other containers.

Another technique for collecting zones or fractions makes use of displacement methods. A very dense medium, such as 80% sucrose or a heavy fluorocarbon (Anderson, 1956) is slowly introduced into the bottom of the centrifuge tube. As the gradient is displaced upward into the neck of the tube, the fractions are removed in sequence. This is done simply with a pipet or syringe, or special sectioning devices can be used. In one such device (Anderson, 1956) the top of the centrifuge tube is fitted into a matching hole in a flat Lucite block and the joint sealed with silicone grease. A narrow-gauge needle passes through the block, bends at a right angle, and passes to the bottom of the tube. As the dense solution is fed through this needle it displaces the gradient upward. A second flat Lucite block, matching the first, but capable of lateral displacement, is then lubricated and placed in position. When the gradient rises through the hole in the lower block and into the matching well in the upper block, the latter is then moved to the side and the solution or zone in the well is thus isolated. The operation is readily observed because the sectioning device is transparent.

A recent device of similar design employs a Lucite shutter which slides between the upper and lower blocks. The fraction isolated by the shutter is removed through a syringe needle mounted in the upper block (Phelpstead and Roodyn, 1957) (see Fig. 10).

In another instrument, a knife edge mounted between the two blocks is used in sectioning the contents of the disposable celluloid tubes commonly employed in high-speed centrifuges. The tubes are inserted into a matching hole in the block, held in position with the desired fraction lying above the plane of the knife edge, and the blade is then forced through the tube, cutting its walls and isolating the fraction (Randolph

and Ryan, 1950). Kuff and Schneider (1954) mention problems of leakage around the blade in this instrument.

4. Some Precautions in Centrifuge Maintenance and Operation

Since centrifugation constitutes such a large part of cell fractionation technique, something should be said about the routine precautions involved in the care and handling of instruments. Most modern centrifuges

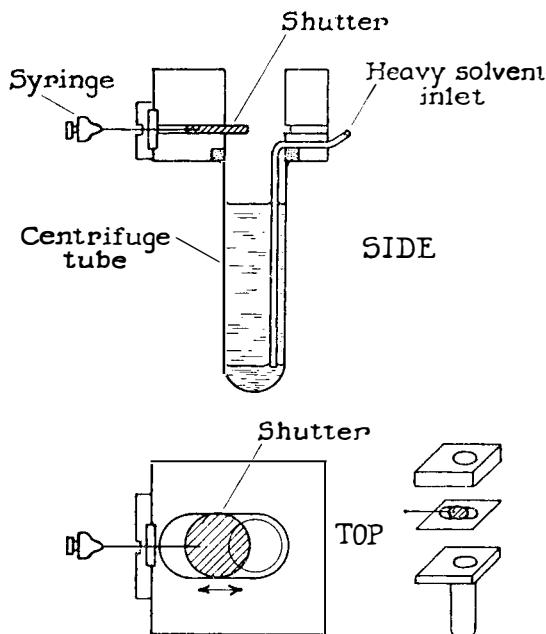


FIG. 10. Sampling device for the collection of layered material following centrifugation. The centrifuge tube is fitted into the transparent plastic block. A heavy solvent which is fed into the bent capillary which leads to the bottom of the tube slowly displaces the layers upward. As the desired fraction passes the shutter assembly, the shutter is moved to the side. This isolates the fraction, which can then be collected using a syringe (from Phelpstead and Roodyn, 1957).

require minimal amounts of care, which is usually limited to occasional oiling, cleaning, and changing of the motor brushes. High-speed centrifuges which may require more attention are usually serviced by the manufacturer. In most centrifuges a careful balance of opposing tubes must be achieved if excessive vibration is to be avoided. In swinging-tube rotors it is advisable to weigh, pair, and number trunnions, shields, cushions, and tubes so that opposing pieces are closely balanced. In angle-head centrifuges the tubes, cushions, and stoppers may need pair-

ing in the same way, but many of the modern angle-head centrifuges have self-centering rotor assemblies so that the tube balance is not critical.*

The importance of slow acceleration and deceleration has already been emphasized. Speed control in most centrifuges is obtained using variable transformers which permit great flexibility in this respect. Since the speed of rotation is such an important factor in cell fractionations, instruments which do not contain a built-in tachometer should be calibrated using mechanical or electrical tachometers or, for high accuracy, a stroboscopic light source and a multiple-sectored disk (Anderson, 1956).

Breakage is often a problem in centrifugation. Chipped or cracked centrifuge tubes should be discarded. Cushioning is an important preventive; rubber jackets or base cushions should be checked for holes, tears, or embedded glass, and should be protected from oil or other rubber solvents. In operations at higher speeds it is advisable to fill shields or tube wells with a liquid which will buoy up the tubes and enable them to withstand greater strain. Glass tubes cannot withstand high centrifugal fields; metal (stainless steel) or plastic (Lusteroid, polyethylene, etc.) tubes are used instead. A further caution is necessary in the centrifugation of organic solvents, such as those used in the non-aqueous isolation procedures. These fractionations are usually carried out in glass or stainless steel tubes to avoid the effects of the solvents on plastic or celluloid tubes. The solvents used differ markedly in volatility; to minimize evaporation and the concomitant changes in density during centrifugation the tubes should be stoppered and the temperature kept low.

Working with radioactive or infective materials introduces special problems in centrifugation, because the probability of breakage must always be considered. Adequate shielding, tight centrifuge covers, sealed tubes, and systems for fast and easy decontamination are required safety precautions.

5. Other Centrifugation Techniques

There are a number of special techniques in centrifugation which have not been considered in this review. A centrifuge permitting the

* Self-centering" direct drive assemblies are now commercially available in a variety of centrifuges. The design and flexibility of the drive assembly minimize vibration caused by rotor imbalance (due to the unequal loading of tubes). Such instruments are fabricated by Ivan Sorvall, Inc., Norwalk, Connecticut; The International Equipment Company, Boston 35, Massachusetts; the Lourdes Instrument Co., Brooklyn 32, New York.

continuous collection of sediment has recently been made commercially available.* Special mention should be made of the newer methods of counterstreaming centrifugation introduced by Lindahl (1948, 1956). In this technique the sedimentation rate of particles in a centrifugal field is modified by allowing the suspension medium to stream in the opposite direction to the centrifugal force. This procedure requires the use of specially designed conical separation chambers in which convection and wall effects are minimized by the use of a density gradient. The counter-streaming centrifuge has been employed in separating yeast cells on a size basis (Lindahl, 1948), in selecting micromeres from other cleavage cells in the sea urchin 16-cell stage (Lindahl and Kiessling, 1950), and for the separation of Ehrlich ascites tumor cells from other cellular elements (Lindahl and Klein, 1955).

IV. SOME REPRESENTATIVE ISOLATION PROCEDURES

To illustrate the practical application of some of the techniques discussed in preceding sections, a small number of representative isolation procedures will now be described. These include the preparation of liver nuclei in citric acid solutions, of thymus nuclei in sucrose-CaCl₂ solutions, and of avian erythrocyte nuclei in nonaqueous media. Also described are the isolations of liver mitochondria and microsome fractions using isotonic sucrose, the preparation of microsomes for amino acid incorporation studies, and the preparation of spinach leaf chloroplasts in saline solution. These particular methods were selected because they have a wide general utility or because they yield subcellular components which have retained a capacity for complex synthetic reactions.

Immediately following each of these detailed isolation procedures there are a small number of references to other, related techniques of preparing similar subcellular components.

A. Nuclei

1. *The Preparation of Liver Nuclei in Citric Acid Solutions*

The following procedure was introduced by Mirsky and Pollister (1946). All operations are carried out in the cold, i.e., at 1–4° C. Fifty

* The "Szent-Györgyi-Blum" continuous-flow centrifuge allows the collection, in one, two, four, or eight tubes, of small amounts of sediment from large volumes of sample, at a flow rate of 200 ml. per minute, in one continuous operation. Material from a reservoir is passed under controlled flow into the rotor tubes; the particles are sedimented, and the clarified supernatant is automatically removed. Speeds up to 17000 r.p.m. are permissible. This instrument is manufactured by Ivan Sorvall, Inc., Norwalk, Connecticut.

grams of fresh calf liver is minced with scissors, suspended in 500 ml. of 1% citric acid, and placed in a Waring Blender or similar mixer. The blender is run at top speed for 6 minutes. (To avoid overheating, the blender vessel should be prechilled and immersed in a freezing-bath; a less satisfactory procedure is to add crushed ice to the homogenate.) A drop of octyl alcohol is added to cut down the foam, and the mixture is centrifuged for 10 minutes at 2000 g. The supernate is discarded. The sediment, resuspended in 750 ml. of 0.2% citric acid, is stirred at low speed (obtained by means of a rheostat) in the mixer for a short time to break up the larger particles. The suspension is strained through a double layer of finely woven towel and then centrifuged at 2000 g. for 5 minutes. Then 750 ml. of 0.2% citric acid are added to the sediment which is again broken up by low-speed stirring in the mixer. This time the suspension is filtered through a double layer of the finest muslin and then centrifuged at 300 g for 5 minutes. No more straining is required, but the nuclei are washed with 0.2% citric acid by alternately mixing at low speed and centrifuging at low speed until the supernatant is clear. No more than three washings after the last straining should be necessary. If the nuclei are to be kept, this is best done while they are suspended in 0.2% citric acid.

This method allows the preparation of very clean nuclei in high yield from a wide variety of mammalian tissues. It is generally advisable to carry out the first steps of the isolation in very dilute suspensions; this reduces the electrolyte concentration and thus minimizes clumping and agglutination of cytoplasmic particles.

For the isolation of nuclei in more dilute citric acid solutions, the methods introduced by Dounce and co-workers may be applied (see Dounce, 1955).

2. *The Isolation of Calf Thymus Nuclei in Sucrose Solutions*

This procedure (Allfrey *et al.*, 1957a) is a modification of that described by Schneider and Peterman (1950). All operations are carried out in the cold. Fifty grams of fresh calf thymus tissue is finely minced with scissors and placed in a blender vessel together with 50 ml. of 0.5 M sucrose solution and 400 ml. of 0.25 M sucrose-0.0033 M CaCl_2 . The tissue is gently homogenized by running the blender at 1000 r.p.m. for 4 minutes. The resulting homogenate is filtered through a double layer of gauze (Johnson & Johnson Type I) and then through a single thickness of double-napped flannelette. The filtrate is centrifuged at 1000 g for 7 minutes, and the supernate is discarded. The sediment is resuspended in 100 ml. of 0.25 M sucrose-0.0030 M CaCl_2 solution with the aid of low-speed stirring, and the suspension is again strained

through flannelette. The filtrate is centrifuged at 1000 g for 7 minutes. The sedimented nuclei are washed in the centrifuge using 100 ml. of 0.25 M sucrose-0.0030 M CaCl₂. Further washings are rarely necessary, since the supernate at this time is usually water-clear. Electron microscopic examination of the isolated nuclei shows a very small amount of whole-cell contamination, of the order of 29 cells per 1000 nuclei.

Thymus nuclei prepared in this way are capable of amino acid uptake into their proteins and can also incorporate purine and pyrimidine precursors into their nucleic acids. A very similar procedure can also be used to obtain functioning nuclei from an AKR lymphoma (Allfrey *et al.*, 1955), but nuclei prepared from spleen, liver, pancreas, and kidney by this procedure are grossly contaminated and cannot take up radioactive amino acids as thymus nuclei do (Allfrey and Mirsky, 1956).

3. The Isolation of Avian Erythrocyte Nuclei in Nonaqueous Media

The following procedure is that of Allfrey *et al.* (1952). Unless otherwise noted, all steps are carried out at 2-4° C.

a. Lyophilizing and grinding. Heparinized chicken blood is centrifuged in the cold as soon as possible after collection. The cells are washed three times with about 4 times their volume of 0.9% saline solution and transferred to lyophilizing flasks. No hemolysis should occur in this gentle washing procedure. The cell suspension is rapidly frozen as a thin film on the wall of the lyophilizing flask by immersing and spinning the flask in a CO₂-acetone slurry. The flasks are then connected to a lyophilizing unit. One hundred grams of the dried cell mass is transferred to a porcelain milling jar containing 450 ml. of petroleum ether (b.p. 60°) and 1400 gm. of irregular grinding stones (2-3 cm. in diameter). The jar cover is locked in position and the jar is mounted on its side on motor-driven rollers and rotated at about 110 r.p.m. for 44-48 hours.

The ground-cell suspension is transferred to 100-ml. glass centrifuge tubes and centrifuged at 900 g for 20 minutes. (A portion of the sediment may be set aside for preparing the "tissue control" as described below.)

b. Fractionation steps. (1) The sediment is resuspended with the aid of an efficient stirrer in about 700 ml. of a 1:1.5 cyclohexane-carbon tetrachloride mixture. The desired specific gravity for the first step in the isolation is 1.290, and the suspension can be adjusted to this density by the addition of small amounts of cyclohexane, if the original specific gravity is too high, or of carbon tetrachloride, if the original density is low. The adjustment is readily carried out with the aid of a small

hydrometer covering the range 1.200 to 1.425 in 0.005-unit graduations. The intercept of meniscus and scale (viewed with the eye at meniscus level) is the specific gravity of the suspension and can be determined to within 0.002 unit. Once adjusted, the suspension is centrifuged at 2000 g for 40 minutes.

The purpose of this first step is to float much of the free cytoplasm and sediment the other components of the suspension. Therefore, the supernatant fluid following the centrifugation should be opaque and not clear, and may contain a small crust. (To remove nuclei which may have been entrapped in this crust, it is sometimes desirable to decant the supernate, redisperse and recentrifuge, collecting the sediment.)

(2) The sediments obtained in the first step are resuspended with rapid stirring in about 700 ml. of 1:1.5 cyclohexane- CCl_4 mixture. The suspension is brought to sp.gr. 1.300 by the addition of small amounts of carbon tetrachloride as required, and centrifuged at 2000 g for 60 minutes. The supernate, which is opaque and contains a crust is decanted. (It may be redispersed and centrifuged again to remove entrapped nuclei.) This second step removes most of the remaining free cytoplasm and a portion of the intact or slightly ground cells.

(3) The sediments obtained at sp.gr. 1.300 are resuspended in 700 ml. of 1:1.5 cyclohexane- CCl_4 , the suspension is brought to sp.gr. 1.310, and centrifuged at 2000 g for 60 minutes. The supernate, which contains many unground or partly ground cells, is discarded.

(4) The sediments at sp.gr. 1.310 are again taken up in 700 ml. of 1:1.5 cyclohexane- CCl_4 , the suspension is brought to sp.gr. 1.320 and centrifuged at 2000 g for 60 minutes. The supernate, which now contains partly ground cells and nuclei with large cytoplasmic tabs, is discarded.

(5) The sediment at sp.gr. 1.320 is then resuspended in 500 ml. of 1:2 cyclohexane- CCl_4 , brought to sp.gr. 1.390, and centrifuged at 2000 g for 60 minutes. The supernate, which now contains the nuclei, is surrounded by a heavy crust which may adhere to the walls of the centrifuge tubes. The crust is detached from the walls with a small spatula, without disturbing the sediment, and the supernate and crust are decanted. Now the sediment, which contains lymphocyte nuclei and heavy debris, is discarded.

(6) Cyclohexane is then added to the supernate to lower the specific gravity to 1.327. The suspension is mixed by rapid stirring and centrifuged at 2000 g for 80 minutes. The supernate, which contains nuclei with large cytoplasmic tabs, is discarded. The sediment is again resuspended in about 400 ml. of the 1:1.5 cyclohexane- CCl_4 mixture, brought to sp.gr. 1.327, and recentrifuged as before. The supernate is discarded.

(7) The sediment at sp.gr. 1.327 is taken up in about 400 ml. of

cyclohexane- CCl_4 , brought to sp.gr. 1.333 and centrifuged at 2000 g for 80 minutes. The supernate, which contains nuclei with cytoplasmic tabs, and some light free nuclei, is discarded. The sedimented nuclei are resuspended at sp.gr. 1.333 and re-centrifuged, again discarding the supernate.

(8) The nuclei are next suspended in about 300 ml. of 1:1.5 cyclohexane- CCl_4 , adjusted to sp.gr. 1.338 and centrifuged at 2000 g for 80 minutes. The supernate, which contains both light free nuclei and heavier nuclei with cytoplasmic tabs is decanted. The sedimented nuclei are washed twice more at sp.gr. 1.338, following the above procedure and discarding the supernates.

(9) After the three washings at sp.gr. 1.338, the nuclei are almost free of cytoplasmic contamination. To remove the last traces of heavy debris, they are suspended in 200 ml. of 1:2 cyclohexane- CCl_4 , brought to sp.gr. 1.390, and centrifuged at 2000 g for 1 hour. The supernate is carefully decanted and the sediment is discarded.

(10) The supernate is adjusted to sp.gr. 1.338, with cyclohexane, and centrifuged for 80 minutes. The supernate, which contains a few tabbed nuclei, is discarded. The sediment, now entirely nuclear, is collected in about 100 ml. of petroleum ether and centrifuged at 900 g for 20 minutes. The nuclei are dried *in vacuo* and weighed.

A "control tissue" preparation, which is useful as a measure of the chemical and enzymatic constitution of the whole cell, and which also indicates losses in enzyme activity due to lyophilization, grinding, and solvent extraction, can be prepared from the original ground-cell suspension. For red cells this is done simply by suspending the broken cells in a 2:1:1 petroleum ether-cyclohexane- CCl_4 mixture (sp.gr. 0.95 at 4° C.) throughout the nuclear isolation procedure. This is dried *in vacuo* at the same time as the isolated nuclear fraction.

The appearance of the suspension at different stages of the isolation procedure is shown in Fig. 11. The preparations are stained with hematoxylin-alcoholic eosin, under which conditions the nuclei appear purple and the cytoplasm red. Beginning with intact cells (Fig. 11, A), grinding in petroleum ether yields the mixed suspension shown in Fig. 11, B), in which the red color of the cytoplasm predominates, and most of the nuclei seem bound to cell fragments. After treatment at specific gravities 1.290, 1.300, 1.310, and 1.320 the nuclei form a larger part of the suspension and most of the free cytoplasm has been removed. Later in the procedure, after washing at sp.gr. 1.327, the suspension consists mostly of nuclei and tabbed nuclei (Fig. 11, C). Sedimentation at 1.338 leaves the nuclei and a few tabbed nuclei (Fig. 11, D). Repeated washings at sp.gr. 1.338 remove the last of the tabbed nuclei and give the final nuclear preparation (Fig. 11, E), which, morphologically, shows negli-

gible contamination. A careful search of the slide may show a few cytoplasmic tabs amid hundreds of nuclei, but in a good preparation such tabs are extremely difficult to find.

The nuclei of many other tissues can be isolated by similar proce-

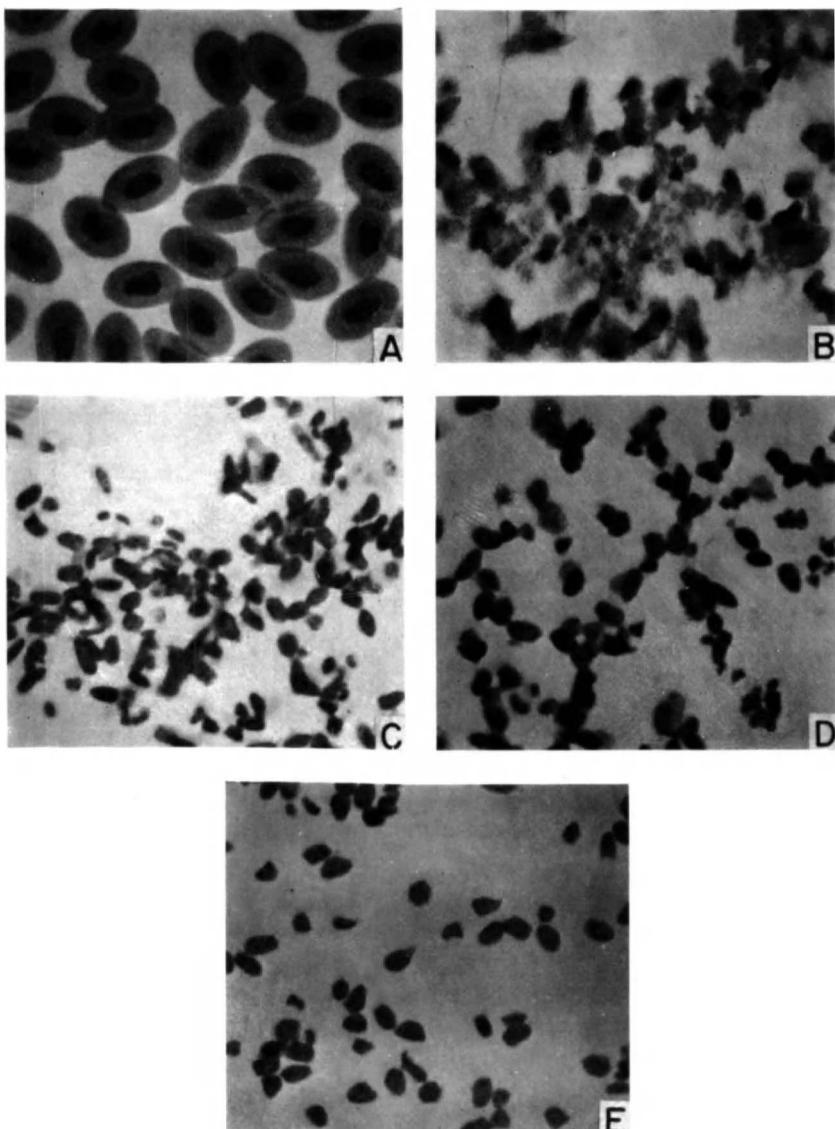


FIG. 11. Stages in the isolation of avian erythrocyte nuclei by cell fractionation in nonaqueous media. All samples stained with hematoxylin-alcoholic eosin; the nuclei stain purple, cytoplasm is red. See text for details. Magnification: $\times 1400$.

dures. In dealing with solid tissues, however, connective tissue fiber may complicate the fractionation. This can be removed by filtering the suspension at successive stages in the isolation, beginning with passage through gauze or 60-mesh screen and progressing through 140-, 200-, and 325-mesh wire screens.

Nuclei prepared in nonaqueous media often meet a very high standard of purity (see Section V, A). Their use as standards of comparison for nuclei prepared in other ways has already been mentioned. They are especially suited to the study of the low molecular weight, water-soluble components of cell nuclei, such as the acid-soluble nucleotides (Allfrey *et al.*, 1955; Kay and Davidson, 1955; Osawa *et al.*, 1957) and the amino acids (Dounce *et al.*, 1950).

4. Other Nuclear Isolations

Other procedures for the isolation of cell nuclei have been considered in connection with isolation media (Section III, D), and standardization procedures (Section V). Nearly all of these methods are concerned with the isolation of nuclei from animal tissues. Plant cell nuclei can also be prepared, in both aqueous and nonaqueous media, from many plant species, including wheat germ (Stern and Mirsky, 1952), coconut endosperm (Cutter *et al.*, 1952), rye germ (Feulgen *et al.*, 1937), and onion root tips (Brown, 1951).

B. Mitochondria

1. The Isolation of Liver Mitochondria in Isotonic Sucrose

The following procedure is based on the methods recommended by Hogeboom (1955) and Schneider and Hogeboom (1950a).

Livers are obtained from rats that have been fasted overnight to remove liver glycogen. Immediately on removal the livers are chilled by immersion in 0.25 M sucrose at 0° C. and then blotted, minced with scissors into pieces about 0.5 cm. in thickness, and weighed. (In earlier procedures the tissue was forced through a masher fitted with a 1-mm. mesh screen that retained the connective tissue framework and allowed the parenchymatous part of the organ to be collected as a pulp.) All subsequent steps in the fractionation are carried out at 0–5° C. 9.0 ml. of 0.25 M sucrose solution is added to each 1.0 gm. of liver and the tissue is homogenized for 1 minute, rechilled, and homogenized for another minute. A coaxial homogenizer is used; its pestle is rotated at 600–1000 r.p.m. by means of a drill-press motor, and the test tube is moved rapidly up and down over the pestle. An appreciable clearance rather than a tight fit between tube and pestle is recommended by

Hogeboom, since a higher percentage of the cells are broken when the tissue suspension can be forced rapidly through the annular space between the tube and the pestle by up-and-down movement of the tube.

Ten milliliters of homogenate in 0.25 M sucrose are centrifuged at 700 g for 10 minutes to sediment the nuclei, unbroken liver cells, connective tissue, and red blood cells. The sediment is discarded. The supernatant from this first centrifugation is then centrifuged at 5000 g for 10 minutes to bring down the mitochondria. The opalescent supernate is removed together with a pink, partially sedimented layer of particles above the firmly packed pellet of mitochondria. (This supernate is saved for the preparation of the microsome fraction.) The mitochondrial pellet is transferred quantitatively to a 10-ml. graduated test tube using 0.25 M sucrose and is redispersed by homogenization in 5 ml. of the sucrose solution. The suspension is centrifuged for 10 minutes at 24,000 g. The sedimented mitochondria are redispersed and centrifuged as before. After three sedimentations the mitochondrial fraction is taken up in isotonic sucrose. The yield of the preparation is about 80%.

In studies of oxidative phosphorylation, liver mitochondria are frequently isolated in 0.44 M sucrose solutions (e.g., Witter *et al.*, 1955) but those prepared in 0.88 M sucrose are inactive. Liver mitochondria which have retained their rodlike shape and are capable of oxidative phosphorylation can be obtained in 0.25 M sucrose-7.5% PVP solutions (Novikoff, 1957).

2. Other Mitochondrial Preparations

Mitochondria have now been prepared from a wide variety of animal tissues, ranging from skeletal muscle (Chappell and Perry, 1954) to brain (Hesselbach and duBuy, 1953). Cardiac muscle tissue is another good source of mitochondria for biochemical studies; heart muscle sarcosomes can be prepared by the technique of Slater and Cleland (1953).

Procedures for the isolation of mitochondria from plant cells have been described by many workers (e.g., Millerd *et al.*, 1951; McClendon, 1952; Laties, 1953; Chayen and Miles, 1953). Some preparations are given in detail by Axelrod (1955). The methods employed usually involve grinding the plant tissue in a mortar with sand, or homogenization in the Waring Blender. Buffered sucrose solutions are commonly employed in such isolations; here the osmotic concentration of the medium can be of great importance. For example, it has been shown that cauliflower mitochondria are extremely sensitive to changes in the tonicity of the extracting medium (Laties, 1954). Reviews on the isolation and

properties of plant mitochondria have been prepared by Hackett (1955) and by Millerd and Bonner (1953).

Insect sarcosomes can also be isolated in sucrose solutions. These interesting structures were studied in insect flight muscles by Watanabe and Williams, (1951, 1953). Their isolation in 0.36 M sucrose solutions is given by Lewis and Slater (1954).

The fractionation of sonic extracts of *Pseudomonas fluorescens* to yield particulates rich in oxidative enzymes has been described by Gunsalus *et al.* (1953).

C. Microsomes

1. The Isolation of Microsomes in Isotonic Sucrose

This is a continuation of the method previously described for the preparation of liver mitochondria.

The combined supernatants and washings obtained in the isolation of mitochondria are brought to a volume of 35 ml. using 0.25 M sucrose and centrifuged at 54,000 g for 60 minutes (in the #30 rotor of the Spinco preparative ultracentrifuge, Model E). The firmly packed, reddish, transparent pellet is resuspended in 12.5 ml. of 0.25 M sucrose and centrifuged for 30 minutes at 148,000 g. The washed sediment is resuspended in a known volume of isotonic sucrose.

This microsomal fraction consists of cytoplasmic material that is too small to be resolved clearly in the light microscope, and its nature and purity must be assessed in other ways (see Section V, A, 1).

2. The Isolation of Microsomes in a Buffered Hypertonic Sucrose Medium

The following method is that described by Keller and Zamecnik (1956) for the preparation of microsome fractions used in amino acid incorporation experiments.

Approximately 18 gm. of rat liver are homogenized, 3.5 gm. at a time, with 2.3 volumes of buffered medium. In carrying out this homogenization, the tissue is placed in a glass vessel surrounded by ice, together with 1 ml. of the medium, and minced for 15–20 seconds with scissors. The mince is transferred to a glass homogenizing tube kept in an ice bath at 0° C. and homogenized for 15–20 seconds with an ice-cold glass pestle. The difference in diameter of pestle and tube is 0.6 mm. and the rate of revolution of the pestle 600 r.p.m. The homogenization medium contains 0.35 M sucrose, 0.035 M KHCO₃, 0.004 M MgCl₂, and 0.025 M KCl (medium A).

The homogenate is centrifuged at 15,000 g for 10 minutes at 0° C. The supernatant fluid containing the microsomes and the soluble cell

fraction is removed and diluted with 3 volumes of an unbuffered medium containing 0.90 M sucrose, 0.004 M MgCl₂, and 0.025 M KCl (medium B). The diluted extract is centrifuged at 105,000 g for 1 hour at 0.3° C. in a Spinco preparative ultracentrifuge. The clear supernatant fluid is pipetted out and can be saved for the preparation of the pH 5-precipitable amino acid-activating enzymes. The inner walls of the centrifuge tubes are carefully wiped dry with filter paper, and the microsome pellets are then transferred to a homogenizer tube. The combined pellets are homogenized with medium A to give a suspension containing about 20 mg. of protein per milliliter.

3. Other Microsomal Preparations

Submicroscopic particulate fractions have now been prepared from a wide variety of animal tissues. A chemical and morphological study of pancreatic microsomes has been made by Palade and Siekevitz (1956b), and the reader is referred to this paper and to an earlier work on liver microsomes (Palade and Siekevitz, 1956a) for a detailed consideration of the elements of microsome structure. There is an extensive literature on the morphology of the cellular structures which give rise to the microsome fraction (see Bernhard *et al.*, 1952, 1954; Weiss, 1953; Sjöstrand and Hanzon, 1954; Palade, 1952; Palade and Siekevitz, 1956a, b). The morphology cannot be considered here, but it is discussed in detail in Volume II (chapter by Palade and Porter).

Microsome fractions which are capable of amino acid incorporation can also be prepared from plant cells (Webster and Johnson, 1955; Webster, 1956).

In recent years a number of techniques have been devised for sub-fractionating microsome preparations, and some of these techniques have had an important role in studies of protein synthesis. The reader is referred to papers by Daly *et al.* (1955), Littlefield *et al.* (1955), Littlefield and Keller (1957), and Simkin and Work (1957).

D. Chloroplasts

1. The Isolation of Chloroplasts in Saline Solution

The following method is that of Arnon *et al.* (1956). It is important that all manipulations be carried out in the cold to avoid losses in activity of the chloroplasts.

Spinach leaves are washed with distilled water, shaken to remove excess liquid, and placed in a plastic bag in a refrigerator to maintain their turgidity. The midribs are removed from the fully turgid leaves and the leaf blades are weighed just prior to grinding. The leaf blades are then quickly sliced into pieces about 0.5 cm.² to facilitate grinding.

In a large ice-cold mortar, 100–200 gm. of sliced leaf blades are ground with 100–250 ml. of cold 0.35 M NaCl and cold sand to assist in the grinding. The slurry is squeezed through a double layer of cheesecloth and the green juice is centrifuged in the cold at 200 g for 1 minute. This removes sand, leaf debris, and whole cells. The green supernatant fluid (leaf homogenate) is carefully decanted and centrifuged in the cold for 7 minutes at 1000 g. This sediments the whole chloroplasts, leaving broken chloroplast fragments and smaller cellular particles in the supernatant fluid, which is decanted.

The sedimented whole chloroplasts are finally suspended in 25 ml. of 0.35 M NaCl solution.

Spinach chloroplasts prepared in this way are able to carry out photosynthetic phosphorylation, but not oxidative phosphorylation. For the latter purpose, the chloroplasts can be prepared in sucrose containing a sodium citrate-sorbitol-borate buffer (Ohmura, 1955; Arnon *et al.*, 1956).

Other chloroplast preparations are described by Granick (1938), Gorham (1955), and Chiba and Sugahara (1957).

Granules which are capable of photosynthetic phosphorylation can also be prepared from pigmented bacterial cells (see Frenkel, 1954; Newton and Kamen, 1957).

E. The Isolation of Components from Cell Nuclei

1. Isolated Chromosomes

Chromosomes have been prepared in quantity from a variety of animal tissues, including mammalian thymus, liver, kidney, and pancreas, and the erythrocytes of birds, fish, and amphibians (Mirsky and Ris, 1947, 1951). The isolation methods involve grinding in a colloid mill or high-speed homogenization in the blender under conditions which rupture the nucleus and release the chromosomes into an isotonic saline medium. Separation of the chromosomes from other cellular constituents is accomplished by straining through fine cloth and by differential centrifugation.

Although chromosomes prepared in this way have suffered some alteration in chemical composition due to the extraction of soluble materials, there is good evidence that they are essentially free of non-chromosomal material. Careful microscopic examination shows that the isolated elements have both the staining properties and the characteristic morphology of chromosomes (Mirsky and Ris, 1951; Ris and Mirsky, 1951; Polli, 1952; Denues, 1953). They contain from 26 to 40% DNA, the amount depending on the cells from which they are derived, and a high concentration of histones. Much of the chemical knowledge of the organization and composition of the chromosome has been derived from the

study of these isolated units (see Mirsky and Ris, 1947, 1951; Mirsky, 1947). Other preparations of chromatin threads have been described by Claude and Potter (1943).

2. Nucleoli

Nucleoli can be isolated from starfish oöcytes by differential centrifugation in sucrose solutions (Vincent, 1952, 1957). These nucleoli contain appreciable concentrations of ribonucleic acid and do not contain deoxyribonucleic acid. A study of P^{32} incorporation into the RNA of these nucleoli has recently been made (Vincent, 1957). The starfish oöcyte nucleolus has also been shown to contain very high concentrations of nucleoside phosphorylase and the DPN-synthesizing enzyme described by Kornberg (Baltus, 1954).

The fractionation of isolated liver nuclei to give particles described as "nucleoli" has been described by Litt *et al.* (1952). These intranuclear inclusion bodies were obtained from rat liver nuclei previously isolated in very dilute citric acid. The nuclei were broken in distilled water using sonic vibration, and the "nucleoli" were isolated by centrifugation in 1% gum arabic solutions. Nuclear fragments isolated in this way may include nucleoli, but they contain only 2-4% RNA as compared to 12-18% DNA (Dounce, 1955). This high DNA content suggests considerable chromosomal contamination, and it is doubtful whether such nuclear fragments should be properly considered to represent isolated nucleoli.

3. Mitotic Apparatus

Mazia and Dan have described the isolation and some properties of mitotic spindles prepared from the dividing cells of sea urchin eggs (Mazia and Dan, 1952; Mazia, 1957). The procedure involves fixation with 30% ethanol at -10°C . and dispersion of the cytoplasm with chemical agents (e.g., digitonin or ATP) that do not disrupt the mitotic apparatus. Differential centrifugation then gives the isolated spindles. It was pointed out by Mazia that the chromosomes tend to dissolve during the isolation.

F. Other Subcellular Particles

A variety of structural elements and storage granules which are localized in the cytoplasm of plant and animal cells can be isolated by special fractionation techniques. These include methods for preparing melanin granules from amphibian liver (Claude, 1948), from melanomas (Riley *et al.*, 1949), and from ox-eye choroid (Stein, 1955), glycogen granules from mammalian liver (Lazarow, 1942a, b; Claude, 1946), orange and red chromatophores from carrots (Straus, 1956), ferritin granules (Stern, 1939), myofibrils (Schick and Haas, 1949; Perry and Horne, 1952), hemosiderin granules from the spleen (Nasse, 1889; Behrens and

Asher, 1933), cell walls from the microorganism, *Chlorella* (Albertsson, 1956), and the granules of eosinophilic leucocytes (Behrens and Marti, 1954).

Elements of the Golgi apparatus have also been obtained by the fractionation of homogenates of epididymis tissue, employing a sucrose-NaCl density gradient (Schneider *et al.*, 1953; Schneider and Kuff, 1954).

In studies of protein synthesis in the pancreas, special interest attaches to the contents of the secretory granules, and methods for obtaining fractions which contain these granules have been described by Hokin (1955).

Lipoprotein complexes ("microsomes") containing alkaline phosphatase, xanthine oxidase, and other enzymes have been prepared from milk (Morton, 1954).

After the injection of protein into an animal, droplets rich in acid phosphatase activity can be isolated from cells of the kidney (Straus, 1954).

The isolation of epinephrine-containing granules from the adrenal medulla has been reported by Hillarp *et al.* (1953) and by Blaschko and Welch (1953). Although these granules resemble mitochondria in their high content of succinic dehydrogenase, most of the epinephrine and norepinephrine of the tissue is associated with this fraction, and these hormones are released on exposure of the granules to hypotonic conditions (Hillarp and Nilson, 1954).

G. Protoplasts from Bacteria and Yeasts

Subcellular components prepared from bacteria have come into increasing use for studies of integrated synthetic systems. Cells which either have been sonically disrupted (Gale and Folkes, 1954) or have had their cell walls removed (protoplasts) have been used. Lysis in a hypotonic medium has been employed to prepare protoplasts from *B. megatherium* (Weibull, 1953). The preparation of protoplasts from *E. coli* is possible when the cells are treated with lysozyme and then diluted into hypotonic media (Zinder and Arndt, 1956). In another technique, the bacterial cells are quantitatively transformed into spherical protoplasts when they are exposed to a medium containing penicillin, magnesium, and high concentrations of sucrose (Lederberg, 1956).

The preparation of protoplasts from yeast has been described by Eddy and Williamson (1957).

H. The Preparation of Whole-Cell Suspensions

A number of methods now exist for preparing suspensions of single cells from whole tissues, peritoneal exudates, bone marrow, and blood. Although this review is primarily concerned with the preparation of subcellular components, a brief summary of some of these whole-cell

preparations will be included because the intact cell is a valuable standard of comparison in many studies of isolated cell fractions.

1. Blood Cells

The separation of blood lymphocytes and granulocytes in a density gradient has been described by Ottesen (1954). Erythrocytes, polymorphonuclear leucocytes, and lymphocytes can also be fractionated on a density basis by centrifugation in 35% serum albumin solutions (Agranoff *et al.*, 1954). Flotation in albumin solutions has also been employed for the separation of free malignant cells (Fawcett *et al.*, 1950). Reticulocytes have been prepared in quantity from the blood of birds (London *et al.*, 1950; Allfrey and Mirsky, 1952a) and from rabbits (Koritz and Chantrenne, 1954). The isolated avian reticulocytes are able to synthesize both heme and globin *in vitro*. The separation of eosinophilic leucocytes and their granules from horse blood has been carried out in nonaqueous media (Behrens and Marti, 1954; Behrens and Taubert, 1952a).

2. Cells in Peritoneal Exudates

Nucleated cells can be separated from the red cells in peritoneal, pleural, and pericardial fluids by centrifugation in 30% serum albumin solutions (McGrew, 1954). A preparation of macrophages from peritoneal exudates has been described by Ehrlich *et al.* (1946) and by Roberts *et al.* (1957). The latter authors also obtained a separation of lymphocytes and heterophiles. The concentration of histiocytes in peritoneal exudates after dye injection and a method for their separation was reported by Hamazaki and Watanabe (1929). Peritoneal mast cells have been prepared by centrifugation in a density gradient (Glick *et al.*, 1956).

Cells from the Ehrlich mouse ascites tumor are widely used in biochemical studies; one method for the separation of such cells as a first step in cell fractionation is given by Littlefield and Keller (1957).

3. Cell Suspensions from Bone Marrow

The separation of different cell types, including macrophages, polymorphonuclear leucocytes, and lymphocytes, from bone marrow and other tissues was described by Fastier (1948). Cell suspensions from bone marrow have also been studied by Ficq *et al.* (1954), Moeschlin and Demiral (1952), and by Porter (1957).

4. Single Cells from Solid Tissues

There are a number of techniques for preparing single cells in suspension from solid tissues such as liver, kidney, lymph nodes, etc. Some of these are mechanical methods which involve mincing or teasing-out

of the tissue, subsequently forcing it through gauze or fine screening (Schrek, 1944; Kaltenbach, 1954). Cells prepared in this way may sustain considerable damage which would preclude their use in many biological systems, but there are cases in which cells so prepared have been of value in studies of their function. Thus viable lymphoma cells can be prepared by screening techniques and used in transmission experiments (Kidd and Todd, 1954). Free cells can be teased out of lymph nodes in a Tyrode's solution containing gelatin and separated by passage through 40- and 80-mesh screens (Harris and Harris, 1954). A similar isolation of cell suspensions from lymph nodes, employing a PVP-macrose medium has been described by Roberts and Dixon (1955). Such cells have been used in studies of antibody formation.

Gentle homogenization is sometimes employed to disrupt the organization of solid tissues and release free cells into suspension. Rat liver, for example, has been dispersed by low-speed homogenization in a mixed salt solution containing 5% glucose (Harrison, 1953a). Kupffer cells were separated from the parietal cells in such suspensions by Kamahora and Kenishi (1954). Perfusion with calcium-binding agents (e.g., citrate or Versene) followed by homogenization in a calcium-free Locke's solution was recommended by Anderson (1953); in this case the softening of the intercellular cement substance in the absence of calcium permitted the isolation of large numbers of liver cells. Similar techniques have been followed by Laws and Strickland (1956) and by Kalant and Young (1957). Again it should be pointed out that even gentle homogenization may destroy or inactivate many of the cells in these preparations.

In other procedures cells are separated from solid tissues with the aid of enzymes. For example, cell suspensions from chick embryonic organs have been prepared by trypsin digestion (Moscona, 1952). This technique has found wide application, and trypsin has been used simply or in combinations with other enzymes, such as hyaluronidase (St. Amand and Tipton, 1954).

A novel technique for the separation of reticuloendothelial cells allows them to first phagocytize small iron granules. The cells can then be separated with the aid of a magnetic field (Beard and Rous, 1934; St. George *et al.*, 1954).

I. Chromatographic Isolations and Other Special Techniques

Although nearly all of the common cell fractionation procedures are based on the differences in sedimentation rates of particles in suspension, there are a number of isolation methods which exploit differences in surface charge and adsorptive properties. Chromatographic separations have been successfully applied in many cases, among them the isolation of the Rous sarcoma virus from tumor tissue (Riley, 1948) and pigment granules from mouse melanomas (Riley *et al.*, 1949). The trans-

missible factor of fowl leukemia has also been purified by chromatography (Pontén, 1954). Bacterial suspensions can be fractionated by chromatographic techniques to give populations of cells with uniform surface properties (Maruyama and Yamagata, 1955). Albertsson (1956) has described the separation of starch grains from the cell walls of disintegrated *Chlorella* by passage through columns of calcium phosphate. Blood platelets can be adsorbed on Dowex 50 and then eluted with isotonic saline (Freeman, 1951).

Chromatography on cellulose ion-exchangers (such as ECTEOLA-SF), has been applied with considerable success to the purification of bacteriophages (Taussig and Creaser, 1957), tobacco mosaic virus (Commoner *et al.*, 1956) and mammalian viruses and rickettsiae (Hoyer *et al.*, 1958).

A partition and partial separation of cell fragments of *Chlorella* has been observed in a two-phase system containing water, a pH 7 potassium phosphate buffer, and Carbowax 4000. A separation of spinach chloroplasts and starch grains could also be achieved using this partition system (Albertsson, 1956).

A very different technique employed by Behrens begins by emulsifying the tissue in paraffin oil. Homogenization under these conditions separates the cell components into discrete droplets which can then be fractionated on the basis of their size or density by centrifugation techniques. Using this emulsification procedure, Behrens prepared nuclei from mouse liver, *Scyllium* erythrocytes, and octopus hepatopancreas tissue (Behrens, 1954). Kopac (1955) has recommended the use of fluorocarbons for this form of separation.

V. STANDARDIZATION PROCEDURES

The isolation of a subcellular component always requires some form of standardization to establish the purity of the preparation, and it is often desirable as well to test the degree of retention of its structural or functional integrity. Such tests are especially necessary if the isolation involves recourse to new or untried techniques. The present discussion is concerned with tests for purity and with the methods which can be employed to establish whether the isolated organelles resemble in their structure and composition the corresponding formed elements of the cell.

A. Tests for Purity

1. Microscopic Examination

In following the progress of cell fractionations, frequent examination under the microscope is an indispensable guide to the investigator. At the outset of most isolations the light microscope is the only measure of

the success of the method: it indicates how many of the cells are broken by homogenization, whether nuclei, chloroplasts, sarcosomes, mitochondria, etc., have been released into the medium, and whether these larger structures have been damaged, broken, clumped, or enmeshed in other cellular debris. This information can then be used to guide subsequent steps in the isolation. Thus, homogenization or grinding techniques may be modified to permit more complete cell breakage; the concentration of the suspension may be lowered to minimize entrapment of particulates, and obvious contaminants (e.g., fiber, whole cells, red cells) can be recognized and removed early in the procedure.

As the isolation progresses the appearance of the desired fraction under the microscope becomes the first criterion of its purity. For larger subcellular components, the light microscope plays a major role in this evaluation; this is especially true in nuclear isolations. Supplemented by the use of phase contrast or by the application of stains and counter-stains, ordinary microscopic examination is a sensitive indicator of whole-cell contamination and of cross contamination of the nuclei by other particulates, connective tissue, or cytoplasmic debris.

In aqueous isolation procedures crystal violet staining provides a rapid and convenient method for following the purification. A drop of the nuclear suspension is placed on a microscope slide beside an equal drop of a 0.1% solution of crystal violet dissolved in the isolation medium. By lowering the cover slip so that it touches the drop of suspension before making contact with the dye solution, one obtains a field containing unstained nuclei at one side and heavily stained material in direct contact with the dye solution at the other. The progress of the dye as it diffuses into the nuclear suspension can be followed under the microscope. This is sometimes useful in disclosing the presence of undamaged whole cells (which are slow in taking up the dye), and it frequently reveals differences in staining properties which facilitate the recognition of other contaminants.

In the nonaqueous isolation procedures a drop of the tissue or nuclear suspension is spread on a microscope slide and dried at room temperature. (Drying is very rapid with the volatile solvents usually employed in such isolations.) A drop of the dye solution (1 mg. crystal violet per milliliter H₂O) is spread over the dried sample on the slide, a cover slip is applied, and the stained preparation is examined and appraised for its content of free nuclei. Under these conditions the nuclei stain an intense blue relative to a light blue cytoplasm and colorless fiber; the difference in intensity of staining together with the characteristic nuclear morphology makes identification easy. As a rule there is no difficulty in distinguishing between free nuclei and nuclei with attached cytoplasmic tabs.

When examining the final stages of a nuclear preparation for cyto-

plasmic contamination, mixed stains offer many advantages. Hematoxylin-alcoholic eosin, or the acetoorcein-Fast Green mixture of Kurnick and Ris (1948) have been employed. The former is a particularly effective stain for avian erythrocyte nuclei (see Fig. 11) but is not as satisfactory for other tissues, such as thymus, in which nuclear-cytoplasmic contrast is rather low. (The absence of contrast may be related to the treatment with organic solvents in the nonaqueous isolation procedures.) The erythrocyte preparations shown in Fig. 11 are stained with Harris' hematoxylin, followed by 0.5% alcoholic eosin, and mounted in balsam. Under these conditions nuclei stain a deep purple; the cytoplasm is bright red.

The acetoorcein-Fast Green mixture stains chromatin red to violet; the cytoplasm is light green, and protein within the nucleus is also green. The green of the nucleus, however, is blended with the contrasting stain for chromatin; cytoplasmic green is light and more uniform. Together with the characteristic nuclear morphology this stain makes it relatively simple to distinguish between a free nucleus and a nucleus with an attached cytoplasmic tab. Acetoorcein-Fast Green staining has been found effective in many isolations (Allfrey *et al.*, 1952) and is readily carried out. A drop of the dye mixture is spread over the nuclei, a cover slip is applied, and, after 2-3 minutes, staining is complete.

With the three staining reactions described above and using high magnifications (900-1400 \times), the detection of cytoplasmic and other contaminants of nuclear preparations is usually simple and direct. In the nonaqueous isolation procedures, contamination usually consists of (1) small tabs attached to relatively large nuclei, (2) fiber, probably originating in connective tissue, blood vessel walls, etc., and (3) a relatively dense "matrix" material which binds the nuclei together in small clumps and thus avoids the specific gravity separation. The latter impurity is sometimes the result of nuclear breakdown caused by overgrinding of the tissue suspension. (This can be demonstrated by Feulgen staining.) In other cases, however, (e.g., ground suspensions of intestinal mucosa) the "matrix" contamination appears to be nonnuclear and cannot be removed by specific gravity separations alone. Although chemical and enzymatic studies of intestinal mucosa nuclei (described below) indicated that contamination does not exceed 4-5%, such contaminants are detected with ease under the microscope. Similar results with other nuclear preparations establish the high sensitivity of the staining reactions as a test and measure of nuclear purity. This sensitivity is not surprising, however, if one considers that hundreds of nuclei are sometimes examined in a single field and contrasting stains for cytoplasm emphasize the impurities.

The use of stains as an adjunct in the microscopic examination of

isolated cell components is not limited to nuclei. In mitochondrial isolations the course of the purification can be followed by staining with Janus Green B [a dye which also selectively stains mitochondria intracellularly (Michaelis, 1900)]. Here again the dye can be dissolved in the isolation medium. Although mitochondria prepared in sucrose solutions stain with Janus Green B, those prepared from water or saline homogenates of the liver do not (Hogeboom *et al.*, 1948). The "sucrose" mitochondria are also deeply stained after osmium tetroxide fixation by Aniline Acid Fuchsin, by hematoxylin, and by basic dyes such as safranine and crystal violet. Mitochondria in sucrose solutions can be distinguished from secretory granules and lipid droplets by their failure to stain with solutions of neutral red (Hogeboom *et al.*, 1948).

In the isolation of chloroplasts, the green pigment itself serves as a useful marker. Safranine staining after osmic acid fixation has also been used (Arnon *et al.*, 1956).

The use of phase contrast also finds wide application in the microscopic examination of cellular components (Anderson and Wilbur, 1952; Novikoff *et al.*, 1953). Its chief disadvantage is the production of "halos" around particulates, which tend to obscure their peripheries. The change of phase which is observed when particles are suspended in protein solutions of increasing concentration has been suggested as a means of distinguishing between whole cells and free nuclei (Barer *et al.*, 1956). In isolated thymocyte nuclei, however, differences in permeability among the nuclei make this (and differences in stainability) a less reliable guide or test for whole-cell contamination than examination under the electron microscope.

For studies of smaller particles, the light microscope, phase contrast, and staining reactions have a more limited role. Light microscopy can be used to demonstrate the absence of other larger contaminants, such as whole cells, nuclei, fiber, etc., but tests for purity and the characterization of the isolated subcellular component must be carried out in other ways. In recent years, increasing use has been made of the electron microscope for this purpose. Thus, electron microscopy has been used to characterize the cytoplasmic particulates obtained from rat liver by differential centrifugation in 0.88 M sucrose (Kuff *et al.*, 1956) and in 0.25 M sucrose containing 7.3% PVP (Novikoff, 1957). A detailed electron microscope study of the pancreatic microsome fraction illustrates the great power and utility of the method, not only as a check on purity, but as one of the main means of identifying and characterizing the elements of the microsome fraction (Palade and Siekevitz, 1956b).

As mentioned above, electron microscopy (together with chemical and enzymatic tests) was a necessary supplementary test to show the

absence of whole-cell contamination in preparations of isolated thymus lymphocyte nuclei (Allfrey *et al.*, 1955; Allfrey and Mirsky, 1955).

Although morphological tests for purity constitute an essential element of isolation procedure, the absence of visible contamination is not, in itself, a conclusive proof of purity. Other tests are required to establish the presence or absence of adsorbed or occluded materials. Even when a subcellular fraction does show visible contamination, the microscope does not usually allow a reliable quantitative measure of its concentration. Such quantitative estimates of the purity of a preparation must be obtained in other ways, which depend on the chemical, enzymatic, or immunological properties of the isolated component or suspected contaminants.

2. *Chemical Tests for Purity*

Chemical analyses can frequently offer the most direct evidence for the presence or absence of many forms of contamination. In the isolation of cytoplasmic particulates, for example, one of the simplest tests for contamination by nuclei or by whole cells, is an analysis for deoxyribonucleic acid (DNA). This substance, an essential element of chromosome structure, is characteristically localized in the nucleus of nearly all cell types. (It should be pointed out that the specific nuclear localization of DNA has been demonstrated by many independent methods, such as Feulgen staining and ultraviolet microspectrophotometry of tissue sections, as well as by chemical analysis of nuclei and chromosomes isolated in different media.) The detection of deoxyribonucleic acid has most frequently been carried out using the diphenylamine reaction of Dische (1930), but higher sensitivities are possible employing the more recent procedure introduced by Webb and Levy (1955).

Although a negative DNA analysis is good evidence for the absence of contaminating chromatin in cytoplasmic fractions, it does not constitute a proof that all nuclear contamination is absent. It has been shown repeatedly that the aqueous isolation procedures are usually attended by large-scale losses of soluble nuclear components (e.g., Allfrey *et al.*, 1952; Dounce *et al.*, 1950). These soluble proteins, nucleotides, etc., may contaminate preparations of cytoplasmic particulates, and they are certainly found in the "supernate" which remains after sedimentation of the microsome fraction.

In the isolation of nuclei, a DNA analysis is one of the first tests for the purity of the preparation, low figures indicating the likelihood of cytoplasmic contamination. As purification progresses, the DNA content of the nuclear fraction will exceed that of the whole tissue by a factor

which depends on the relative size of the nucleus and the cell. In calf kidney, for example, the nucleus comprises 20% of the cell mass, and the DNA content of the final nuclear preparation will be five times that of the whole tissue homogenate (Allfrey *et al.*, 1952). A supplementary analysis for ribonucleic acid (RNA) can strengthen the evidence for purity of the nuclei. Since the ribonucleic acid content of the nucleus is usually only about one-tenth of its DNA content, a high concentration of RNA in the nuclear fraction is good evidence for the presence of cytoplasm, particularly of elements of the microsomal fraction. This conclusion is based on the high ribonucleic acid content of isolated microsomes, and on the cytochemical evidence (basophilia and ultraviolet absorption) for localization of ribonucleic acid in the ergastoplasm (or endoplasmic reticulum), structures which yield the microsome fraction. Similarly, because pure preparations of isolated mitochondria have relatively low RNA contents, RNA analyses can be used to indicate the presence of appreciable amounts of microsomal contamination. The most common procedure for ribonucleic acid estimation employs the orcinol reaction (Schneider, 1945), but a more specific reagent which does not react with DNA has recently been described by Webb (1956).

A number of other tests for the purity of nuclei are possible, the nature of the test varying with the source of the nuclei. There are many cases in which a protein which is present in high concentration in the cytoplasm does not occur in detectable amounts in the nucleus. Beef heart nuclei isolated by the nonaqueous method, for example, do not contain myoglobin, since their total iron content (0.0098%) can be completely accounted for as nonheme iron (Allfrey *et al.*, 1952). Yet myoglobin is one of the characteristic soluble proteins of beef heart tissue. Its absence from the isolated nuclei can be taken as evidence that a general migration of cytoplasmic components into the nucleus cannot occur when the tissue is frozen prior to isolating the nuclei in nonaqueous media.

The absence of myoglobin in beef heart nuclei is to be contrasted with the occurrence of hemoglobin in appreciable quantities in nuclei isolated from avian erythrocytes by similar procedures. To test the possibility that this nuclear hemoglobin represented cytoplasmic contamination which was ground into the nuclei during the milling procedure (since it was not evident in microscopic examination), calf thymus nuclei were prepared from a mixed suspension of thymus tissue and avian erythrocyte debris. The suspension was ground and fractionated in the usual way for the isolation of calf thymus nuclei. The isolated nuclei were white, in contrast to the dark brown coloration of erythrocyte nuclei, and their normal iron content (0.0066%) indicated that no contamination by extraneous hemoglobin had occurred.

It has already been pointed out that the nature and the extent of cross contamination in cell fractionations depends to a very large extent on the nature of the suspension medium. Changes in the distribution of cytochrome c between mitochondrial and microsome fractions can be observed depending on whether the fractionation is carried out in water or in isotonic saline (Schneider *et al.*, 1948). When radioactive cytochrome c was added to liver prior to homogenization and separation of the particulates, the extent of adsorptive contamination of the microsome fraction was found to vary greatly depending on whether isotonic sucrose, saline, or water was used as the isolation medium (Beinert, 1951).

The recognition and estimation of contaminants is greatly simplified when they are pigmented. Thus a visual examination of liver nuclei prepared in 2.2 M sucrose solution shows some pigmentation which has been tentatively identified as hemoglobin (based on the changes in its absorption spectrum when reduced and when combined with CO (Bellin and Hopkins, 1957). Dounce (1955) has reported that liver nuclei prepared in 70% glycerol solution are also contaminated by hemoglobin.

Chemical tests for the purity of isolated fractions can be supplemented in many ways by using immunological, enzymatic, or tracer techniques.

3. Immunological Tests for Purity

One example of an immunological test for the purity of an isolated subcellular component is the demonstration that calf thymus nuclei isolated in a nonaqueous medium were free of contaminating serum proteins. To test for such contaminants, antisera were prepared (in the rabbit) which would precipitate bovine serum albumin and bovine serum globulin. Saline extracts of the isolated thymus nuclei, and of the whole tissue, were then tested at different dilutions for their precipitin titers against these antisera. A comparison of the precipitin titers showed that the isolated nuclei contained less than 10% of the serum protein concentration found in the tissue. Since, in calf thymus, the nuclei comprise about 60% of the tissue, it follows that the serum protein content of the isolated nuclei is less than 5% of the concentration in the non-nuclear portion of the tissue.

The same test was performed "in reverse" by preparing an antiserum against calf thymus nuclear extracts. This antinuclear serum was then tested for its precipitin titer against bovine serum globulin and serum albumin. In this case no precipitate was obtained against serum albumin and only exceedingly faint reactions were obtained with serum globulin (Allfrey *et al.*, 1952).

The absence of serum protein is one index of the purity of isolated

subcellular fractions. Other suspected contaminants which are antigenic can be tested for in similar ways. But because the immunological tests are often relatively difficult or time-consuming, they play a secondary role in the study of isolated cell components. A far more common procedure is to test for the presence or absence of enzymes of known intracellular localization as described below.

4. Enzymatic Tests for Purity

The way in which enzyme assays can be used as a criterion of purity can be illustrated by reference to studies on the composition of mammalian cell nuclei isolated in nonaqueous media (Allfrey *et al.*, 1952). In this work a number of enzyme activities known to occur in characteristically high concentrations in different tissues were sought for in the nuclei prepared from those tissues. The tests compared the enzyme activity (in units per milligram) of the isolated nuclei with the activity of a "control" tissue preparation; i.e., tissue which had been lyophilized, ground, and extracted with the solvents used in preparing the nuclei. The results of such comparisons were particularly striking in demonstrating the nearly complete absence of many cytoplasmic enzymes from the nuclear fraction. For example: (1) There was no uricase activity detectable in nuclei prepared from calf liver and kidney, and equine liver nuclei showed only 2-5% of the uricase activity of the tissue. (2) There was no catalase activity in nuclei isolated from calf kidney or chicken kidney. (3) The arginase activity of the latter nuclei was only about 6% of that observed in the tissue control. (4) Beef pancreas nuclei had only 7-8% of the amylase and lipase concentration of the tissue and horse pancreas nuclei had even less (1.4-2.5%). These latter figures stand in sharp contrast to the results obtained in beef pancreas nuclei isolated in citric acid, in which case nuclear lipase activity reached 50% of the tissue concentration (Dounce, 1950b). The latter figure is probably the result of selective nuclear adsorption of the enzyme from the aqueous tissue homogenate. Similar evidence for the adsorptive contamination of pancreas nuclei prepared in strong sucrose solutions stems from the observation (Lang *et al.*, 1953) that such nuclei contain a higher concentration of trypsin than does the cytoplasm! It has been found that pancreas nuclei prepared in nonaqueous media contain only a fraction of the tryptic activity of the cytoplasm (Mirsky, unpublished observations).

The foregoing examples illustrate the application of enzyme activity measurements in demonstrating the presence or absence of cytoplasmic contaminants in nuclear fractions. A great many measurements on nuclei isolated by the nonaqueous procedures have supported the chemical and

immunological evidence that such nuclei were free of serious cytoplasmic contamination. Furthermore, since the enzymes considered were in some cases soluble (e.g., amylase and catalase) and in other instances insoluble (e.g., uricase), their absence from the nucleus supports the general conclusion that enzymes are not transported from cytoplasm to nucleus during the isolation in nonaqueous media. It follows that the Behrens procedure is an effective and reliable method of approaching the problem of intracellular enzyme distribution. (The rather unlikely but conceivable possibility should be mentioned that the absence of certain enzyme activities in isolated nuclei may represent a selective destruction or selective inhibition of these enzymes when they occur in the nucleus; such inactivation or inhibition need not occur in the cytoplasm of the "control tissue" preparation. This possibility, however remote, cannot be disproved simply by measuring relative enzyme activities, but requires a direct chemical test. For example, the absence of myoglobin in isolated heart muscle nuclei could be demonstrated by direct analysis for iron and heme pigments.)

Enzyme activity measurements can also be applied in testing the purity of isolated cytoplasmic particulates. It has been observed, for example, that practically all of the cytochrome oxidase and succinic dehydrogenase of the liver cell are confined to the mitochondria (Schneider and Hogeboom, 1950a; Hogeboom *et al.*, 1952). The presence of these oxidizing enzymes in other fractions, e.g., the microsomes, is thus good evidence for contamination by mitochondria. Conversely, glucose-6-phosphatase activity is largely confined to the microsomal fraction (Hers *et al.*, 1951), and its presence in isolated mitochondria is an indication of contamination by microsomes. Similarly, the observation that liver nuclei isolated in sucrose solutions contain nearly all of the DPN-synthesizing enzyme of the cell (Hogeboom and Schneider, 1952a) permits another test for nuclear contamination of mitochondria or microsome fractions.

The number of such tests that can be applied to isolated nuclei, mitochondria, and microsomes is now very considerable, and it is not the purpose of this review to enumerate all the possibilities. Some indication of the wide range of activities which have been observed in fractions prepared by differential centrifugation is given in the review by de Duve and Berthet (1954).

Because isolated cell fractions are now so widely used for studies of intracellular enzyme distribution and because the results obtained are so often in conflict, a discussion of the methods used and their limitations comprises an important topic within the scope of a review on cell fractionation procedures.

B. Problems Arising in the Study of Intracellular Enzyme Distribution

Few problems in cell physiology possess more inherent interest than the question of the intracellular localization of enzymes, their substrates, cofactors, activators, and inhibitors. A direct approach to this problem is certainly to be had in the analysis of the isolated subcellular components, and this approach has found wide and often uncritical acceptance. Its widest application has been the study of enzyme distributions in fractions prepared from sucrose homogenates of mammalian tissues.

In the common "four-step" sucrose fractionation schemes, differential centrifugation of the tissue homogenate yields (1) a nuclear fraction (usually heavily contaminated by whole cells and adhering cytoplasmic debris), (2) a large-granule fraction containing the mitochondria [these are often contaminated by lysosomes (de Duve *et al.*, 1955)], (3) a small-granule or microsome fraction (which, because of its high ribonucleic acid content tends to adsorb soluble proteins, such as cytochrome c), and (4) a final supernate which contains all the soluble and non-sedimentable components of the homogenized tissue. In practice, the enzyme activities observed in these fractions are usually compared with that of the original homogenate, which is taken as representative of the whole tissue. When the comparison is carried out in a quantitative manner it is also customary to introduce a final check on the assay in the form of a reconstituted homogenate, made up by recombining the four isolated fractions in suitable proportions. This method allows the construction of a "balance sheet" which shows whether the total enzyme activity of the tissue is recovered as the sum of the activities of the separate fractions. This "balance sheet" computation of enzyme distribution has advantages in detecting contamination and in discovering localized inhibition of enzyme activity.

The results of enzyme distribution studies carried out on cell fractions prepared in sucrose solutions are sometimes clear and unequivocal. This is especially true when all the enzyme activity finally appears in one particulate fraction. The localization of cytochrome oxidase and succinic dehydrogenase in mitochondria, of a DPN-synthesizing enzyme in nuclei, and of glucose-6-phosphatase in microsomes all illustrate the positive achievements of the method.

The results are far less satisfactory and hardly convincing when they show an enzyme to be localized in the supernatant fraction, or when a soluble enzyme is reported as missing from the nuclear or microsomal fraction. In these cases all the obvious defects of working in an aqueous medium are compounded. Any soluble component, leached out of nuclei, damaged mitochondria, lysosomes, or other cytoplasmic particu-

lates will appear in the supernate; its presence there does not constitute any proof that it was localized in the soluble phase of the cell. The extraction of water-soluble components from the nuclei of cells homogenized in sucrose solutions has been observed repeatedly. Nucleoside phosphorylase, for example, appears in the supernate after differential centrifugation of liver homogenates (Schneider and Hogebloom, 1952) and is absent from the nuclear fraction. Yet the analysis of many nuclei isolated in nonaqueous media shows the enzyme to be present in the nuclei at concentrations equal to or greater than those observed in the cytoplasm (Stern *et al.*, 1952). The loss of proteins and of nucleotides from nuclei isolated in sucrose solutions has already been mentioned, but it should be emphasized that the proven redistribution of soluble enzymes constitutes a major disadvantage of aqueous cell fractionations and can often lead to a completely erroneous picture of intracellular enzyme distributions.

The redistribution of soluble enzymes is only one of the experimental difficulties which attend the four-step fractionation scheme. Other difficulties facing the analyst are equally formidable. The nuclear fraction is usually grossly contaminated by whole cells and by cytoplasmic debris. [The nuclei can be further purified by "layering" techniques which eliminate much of the contamination (Wilbur and Anderson, 1951; Hogebloom *et al.*, 1952)]. The mitochondrial fraction, as usually prepared, may contain other large granules, the lysosomes, which are rich in hydrolytic enzymes (including cathepsins, nucleases, acid phosphatase, and β -glucuronidase) (de Duve *et al.*, 1955). There is reason to believe that 20–30% of these enzyme-rich granules are damaged in a complete fractionation, and that they release their enzymes into the suspension medium, where they remain or become adsorbed onto other particulate fractions (de Duve and Berthet, 1954). This artificial solubilization and transfer of particulate-bound enzymes further complicates the study of their distribution. The mitochondrial fraction may also contain a variable amount of microsomal contamination; this is indicated by a high RNA analysis. The microsomal fraction itself, because of its high ribonucleic acid content tends to attract and adsorb oppositely charged components of the suspension. The adsorption of cytochrome c by microsomes has already been cited.

The question of the purity of the separate fractions is further complicated by the presence of cell membranes, the ghosts of damaged particles, and connective tissue or blood vessel debris. Since even trace amounts of contamination may considerably influence an enzyme assay (see below) extreme caution must be used in interpreting the results.

An obvious limitation of the four-step fractionation scheme is im-

plicit in the method. There are, of course, more than two types of cytoplasmic granule. Other structures, such as lysosomes, secretory granules, centrosomes, the Golgi apparatus, etc., follow the fractionation in one way or another and contribute to the final pattern of enzyme distribution. The microsomal fraction has been further subdivided in ways significant to both its function and morphology (Keller and Zamecnik, 1956; Palade and Siekevitz, 1956b; Chauveau, 1954), and the subfractionation of mitochondria to give particles of differing enzymatic constitution has been repeatedly described (e.g., Novikoff *et al.*, 1953; Kuff and Schneider, 1954). There appears to be both a multiplicity and a heterogeneity of granules in each of the fractions.

Apart from these questions of the nature and purity of the isolated fraction, there are additional difficulties which are encountered when one attempts to estimate enzyme activities in such extremely complex systems. The artifacts associated with enzyme assays have been treated in other reviews (e.g., de Duve and Berthet, 1954) and need not be given in detail here. They include changes in enzyme activity due to the breaking and partition of the cell. This may give rise to autolytic changes, to enzyme inactivation or denaturation, and may result in a separation of an enzyme from related coenzymes, activators, and inhibitors. The assay method itself may be misleading when enzymes occur in the zymogen form or when they exist in enclosed structures which prevent access of the substrate. Furthermore, the substrate may be diverted into other pathways by competing enzyme systems, or may not be sufficiently specific. There are examples in the literature which illustrate the occurrence of each of the artifacts mentioned (see de Duve and Berthet, 1954).

In view of the experimental difficulties which have been described in the preceding paragraphs it is hardly surprising that many results on the intracellular distribution of enzymes are treated with skepticism. Except in those cases where all or a very large part of the total tissue activity is recovered in a particular fraction, or when the enzyme studied is shown to be an insoluble component of larger structures, quantitative estimates based on four-step fractionations in sucrose are hardly convincing.

Fortunately, other experimental approaches frequently permit a more definite answer to the question of the relationship between the function of an intercellular structure and its enzymatic composition. One approach to this problem consists in the analysis of single fractions isolated under conditions which permit an estimate of their nature and purity. In this way many of the artifacts associated with complete cell fractionations may be avoided. This approach can be best illustrated by experiments on the enzymatic composition of isolated nuclei.

Nuclei have now been isolated from a variety of animal and plant tissues by the Behrens nonaqueous isolation procedure (Behrens, 1938) or its modifications (e.g., Allfrey *et al.*, 1952). As was previously pointed out, isolations of this sort preclude a transfer of water-soluble components from the cytoplasm to the nucleus, or vice versa, and give the most reliable preparations for an analysis of nucleic acids, total proteins, or nucleotide content. The great utility of the nonaqueous isolation method also extends to studies of the enzymatic constitution of the isolated nucleus. Its use for this purpose is, however, limited by the fact that only some of the enzymes of the cell can resist lyophilization and exposure to organic solvents. The validity of the results obtained then depends on the demonstration that the enzyme under investigation is not inactivated during the course of the isolation. One way in which this can be done is to compare the activity of the fresh tissue (in units of activity per unit dry weight) with that of the tissue after lyophilization, after grinding, and after extraction with the solvents used in the isolation of the nuclei. Such comparisons indicate which enzymes resist the treatment, and in cases where partial inactivation is observed, it may be possible to correct for the loss of activity during preparation of the nuclei. In fact, many enzymes have been found to retain their activities despite lyophilization and prolonged exposure to petroleum ether, cyclohexane, and carbon tetrachloride. The study of these enzymes in nuclear fractions which have been shown to be free of cytoplasmic contamination by chemical, immunological, and other enzymatic tests leads to many interesting conclusions about nuclear composition and nuclear function. Thus the differentiation of the nucleus and its capacity for change in altered physiological conditions could be demonstrated by measurements of its activities in a variety of enzymatic systems (Stern *et al.*, 1952).

Enzyme activity measurements on a single fraction, such as isolated nuclei, have sometimes drawn criticism because the yield of the fraction may be low, especially when all the emphasis is placed on purity and high yields are a secondary consideration. Low yields introduce the possibility that the methods of isolation select a nonrepresentative fraction of the nuclei. This is indeed a possibility, but a number of observations of nonaqueous nuclei show that they comprise a better sample than may have been supposed: (1) This is seen at once when nuclei isolated from liver are examined microscopically, for a fair representation of the various morphological types are seen. (2) Two nuclear fractions, differing in specific gravity, have been prepared from liver, and yet enzyme assays gave the same results for both fractions. The same enzyme activities were also found for two thymus nuclear fractions of different specific gravities (Allfrey *et al.*, 1952). (3) Thymus nuclei

isolated in nonaqueous media and by means of sucrose solutions have quite similar enzyme compositions (Stern and Mirsky, 1953). In these cases, at least, the selection of a nonrepresentative nuclear sample does not seem to have occurred.

Another criticism which has been directed against enzyme studies of isolated single fractions is based on the contention that such assays have no significance until all the fractions of the tissue have been analyzed and a "balance sheet" shows that the sum of the activity of all the fractions equals the activity of the whole tissue (Schneider and Hogeboom, 1951). This contention has generated considerable controversy, largely because of the differences in emphasis and interests of different investigators (see Stern *et al.*, 1952; Dounce, 1955; Green, 1952; Hogeboom *et al.*, 1953). The value of a "balance sheet" in detecting contamination and localized enzyme inhibition has already been mentioned in connection with the "four-step" fractionation procedures in sucrose. The difficulties arise when the "balance sheet" calculation becomes the basis for the notion that the activity of a given fraction is not significant unless it represents a large part of the total enzyme activity of the tissue. According to an extreme view, the occurrence of an enzyme in the liver nucleus at a concentration equal to that found in the cytoplasm would have no significance because the nucleus is only about 10% of the cell (Hogeboom *et al.*, 1953). This viewpoint is of course erroneous because in biological systems the presence of a component is itself significant. This is especially true for the nucleus, where genetic evidence shows its predominating influence on cellular organization and function. It is inconceivable that high enzyme activities within the nucleus have no significance because the enzyme also occurs in the cytoplasm. On the contrary, where the absence of contamination has been established by independent tests, the presence of any enzyme activity in isolated nuclei must be regarded as significant to the function of the nucleus and may serve as a clue to the nature of the interaction between the nucleus and the cytoplasm which it controls.

There is now little reason to doubt that an experimental approach that measures the enzyme activities of isolated nuclei or of other purified single fractions has an important role in research on the structure and function of the cell. Doubts may arise in connection with the purity of the fraction or changes in its composition during isolation, but these are questions which are often subject to direct test.

In summary, the study of intracellular enzyme distribution based on the analysis of isolated cell fractions can be of value in many ways. But it is the responsibility of the investigator using this type of analysis to consider the many possibilities of cross contamination and to present

good evidence for the purity of his preparations and for the absence of artifacts associated with their isolation.

C. Tests for Retention of Function

The standardization techniques so far considered (microscopic examination, chemical, immunological, and enzymatic tests for suspected contaminants) are all measures of the purity of isolated subcellular components. Another important aspect of standardization procedures is the demonstration that the isolated organelle has maintained its structural and functional integrity.

There are many instances in the literature where the retention of function has been demonstrated in a clear and unequivocal manner. Nuclei, mitochondria, chloroplasts, and microsomes have all been found to retain a capacity for complex synthetic reactions. To illustrate the methods used and some of the variables which must be considered, reference will again be made to studies of isolated nuclei.

It has already been pointed out that nuclei isolated from the thymus in 0.25 M sucrose solutions containing 0.002–0.003 M CaCl₂ compare favorably with the "standard" thymus nuclei prepared in nonaqueous media. The comparison extends even to the observation that the thymus "sucrose" nuclei have retained their complement of low molecular weight nucleotides (Osawa *et al.*, 1957). It was found that when these nuclei are rapidly isolated and then incubated in the sucrose medium, there is a rapid conversion of adenosine monophosphate (AMP) to adenosine triphosphate. The conversion to the energy-rich triphosphate form takes place only in the presence of oxygen; in this respect it resembles oxidative phosphorylation in mitochondria. However, the aerobic synthesis of ATP in the nucleus was found to be insensitive to a number of substances, such as carbon monoxide or Janus Green B, which are known to block oxidative phosphorylation in isolated mitochondria (Osawa *et al.*, 1957).

A second test for function in isolated nuclei reveals some interesting information about the effects of the isolation medium on nuclear activity. In these experiments the capacity of the nucleus to incorporate radioactive amino acids into its proteins was used as a measure of its function under different conditions of incubation. It was first observed that amino acid uptake into the proteins of the nucleus was negligible in the absence of sodium ions and that a well-defined optimum existed at sodium (chloride) concentrations near 0.07 M. The sodium ion requirement was specific; equivalent amounts of potassium had no stimulating effect on the incorporation of radioactive amino acids (Allfrey *et al.*, 1955).

The extreme dependence of the synthetic capacity of the nucleus

on sodium ion concentration made it necessary rigorously to control the sodium level of the incubation medium, and this introduced a related problem, namely the osmotic balance between the synthesizing structures and the suspending medium. It was soon found that nuclei exposed to high or very low sucrose concentrations lost their ability to incorporate amino acids into protein. A closer study of the effect revealed that there was a fairly sharp optimum at a sucrose concentration near isotonic, i.e., at 0.20 M in a medium which also contained sodium phosphate buffer (0.025 M), glucose (0.02 M), and NaCl (0.03 M). Although the effects described bear some resemblance to the osmotic properties of semipermeable membranes, it should be pointed out that isolated thymus nuclei are not enveloped by an intact semipermeable membrane and that they are freely permeable to large molecules, such as ribonuclease, deoxyribonuclease, nucleic acids, histones, protamines, and basic dyes. The marked effects of sucrose concentration on nuclear activity suggest that the fine structure necessary for nuclear protein synthesis is in osmotic balance with its environment, and that nuclear function can vary with that balance.

This conclusion finds support in parallel observations on the effect of varying sucrose concentrations on the capacity of thymus nuclei to incorporate orotic acid-C¹⁴ into the pyrimidines of its ribonucleic acids. Here, too, there was a well-defined optimum in sucrose concentration which yielded maximum C¹⁴ incorporation; but the sucrose optimum for orotic acid incorporation (0.13 M) was below the concentration optimum for protein synthesis in the same nuclei (0.20 M) (Allfrey and Mirsky, 1956). Since an important part of nuclear RNA synthesis occurs in the nucleolus (Allfrey and Mirsky, 1957b), while a large part of the amino acid incorporation occurs along the chromosomal protein, the differences observed may reflect the different osmotic properties of these intranuclear structures.

There are a number of observations which suggest a special significance of sucrose in the isolation medium. The most direct evidence comes from experiments which show that thymus nuclei isolated in 0.25 M sucrose and then washed with isotonic saline lose their capacity for amino acid incorporation. Dilute phosphate buffers (0.1 M) have the same effect; yet phosphate buffers in 0.25 M sucrose do not cause inactivation. A related observation of some interest is the fact that when thymus nuclei in sucrose solution are treated with deoxyribonuclease, they lose up to 90% of their DNA and yet no more than 15% of the histone is released, although histones are water-soluble and known to be attached to the DNA in the nucleus. This contrasts strongly with the properties of the same nuclei in a saline medium, for when DNA is

removed and sucrose is not present, half of the histone is released from the nuclei. Similar effects are observed when one compares RNA and nucleotide retention in sucrose and in saline solutions.

The role of sucrose in stabilizing nuclear composition and in preserving nuclear function raises the question of its specificity. This was tested in a study of amino acid incorporation by thymus nuclei isolated in other sugar solutions. It was found that the nature of the sugar made little difference to the synthetic capacity of the isolated nucleus. Other disaccharides (maltose, lactose) in 0.25 M solutions permitted the isolation of nuclei which were just as active as those prepared in sucrose; the monosaccharides tested (fructose, glucose) also gave preparations which were the equivalent of sucrose nuclei in this respect. However, neither glycerol nor ethylene glycol at isotonic concentrations could preserve the synthetic capacity of the nucleus.

The importance of the suspension medium to the function of isolated cell components is not peculiar to nuclei; it has also been demonstrated in mitochondria, microsome, and chloroplast preparations. Specific ion requirements are frequently observed. Plant cell microsomes, for example, require potassium for amino acid incorporation (Webster, 1957), and isolated chloroplasts need magnesium for photosynthetic phosphorylation (Arnon *et al.*, 1956). Osmotic balance is an important factor in oxidative phosphorylation by liver mitochondria. Thus little or no ATP synthesis is obtained in mitochondria isolated in 0.88 M sucrose, while high P:O ratios are observed if the isolation is carried out in 0.25 or 0.44 M solutions. The dependence of phosphorylation rate on sucrose concentration is very marked in insect muscle sarcosomes (Lewis and Slater, 1954).

Tests for function of isolated cell components are frequently negative unless specific cofactors are added. In the study of photosynthetic phosphorylation by isolated chloroplasts it was observed that even the broken chloroplast would synthesize ATP if the requisite cofactors (flavin mononucleotide, vitamin K, and ascorbate) were added (Arnon *et al.*, 1956). In experiments on amino acid incorporation by isolated liver microsomes, ATP or an ATP-generating system must be supplied or very little uptake occurs (Siekevitz, 1952; Keller and Zamecnik, 1956). It follows that negative results in functional tests of isolated fractions need not indicate irreversible damage due to fractionation, and that the right combinations of cofactors or supplementary enzymes may restore the activity in systems which would otherwise be inert. This is a hopeful and promising aspect of research on subcellular components.

One of the most curious findings in testing the function of isolated organelles is the poor correlation between the morphology of the

structure isolated and its capacity to carry out synthetic reactions. There are a number of instances in which an isolation medium was devised to preserve the normal intracellular appearance or the fine structure of a subcellular component. The isolation of rod-shaped mitochondria in 0.88 M sucrose (Hogeboom *et al.*, 1948) and the preparation of optically homogeneous (nongranular) nuclei in 70% glycerol (Schneider, 1955) or a glycerol-glycerophosphate-salt solution (Philpot and Stanier, 1956) illustrate the approach. But in these cases the appearances are deceptive; mitochondria prepared in strong sucrose solutions do not phosphorylate AMP, and thymus or liver nuclei prepared in hypertonic glycerol solutions do not incorporate amino acids into their proteins. On the other hand, the rounded mitochondria obtained in 0.25 M sucrose do carry out oxidative phosphorylation, and the rough, granular thymus nuclei prepared in sucrose-calcium chloride solutions do make labeled proteins and RNA from radioactive precursors. Clearly, morphology alone can be a deceptive guide when the aim of the isolation is to prepare an active, functioning organelle. Nevertheless, there is good reason to persist in a search for methods which preserve the organization and fine structure so important to the normal life and function of the cell, and which also permit biochemical studies of the metabolic pathways and synthetic reactions which characterize the isolated units.

One of the great advantages of isolated cell fractions for biochemical studies is the opportunity which they afford to test the effects of different enzymes on the activity of an "exposed" synthetic system. This is illustrated by experiments on amino acid incorporation by microsomes and by isolated cell nuclei. It was first observed that treatment of liver microsomes with ribonuclease destroys their capacity for amino acid incorporation (Allfrey *et al.*, 1953). Subsequent studies by Hoagland *et al.* (1957) showed that the ribonuclease does not affect the process of amino acid activation but does block the transfer of the activated amino acid to a "carrier" ribonucleic acid. A similar inhibition of amino acid incorporation by ribonuclease treatment has been demonstrated in plant microsomes (Webster, 1957) and in bacterial preparations (Gale and Folkes, 1955; Beljanski, 1954). (It is of interest in this connection that treatment of the microsome fraction with ribonuclease does not impair its capacity to incorporate C¹⁴-labeled acetate into cholesterol (Bucher and McGarrahan, 1956).

In isolated calf thymus nuclei it is deoxyribonuclease which prevents amino acid incorporation into the protein of the nucleus. The enzyme has little or no effect on amino acid uptake by intact thymus cells (Allfrey *et al.*, 1957a). It is this "exposed" nature of the isolated cell structure which makes it so suitable for tests of this sort which utilize

specific enzymes as a tool for chemically dissecting a complex system and observing the changes in its function.

The isolated subcellular component offers another considerable advantage. It permits a great simplification of experiment. Substrates and activators become more accessible; they are less liable to side reactions; and their concentrations can be controlled with accuracy. This is especially important when tracer techniques are employed. The effects of different inhibitors, temperature changes, radiation, etc., can also be tested directly on the isolated cell component. Moreover, the products of isolated metabolic systems are less liable to side reactions and are more readily accessible to trapping reagents or added enzymes. These are all factors which add to the innate desirability of working with parts of cells as a means of understanding cell function and organization.

D. Micromethods in Standardization

An important aspect of standardization, not touched upon in the previous discussion, concerns the use of other techniques (such as ultra-microchemical analysis, ultraviolet microspectrophotometry, specific combinations with fluorescent antibodies, radioautography, specific quantitative staining reactions and other histochemical tests), all of which permit a direct approach to the analysis of single structures in or outside of the cell. These techniques have already found some application in studies of cell components before and after isolation. Thus, isolated cell nuclei have been used as "knowns" in developing a quantitative Feulgen staining reaction for measuring the deoxyribonucleic acid content of a single nucleus (Ris and Mirsky, 1949). Radioautographs were employed to show that free isolated cell nuclei can incorporate radioactive amino acids into their proteins (Allfrey *et al.*, 1957). Ultraviolet absorption measurements have been used as an index of DNA concentration in isolated sperm heads (Leuchtenberger *et al.*, 1952a, b); and X-ray microradiography has made it possible to determine the dry mass and the content of lipids, ribonucleoproteins, and proteins in single nerve cells (Brattgård and Hydén, 1952).

It is certain that the further application of such techniques and the development of new microanalytical methods will greatly expand the range and the accuracy of future chemical studies of isolated subcellular components.

VI. SOME GENERAL CONCLUSIONS

The previous pages summarize some of the information which bears on the problems of cell fractionation; in the main these are problems

of method: how to break cells, how to select an isolation medium, and how to separate particles in suspension. Particular emphasis has been placed on the importance of testing isolated subcellular components for their purity and the need for a critical awareness of the limitations of the isolation method employed. The investigator should know whether the isolated component has changed its composition during the course of the isolation, if it has lost materials or not, and whether adsorption or occlusion of other substances from other parts of the cell has occurred. It has been shown that information of this sort can often be obtained by comparing elements isolated in different ways and particularly by comparing the results of fractionations carried out in aqueous and non-aqueous media. In some instances the composition of the isolated component may be compared with that of the corresponding structure in the cell by employing specific quantitative staining reactions, ultraviolet absorption measurements, or other microanalytical techniques.

In many cases isolated cell structures can be tested for their retention of function, i.e., for their capacity to mediate or carry out complex synthetic reactions *in vitro*. This is often the reason for their isolation, but a test for function can also serve as a test of the isolation method itself. For this reason the isolation procedures which have been shown to yield functioning subcellular components have been emphasized in preference to methods which yield the same components in an inactive state.

In the development of new isolation methods and in the application of known methods to new, untried materials, tests for function can serve as a guide in the choice of techniques for cell breakage, in the selection of the medium, and in many subsequent steps of the fractionation procedure.

From small beginnings, the use of isolated subcellular components has come to play a very important role in the study of cellular organization and function, and it is certain that a large part of future experimentation will involve the isolation of parts of cells. Considering the past successes of this approach, it is clear that the future possibilities of the various fractionation methods, if critically applied, are virtually unlimited.

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CHAPTER 8

The Cell as Organism. "Tissue Culture," Cellular Autonomy, and Cellular Interrelations

By PHILIP R. WHITE

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I. INTRODUCTION

The cell is to biology what the molecule is to chemistry—it is the lowest common denominator of all life, the unit beyond which one cannot pass in simplification without losing the essential capacity for continued survival and reproduction. One can of course isolate enucleate cells such as erythrocytes, cell inclusions such as plastids and mitochondria, and subcellular viruses, which are capable of limited “survival” and a limited degree of function, yet in an isolated state none of these can reproduce its kind and any function it may perform is grossly incomplete, being in abeyance in viruses, and at best of brief duration in such things as mitochondria. Unless the unit is complete in all its parts—a semi-permeable membrane of some sort, a nucleus, an adequate amount and quality of cytoplasm with its complement of specific inclusions, granules, vacuoles, chondrosomes, etc.—unless it possesses all these in suitable proportions, it is an impaired organism. If it does possess them in proper balance, and if it is placed in a suitable environment, it is a competent organism—competent for all the processes of living. And this is true whether the cell is a *complete* organism, as is true of the fertilized egg, or whether it is a unit taken out of one of those multicellular societies which we call an animal or plant.

What we more commonly call an “organism,” a plant or animal, is a complex society of these cells. Within that society each cell is assigned a limited range of those functions which are essential to the healthy operation of the community: *protection* to the epithelium of an animal or the corky bark of a tree, *support* to bones or wood, *movement* to muscle, *sensation* and *communication* to nerves, *assimilation* to the intestinal lining, or to the leaf mesophyll, or to the root hair, *elimination* to kidney or sweat glands, *storage* to fat and pith, *reproduction* to still more specialized cells. In any individual cell some one or more of these functions can be recognized though one will seldom find all of them in any single cell.

In the present chapter we shall be interested exclusively in the vital, functional aspects of these elementary units, the cells, rather than in any details of structure or of location. More specifically, we shall be interested in one particular method of studying these aspects. This is the method commonly known as “tissue culture,” the method or methods by

which these elementary units are removed from the complex in which one normally finds them, that is the body, and are then provided with suitable substitute environments which shall, ideally, in no way impair their normal functional capacities. They are thus made available for direct observation and experiment, so that we may, in a sense, walk around them, examine them from all sides, observe and manipulate their behavior, place them in more and more complex situations, bring cells of different sorts into relation with one another, permit them to multiply and to build up systems which may be simplified segments of an organism or may constitute entirely new organizations, disaggregate, reaggregate, and rearrange them and thus learn how, in fact, a community of cells develops from a single egg into a complete, independently functional creature, and how, once developed, it continues to maintain its assigned functions in satisfactory balance.

It is clear that in no other way can we hope to understand fully what an organism really is. The histologist and cytologist can examine the skeleton, the framework, the variety of artifacts which are left after the life of the cell or organism has been destroyed. In some cases he may be able to examine cells in a state of suspended or limited animation, by a variety of extremely effective methods. From such examinations he may be able to infer processes and functions. But cytological and histological methods give us only a spatial and temporal *section* of reality, from which extrapolation is at best hazardous. The physiologist, biochemist, and biophysicist may likewise apply a variety of precise measures to the operation of another set of limited segments and aspects of reality, taking either the organism as a whole as his framework, or the operation of a system such as is represented by a set of enzymes. But only when all these method are brought to bear on the lowest common denominator of life, the cell, over long periods of time, without gross or continued impairment of function, can we hope to grasp reality in anything like completeness and true perspective. This is the sort of study for which "tissue culture" provides the essential means.

II. HISTORY

What, then is "tissue culture"? It is not *a* method but a variety of methods, bound together by a common objective. It can perhaps best be understood from a brief historical survey. Ever since Schleiden (1838) and Schwann (1839) first recognized in formal fashion the ubiquity and fundamental importance of the cell as the "elementary organism," the simplest common denominator of all complex organisms, the importance of studying that elementary unit has been implicit in much of our thinking. Attempts to carry out such studies, however, commonly resulted

only in destroying the unit, that is, killing it. The problem of duplicating the environment or of providing suitable substitute environments proved to be a thorny one. Apparently one of the first successful attempts was that made by Arnold (1887). Taking thin slices of elder pith, he soaked them thoroughly in aqueous humor from the frog's eye. He then implanted them in the peritoneal cavities or lymph spaces of other frogs. Here the leucocytes invaded the aqueous humor trapped in the pith. After a few days the slices of pith were removed and placed in a dish containing either a saline solution or a second supply of aqueous humor like that originally used. The entrapped leucocytes, finding this fluid environment similar to that in which they had previously found themselves, continued for some time to behave "normally" and Arnold was able to follow, under the microscope, their migration into the fluid, their division into new cells, the processes by which they engulfed bits of debris, etc. His drawings are quite remarkable for the date (1887). At that time, however, these observations answered no specific and urgent questions and hence attracted little attention.

The idea of isolating cells and growing them for the avowed purpose of studying cellular autonomy and interrelations had not been formulated by Arnold as a general objective. It was perhaps first expressed in clear fashion fifteen years later, by Haberlandt (1902). But, although Haberlandt carried out many experiments with the aim of establishing just such cultures as those for which he had foreseen the need, his studies were not successful. They again attracted only a passing philosophical interest.

In 1907, however, a method similar to that of Arnold *was* used to answer a crucial question and, since the experiment, unlike Haberlandt's, was successful and was followed by a brilliant statement of principles for which the time was by then ripe, this time the idea did take root.

At that time the origin of the nerve fibers, which, in the body, connect the ganglia in the central nervous system with the end organs at some distance therefrom, was a matter of bitter controversy. Classical histology had clearly demonstrated their existence but could not determine whether they arose by precipitation along intervening paths of some sort, or by growth from the ganglia, or by growth from the end organs, or by some combination of these methods. Each theory had powerful proponents. Transplantation of parts within the embryo, in Amphibia for example, had failed to produce an unequivocal answer (Harrison, 1904). Faced with this dilemma, Harrison (1907, 1910, 1912) turned to what was essentially the same method as that used by Arnold. He isolated bits of embryonic frog ganglia in drops of lymph drawn from the frog, where they could be watched under the microscope, and,

as Arnold had observed the migration of leucocytes, Harrison was able to *see* these ganglion cells spinning out nerve fibers similar to those seen in histological sections. He was able to prove that nerve fibers arise, not by precipitation *de novo*, nor by growth from the end organs, but solely by growth from the ganglia.

The answering of this one moot question presented in dramatic fashion the possibilities inherent in this method of living dissection without destruction of function. Not all important histological questions can be answered as simply or as definitely as was this one. The method was slow to develop for a variety of reasons. Nevertheless Harrison had made certain that it was firmly and soundly rooted, and the last half century has seen its gradual growth in precision and usefulness. The method of tissue culture will forever remain a monument to Ross G. Harrison.

III. THE SUBJECT MATTER

What, then, are the questions which we may hope to elucidate by this general method of approach? They fall, in general, into three main classes.

A. *Morphogenesis*

The first of these we may designate as questions of morphogenesis, the origin and progressive expansion of form and function, the unfolding of an organism from the relative simplicity of a single fertilized egg cell to the incredibly complex community of cells which is an adult man or a mature tree. All of the cells of an adult have come from a single source, the fertilized egg. And they have arisen by a simple process, cell division. Are they then, within a given organism, all alike? Certainly they are not alike in form, nor do they perform like functions. There has been a division of responsibilities and of superficial characteristics. Has there, however, been a segregation of fundamental qualities so that the cells are no longer, as Schleiden (1838) had thought, and Haberlandt (1902) assumed, complete organisms? Is a muscle cell irrevocably different from a cell of a kidney glomerulus, and a pollen-mother-cell irrevocably different from a root hair? Or have these differences in function and form resulted from divergences within the developing body, divergences either of inherent origin or produced by the spatial and temporal dichotomies of local environment of the cells, which dichotomies might be capable of reversal? If the segregation is truly an irreversible one, at what stage did it become so? And what is its mechanism? Not all types of cells found in the adult are present even at birth. Is this segregation of function still going on in adult life? Certainly it

is most obvious during prenatal development, but when, if ever, does it stop? And how can it be modified, directed, halted, or reversed?

1. Differentiation of Epithelium and of Bone

Few indeed of these questions have yet been answered. But a clue to the probable nature of the answers to be expected in animals, as well as an indication of one method of attack, we do have, in certain culture work from Dr. Fell's laboratory in Cambridge, England. The particular question is this: the ciliated epithelium of the nose, the absorptive epithelium of the intestinal villi, and the protective epithelium of the skin are all variants of a single class of cells, to which are also related such dissimilar members as the nerves. Similarly, the cell of a mature functioning bone and of a bone in osteoclastic arthritis are of like origin. Why, then, are they functionally and structurally so different? A clue to both questions has been given by the clinically observable effects of certain glandular diseases and nutritional deficiencies. Excessive keratinization of skin, and weakness of bone, are characteristic of animals in states of vitamin A deficiency, brought about either by simple malnutrition or by thyroid dysfunction (myxedema). Yet since these defective conditions are generally evident at widely distributed regions of the body, the clinical evidence gives us no clue as to whether the vitamin acts directly on the cells of the body or whether, instead, it alters some intermediary characteristic of the blood stream or other element in the environment of the finally reacting cell. An obvious approach to this problem was therefore to isolate skin and bone under conditions where their supplies of vitamin A could be manipulated without the intermediary of a complex digestive and distributive system. This Fell and Mellanby (1952, 1953, 1955) have done. It has been possible to produce keratinized skin on the one hand, or mucus-secreting and even ciliated epithelium, on the other hand, by providing low or high levels of vitamin A supplied to the culture. Normal, solid bone with typical marrow can be exchanged for gelatinous osteoclastic degeneration, or the reverse, by manipulation of this same vitamin. In the case of skin this lability persists at least well toward the end of the embryonic period (13-15 days in the chick) and probably on into adult life (Fell, 1957). The method has established the fact that at least one effect of vitamin A, in both skin and bone, is direct, on the epithelial cell or the osteoblast itself, not on some more remote, secondarily involved system. It tells us that the difference between keratinized and ciliated epithelium is *not* irreversible at the particular developmental stages examined. It tells us that we must look to differences in *local supply*, to differential *absorption* and distribution of vitamin A from the blood stream, as a crucial factor in morphogenesis. It tells us nothing about how this graded supply

ply, which is after all a function of the organism as a whole, not of its individual cells, is brought about, but it poses the critical questions in new and hence useful forms.

2. *Differentiation of Plant Stem Growing Points*

Or we may frame a similar set of questions with regard to plants. What are the processes by which a leaf is organized and ultimately provided with a vascular connection to the roots? The growing apex of any stem is a simple, low dome of cells which appear to be all alike and to be indistinguishable from the corresponding cells of a root. If such an apex from a fern is isolated as a block 0.5 mm. or less on a side and is placed on a suitable nutrient substratum, it will form leaves and roots and develop into a complete plant (Wetmore, 1954). Its cells are at this stage clearly essentially totipotent.

Below the smooth dome of the apex are formed a progressive series of folds which develop into leaves or flowers. If one of the first five recognizably distinct folds (in the cinnamon fern, *Osmunda cinnamomea*) is excised, it will grow into a complete plant as does the apex (Steeves, 1957). Its cells are still totipotent and have as yet come under no specific differentiating "field." But if any fold further toward the base than about the fifth is excised, it does not develop into a plant but into a leaf. Its potencies have been segregated and oriented in a particular way. Yet it still retains a certain degree of morphogenetic plasticity, for whether it is to develop into an unlobed juvenile-type leaf or into a complex pinnatifid adult form, can be controlled by varying the concentration of carbohydrate in the nutrient (Wetmore, 1954).

Below the apex and inside the ring of leaf-primordia folds there is commonly a considerable bulk of parenchymatous tissue. If a corresponding block of this tissue is isolated from the apex of a plant of lilac (*Syringa vulgaris*) it will not grow at all on the nutrient suitable for stem apex or leaf primordium. But it can be caused to grow as a disorganized mass by addition of a chemical growth factor, an auxin (Wetmore, 1957). If the auxin is supplied in the substratum, there will be formed in the mass, scattered and unoriented nests of vascular cells, a beginning, though abortive, organization. Such a mass can be grown for years, and can be repeatedly divided and subcultured, without any further differentiation. It appears to have lost most of its potencies except that of reproduction (division). If, however, auxin is supplied at a restricted point on the top of the mass, these vascular cells form in a definite ring—a cylinder whose distance from the center depends on the concentration of the auxin supplied—but no roots arise. And if, finally, in place of the localized supply of auxin, we graft into the apex of the parenchymatous mass a bud of the type formed from a stem apex, the

vascular ring then progresses upward until it becomes continuous with the vascular elements of the bud, there are formed roots from the base of the ring, and we have again a complete plant (Camus, 1949; Camus and Gautheret, 1948a, b; Wetmore, 1957). The progressive steps from a growing but disorganized mass of parenchyma to a fully organized stem can be controlled by varying the nature, concentration, and direction of supply of recognizable chemical substances, auxins, in the same way that vitamin A served to control the direction and degree of differentiation of certain animal cells. The apparent loss of totipotency is spurious and can be reversed, once we recognize its origins. And tissue culture has furnished the means of clarifying the issue.

B. Physiology

A second group of problems in biology for which tissue culture offers unique opportunities involves questions of physiology. While physiological processes are at the foundation of all morphogenesis, there are many physiological functions which do not result in changes in form.

1. Origin of Heartbeat

Such is, for example, the simple question of heartbeat. What causes the heart to beat? The beat can be accelerated, retarded, blocked, or renewed by processes in which the nerves are involved. But are the nerves responsible for the beat? Does the beat only begin after innervation? Some of these questions can be answered by direct observation *in situ*. But there are others for which tissue culture methods offer more satisfactory, or even the only, answers. One can take a heart and separate it into its constituent cells—muscle, connective tissue, nerve, pericardium, etc.—and can follow the behavior of one type of cell in the absence of the others. And one can show that each heart-muscle cell is capable of independent rhythmic contraction in the absence of all other cells (Lewis, 1923, 1929). The beat may be modified by the nerves. The various beats of many discrete cells may be synchronized as a result of their close association, with or without nerves. But the beating cell is functionally an autonomous unit. The method does not yet tell us the origin of the contraction, nor does it elucidate the differences between a rhythmically contracting heart-muscle cell and a skeletal-muscle cell whose contractions are much more random, but it does serve to definitely eliminate some possibilities and to pose our questions in new and more effective fashion.

2. Origin of Specific Substances: Lignin

Or we may wish to elucidate the mechanisms by which a particular substance arises. Lignin, for example, is a substance of structural importance in many woody plants. It is of particular importance in the

utilization of plant materials in such industries as the manufacture of paper since its presence interferes with the preparation of a satisfactory product. Herbaceous plants often do not form lignin, but are likewise minor producers of the desired celluloses. What is the mechanism of lignin formation? Inferences can be drawn from painstaking analyses at various stages, in which such a product does or does not appear, but these inferences cannot be put to a crucial test in the intact plant. It has long been known that coniferin is a common metabolic product in the early stages of many lignin producers, but whether or not it is, in fact, a precursor of lignin was not proved. Wacek *et al.* (1953) however, reasoned that if the tissue of a plant which does not normally produce lignin in quantity, could be grown in the presence of coniferin, it might give us some answers to this question. Unorganized tissue of carrot, which does not normally become lignified, proved to be a suitable experimental material. When placed on a nutrient containing coniferin, carrot tissues did, in fact, form lignin. Evidently the carrot tissue possesses the enzymes necessary to transform coniferin into lignin, but does not possess the enzyme systems needed to perform the synthesis of coniferin from suitable antecedent metabolic materials. A similar partial set of enzymes has been demonstrated in roots by similar tissue culture methods, tomato roots possessing the capacity to unite pyrimidine and thiazole into thiamin, but apparently lacking the capacity to synthesize pyrimidine itself (Robbins and Bartley, 1937). We have thus arrived at a useful answer by shunting into the metabolic pathways at "unnatural" points, by means of tissue cultures. The introduction of a competitive analog of coniferin might, therefore, block the formation of lignin and prove to be of great importance to the paper industry.

3. Cell Division and Cell Migration

Or one may wish to clarify the facts as to the details of so universal a process as cell division. Although the framework of our knowledge of this process was certainly laid in observations on such materials as the sea urchin egg, which require no such complex manipulations as "tissue cultures," there can be little doubt that the culture of metazoan cells, and particularly their study by methods of cine-micrography (see Section IV, B, 3), have contributed, particularly in the past decade, more than any other method, to our understanding of mitosis. Tissue cells are much smaller and more transparent than, hence optically superior to, marine or amphibian eggs. Classical methods long left in doubt the questions of whether, for example, the spindle fibers, the centrosomes, the Golgi apparatus, mitochondria, etc., were real "structures" or were artifacts resulting from fixation. But when the process of cell division of a human epithelial cell, for example, is followed in a sheet of cells spread out as a single layer against a flat glass surface, using modern phase contrast

optics and time-lapse cinematography, and the resulting film is projected at accelerated speed and studied as single frames, it is possible to establish the reality of all these "artifacts," and much more besides. The peculiar rotation of the nucleus, which in some cells may "spin" quite noticeably (Pomerat, 1953); the permutations of the mitochondria in shape, passing from spheres to threads to forked structures (Frédéric and Chèvremont, 1952); the way in which a mitochondrion in its movement through the protoplasm may become "caught up" on a water droplet, bent, and perhaps broken; the "boiling" of the cell surface following cell division (Canti, 1927) and again characteristically at the death of enucleate fragments of cytoplasm (Goldstein *et al.*, 1957); the ways in which cells respond to pharmacological agents and to such other agents as X-rays (Canti, 1927); the engulfing of solid particles and the drinking of water (Lewis, 1931, 1937)—all are shown in tissue cultures with a clarity and vividness which can never be attained *in vivo* or in histological preparations. Certainly no one who has watched accelerated time-lapse movies of the developing fish egg (Lewis) can have any remaining uncertainty about the synchrony of blastomere divisions or of the migration of cells into the mouth of the blastopore; no one who has watched the scurrying of monocytes hither and yon among the more sluggish fibroblasts (Lewis *et al.*, 1941) can further doubt their scavenging function; no one who has observed a nerve fiber spun out from a neuroblast (Lewis and Lewis, 1911) or a Schwann cell spiraling around the surface of a neurone (Peterson, 1950), or a parathyroid cell gnawing its way into a bit of bone (Gaillard, 1955a, b, 1957) can ever again harbor a static picture of the structure of an organism. One of the most startling sights is to watch the behavior in tissue culture of a "pearl" of pulmonary epithelium. If the ciliated surface happens to be turned inward, we have a kaleidoscopic internal activity in a mass lying quietly on the floor of the drop. But if the ciliated surface is turned outward, the pearl spins crazily across the field like an extraordinarily active protozoan (White, unpublished observations). The lung "comes alive" to the observer as it does in no other way. Or if we watch the effect of morphine, for example, administered in a tissue culture in a perfusion chamber and then washed out, and follow the vacuolization in the presence of the drug and the subsequent recovery, we have quite a different idea of narcosis from that obtained in the clinic (Buchsbaum, 1954).

4. Glandular Activity

Still another process capable of elucidation by these methods is that of glandular activity, and here we can match our observations in plants as well as animals. The embryonic and developing kidney tubule can

be isolated, and the sequence in which functions of the various parts emerge can be followed under observation (Chambers and Cameron, 1944; Chambers *et al.*, 1935) and can be modified at will. One of these functions is the active secretion of water, by which the large volumes of fluid which are filtered through the glomerulus in passive fashion, under the pressure head of the blood stream, are then driven back into the blood stream against this head by the activity of the tubule cells. This is one of the most important processes in all vertebrate physiology and is a model for all other secretory processes. Interestingly enough a corresponding process appears to exist in plant roots. The apex of a root consists of growing cells, which have the rich cytoplasm typical of other sorts of active cells, including glands. Basipetal to this region there is differentiated a conducting strand suggestive of a channel for flow of fluid. Such a basipetal flow has in fact been demonstrated in growing excised roots of tomato, in tissue (or organ) culture. The force with which water is secreted into the conducting strands by the activity of cells in the apex has been measured, and some indications of the role of metabolizing cells in this secretion have been studied (White, 1938, 1942a, b). The functions of various endocrine glands have also been studied in organ cultures (Martinovitch, 1938, 1951; Gaillard, 1957; Trowell, 1954).

These are only scattered examples of the use of tissue cultures in the study of physiological problems.

C. Pathology

A third group of problems to which tissue culture can bring useful answers lies in the field of pathology. And here the possibilities are indeed varied. How does a cell respond to an invader such as a virus which is not immediately lethal; and if lethal, what are the progressive stages of cell destruction? How does a cell ward off damage from an infection? Where does immunity reside, especially that sort of "natural" resistance which does not depend on previous exposure and the formation of antibodies? How and where do antibodies themselves arise? In cases where a cellular defect cannot be traced to an invader, how does that defect arise? Cancer, anemias, arthritis, muscular dystrophy are examples of this sort. How far back in the differentiation of cells can such defects be recognized and by what means? All of these questions can be approached by removing the cells in question from the body, isolating them from extraneous influences of other cells, either of the same sort or of other sorts, treating them as independent units, and following their responses step by step.

In many of these problems one can, in turn, consider the cell itself at two quite distinct levels of importance. In studies of noninfectious

maladies such as cancer, nephritis, arthritic conditions, or blood defects the cell will commonly be the primary object of study, *per se*. But when we come to study conditions in which the cell is attacked by an external agent such as a bacterium or a virus, our attention may be divided between the cell and the invader and we may, in fact, find it expedient to use the cell merely as a tool through which to focus our attention on the parasite.

1. *Cancer*

Precancerous states are generally strictly localized and give the impression that the disease is of local origin, representing a defect, whatever its origin, of single cells. From this point of view cancer is looked upon as a *cellular* disease. When, however, this local, benign condition passes into the malignant phase it generally does so through the appearance of metastases, cells from the original site moving through the blood stream and lymph and becoming reimplanted so as to establish new centers of growth elsewhere in the body. Since the malignant cells almost certainly do not *suddenly* appear in the blood stream and *at the same time* begin to establish themselves elsewhere, there is a strong presumption that metastasis involves not so much the acquisition of a new capacity in the cancer cells, as a breakdown of the body's powers of resistance to reimplantation. At this stage there thus emerges a generalized state, which is certainly not solely one of the invading cells, and may be either a *cellular* breakdown of resistance at the site of implantation or a *humoral* state represented by the condition of the entire organism. The first stage, the transition from a normal to a cancer cell, appears to be a subject ideally apt for study by tissue culture methods. The second, the transition from a fixed cancerous cell to a free-moving and freely reimplanting metastatic cell, may well be examined by such methods, such analysis promising possible answers to the relative importance of cellular versus organismal factors.

The first cells to be established in tissue culture were normal embryonic cells (Harrison, 1907; Burrows, 1911; Carrel, 1912; Lewis and Lewis, 1911). Tumor cells were early tried (Carrel and Burrows, 1911), and it was noted that these commonly differed from normal cells, even embryonic ones, in tending to liquify (digest) a plasma substratum and in being in general less fastidious in their nutrient requirements. This difference has been repeatedly confirmed and is responsible for the frequently observed ability of cancer cells *in vitro* to invade and replace a colony of normal cells. A similar difference in fastidiousness exists between normal and cancerous cells of plants (de Ropp, 1947; Gautheret, 1947, 1948a, b). These facts being known, tissue culture would seem to

offer a method of following the appearance of tumor characteristics. One of the first carefully planned approaches to this question was by Earle (1943).

a. Cellular instability. Unfortunately, in this case an unexpected property, since repeatedly confirmed, became apparent. When cultures were carefully treated with substances known to be clinically carcinogenic, such as methylcholanthrene, and suitable untreated controls were maintained, malignant characteristics appeared in the controls as well as in the experimental cultures. Firor and Gey (1945) observed the same phenomena in untreated cultures. This has happened so often that it now appears doubtful if there exist any strains of animal or human cells which have been maintained for long periods, that have not produced malignant lines, with the possible exception of Carrel's original strain of chicken fibroblasts (Carrel, 1912). Such divergence has even been documented in strains derived from a single cell (Sanford *et al.*, 1954). For this reason, tissue culture has not satisfactorily fulfilled its early promise in the study of the origin of malignancy. Such divergences have likewise been noted in plant tissue cultures, of ferns (Steeves *et al.*, 1955) and spruces (Reinert and White, 1956). And the phenomenon of "anergy" (Gautheret, 1955) in plant tissue culture, in which a tissue strain appears having quite different nutritional capacities, usually an increased synthetic capacity and hence decrease in nutritive fastidiousness, seems to be quite similar. On the other hand many strains of plant tissues appear, like Carrel's chick fibroblasts, to be remarkably stable.

b. Stepwise origin of plant tumors. While tissue culture has not answered the question of how an animal cell becomes cancerous, it has given us much valuable information on the characteristics of cancer cells and, in the case of plant cancers, has permitted considerable progress in following the steps through which cancerous changes do occur. In the crown gall disease of plants, a tumorous overgrowth is induced by the action of an intracellular bacterium, *Phytomonas tumefaciens* (Smith and Townsend, 1911). Once the tumor is established, the infecting organism can be destroyed without diminishing the malignancy of the affected host cells. This destruction of the invader may take place naturally (White and Braun, 1942), or it may be brought about by agencies such as heat, since the bacteria are less resistant to high temperatures (40° C.) than are certain host cells (Braun, 1947). The final result is the same—a tumor tissue free of living bacteria. Such tumor tissues can then be transplanted into new hosts (artificial metastasis), producing new, completely aseptic tumors. Presumptive evidence of these facts was established long ago by Smith (Smith *et al.*, 1912), in the Shasta daisy, and by Jensen (1918), in the sugar beet. Conclusive

evidence of the sterility and malignancy of plant tumor tissues was, however, first obtained in 1942 by White and Braun (1942) by the use of tissue cultures from secondary tumors of sunflower. Tumor tissues were isolated in tissue culture, grown on nutrients suitable for support of crown gall bacteria, to establish their sterility, and then reimplanted into healthy hosts to establish their malignancy. Tissue cultures have since been used by Braun, Klein, de Ropp, and others to establish the role of temperature in the phenomenon of tumor induction (Braun, 1947), to define the characteristics of the material transmitted by the bacteria to the host cells (Klein, 1953), to separate the "tumor-inducing principle" now defined as a self-reproducing nucleic acid (Klein *et al.*, 1953), etc. While we do not yet understand all the steps in tumorigenesis, we do have a fairly clear outline of the process, in plants, and in view of the role of tissue culture in this work we have every reason to anticipate that the method will continue to be an important factor in its further elucidation.

The same methods have been used to elucidate the nature of other types of plant tumors. Besides the crown galls, there are two other types of malignant growths in plants. First is the genetic tumor arising in certain hybrid tobaccos, particularly the cross *Nicotiana glauca* \times *N. langsdorffii*. Tissue cultures of these were isolated in 1937 by White (1939) and were shown to be malignant (transplantable in series without loss of tumor characteristics) (White, 1944). It has the same increased capacity for synthesis of auxin which characterizes the crown gall tissues (de Ropp, 1947) and Gautheret's "anergized" tissues of carrot, grape, etc. (Gautheret, 1947, 1948a, b). The second is the virus tumor of *Rumex*, sweet clover and other plants, first described by Black (1945). These have also been maintained in tissue culture. They too differ metabolically from their normal (nontumorous) counterparts. They are characterized by an abnormally high phosphate requirement, which may well be bound up with the excessive synthesis of nucleic acid in virus-infected cells (Nickell, 1954), and a unique capacity to synthesize amylase (Brakke and Nickell, 1951) which permits virus-tumor tissues to grow on sugar-free but starchy substrates on which tissue cultures of all other plant tissues tested so far would soon starve. Since these tissues are probably never quite free of virus they cannot be called true cancers on the basis of present evidence. They can, however, give us much valuable information.

c. *Diagnosis.* Although the use of tissue cultures has been somewhat disappointing in failing to elucidate the nature of animal cancers, there is one direction in cancer research in which it has proved of great practical importance. That is in diagnosis. Certain neuromas are noto-

iously difficult to diagnose accurately because of the anatomical complexity of the cell relations involved and the difficulty of identifying the affected cells in the anatomical complex. When, however, biopsies from suspected tumors of this class are placed in tissue culture and are allowed to grow, the different cell classes grow out at different rates and in characteristic patterns, so that the diagnostic picture is simplified and clarified and a decision made much easier and more certain (Murray and Stout, 1947). While this method has, to date, been particularly successful with neuromas it should theoretically find much wider applications. Leighton (1954a, b) has, in fact, made such a beginning with other types of tumors.

d. Screening of chemical agents. Tissue culture is likewise being very largely used in the screening of many of the carcinostatic chemicals currently under scrutiny. In view of the evident instability of pure lines of cells in culture it has seemed best to use, for these studies, fresh isolations representing mixtures of cell types (Bieselet, 1954) and to maintain them for relatively short periods only.

2. *Viruses*

a. Propagation. One field, in which tissue culture has in recent years taken a commanding role, is in the maintenance and study of the viruses pathogenic for man and other animals. The virus-induced Rous sarcoma of chickens was cultivated quite early in the history of tissue cultures (Carrel and Ebeling, 1926), and the method was employed in the study of vaccinia (Carrel and Rivers, 1927). It was used in the preparation of a vaccine (Li and Rivers, 1930; Rivers and Ward, 1935) which, however, proved unsatisfactory. Recently, however, it has taken on new importance. In 1948 Enders and his associates found that the virus of poliomyelitis could be propagated *in vitro* in human or monkey tissues of nonnervous origin (Weller *et al.*, 1949). This provided a veritable "break-through" in the study of this and other virus diseases and was quickly extended to include the viruses of herpes, mumps, measles, vaccinia, various encephalitides, rabies, pseudorabies, and many other diseases. The method has been used for the preparation of massive quantities of viruses. One of the most spectacular uses to date has been in the preparation of the Salk vaccine for immunization of a considerable part of the people of the Western World against poliomyelitis (Salk, 1953). This vaccine was prepared largely in tissue cultures of monkey kidney cells. Many millions of effective immunizing doses of vaccine have been prepared in this way.

b. Identification. Tissue cells have likewise been used as a means of quick identification of viruses. If human tissues are immunized by being

grown in a series of nutrients containing sera from individuals known to have had infections of, and hence to carry antibodies against, each of the known strains of poliovirus, or in nutrients containing antisera against these strains prepared by cultivation of immune cells *in vitro*, and these cultures are then challenged with cells from individuals infected with viruses of unknown identity, the challenged cultures will continue to grow in the presence of inocula from individuals whose viruses represent those against which the culture serum possessed immunizing qualities, but will be quickly killed by all other inocula, providing a clear identification of the unknown (Syverton and Scherer, 1954). A large literature has grown up on this approach. The whole process of immunity is under restudy, using methods of this sort.

c. Pathology. In these studies, primary interest has centered around the virus itself, and the cultured cell has served merely as a vehicle for the virus. The relation of virus to cell has likewise attracted much study. It has been possible to follow step by step, both by optical observation with phase contrast illumination and with the electron microscope (Bang, 1953), the invasion of a cell by the virus, the breakdown of cell constituents, the formation of inclusion bodies and other disease products, and the final release of virus. These processes could scarcely have been studied with like precision *in vivo*.

d. "Orphan" viruses. One curious outcome of all this study of viruses in tissue culture has been the discovery of large numbers of viruses for which no known diseases have been recognized. These Duran-Reynals has called "orphan viruses"—viruses in search of disease (Duran-Reynals, 1954, quoted in Committee on the ECHO viruses, 1955). Some of these undoubtedly are the causative agents of hitherto obscure diseases. One at least of the adenoviruses isolated from adenoidal and tonsil tissues thus seems to be the agent of a fairly common aseptic meningitis (Meyer *et al.*, 1955). One has the suspicion, however, that there may exist large numbers of viruses which do not, in fact, produce overt disease of any sort, but which may well modify the behavior of the host toward other agencies. Such "masked" viruses are well known in plants, and masked stages of viruses are not unknown in animals, as in the case of the Shope papilloma of rabbits (Rous *et al.*, 1952). And some have very peculiar effects. There is, for example, a virus disease of the fruit fly, *Drosophila*, whose only recognized symptom is a greatly increased sensitivity to carbon dioxide (Brakke, 1957). Such "silent" viruses might have profound effects in altering the course of individual development and even the evolution of species. Further elucidation of these facts, by tissue culture methods among others, will be anxiously awaited.

Other disease agencies can likewise be studied through the use of

tissue cultures. The rickettsial diseases have been particularly successfully handled in this way (Rivers, 1948). Attempts to grow the bacillus of Hansen's disease (leprosy) have been less successful (Hanks, 1947), but not without interest.

3. Pathogenic Fungi

Fungus diseases of man and of animals are of relatively minor importance. Few are fatal. Although often annoying, as in the case of the *dermatophyoses* (athlete's foot, etc.), the others are not of serious clinical or economic importance. This statement may prove to be untrue, for there certainly do exist tuberculosis-like infections of fungus origin (torulosis) which may prove to be of more importance than is commonly recognized. But, though other fungus diseases of animals may be unimportant, the same can certainly not be said of plants. There are hundreds of fungus diseases of plants of very great economic importance. And many of these are caused by obligate parasites which have not, to date, been grown on laboratory media. Many details of their physiology and pathology thus remain obscure. The rusts, with their complex multi-host life-cycle sequences, and the mildews are representative. These offer a challenge to the tissue culturist, which has to date been met to only a minor degree. Morel has successfully grown the grape-mildew fungus, *Peronospora viticola* on tissue cultures of grape (Morel, 1944, 1948; Morel and Gautheret, 1947). There is a brief report, unconfirmed, of the cultivation of the wheat-rust fungus on tissue cultures of the alternate host, *Crataegus* sp. (Hotson, 1953; Hotson and Cutter, 1951; Cutter, 1951). The virus of tobacco mosaic disease has been grown in isolated roots of tomato (White, 1934b) and in tissue cultures of tobacco (Morel, 1948; Ségréatin, 1948). These however, are only indications of the possibilities of a field of investigation as yet scarcely touched.

4. Noninfectious Pathology

There are, of course, many maladies of noninfectious origin, besides cancer. Probably the four most important causes of human debility, barring none, are (1) old age, (2) failure of the circulatory system (heart failure, arteriosclerosis, dropsy, etc.), (3) the various arthritic and neuritic states, and (4) organic insufficiency due to destruction of an organ—by accident, by infection, or by hereditary defect. Perhaps one might reduce them to three by equating the first with all the others.

a. *Aging.* What is aging? If taken out of the body, all cells which are capable of *multiplication* can be immortal. *Strains* of such cells do not age, at least not in tissue cultures. Those cells which do not multiply *in vitro* are certainly not the first to fail in the body. It is well recog-

nized, medically, that the heart *muscle* seldom shows signs of failure even in extreme old age. It is the integrative members of the system—the walls of the blood vessels, the kidneys, the liver, the endocrine glands, the lungs, the nerve sheaths—which seem to wear out first, and these, in contrast to the heart muscle and the nerves, are just the body members which retain their proliferative capacity. Apparently, although proliferation is essential to the longevity of cell strains, it is not essential to the longevity of cells in the body, nor for the functioning of such cells. Just as continued proliferation of “normal” cell strains in tissue culture seems always to give rise, sooner or later, to malignant lines, it appears that this same tendency toward divergence must, in the body, gradually throw the machinery out of balance. How soon this will happen will depend on many factors, not the least being the inherent stability or instability of the genetic constitution of the cells themselves.

It is thus that we may hope to attack many of the problems of aging by studies of this sort. If it is possible to direct the differentiation of epithelial cells into different channels by controlling the level of available vitamin A, as Fell and Mellanby (1952, 1953) have shown to be the case, then it should be equally possible to restore other balances, as well. Collagen is an important factor in the condition of the bones and tendons, in arthritic conditions. And collagen is produced in large quantities in tissue cultures of fibroblasts, in certain environments, as Merchant (1957) and others have shown. The factors involved in this production are not yet clearly defined. They should be. Many neurological maladies are caused by failure of the myelin sheath, and myelin also can be formed *in vitro* (Peterson, 1950). Muscular dystrophy is a disease in which the muscle fibers gradually disappear and are replaced by adipose and connective tissues. Adipose tissue is not easily cultivated in tissue culture, but muscle is, and we are beginning to know something about the way in which muscle cells are formed, for example by the work of C. M. Wilde (unpublished).

b. Glandular dysfunction. Glandular failures are common—in diabetes, in Addison's disease, in hyper- and hypothyroidism, in parathyroid tetany, in ovarian and testicular insufficiency, in prostate disease, in nephritis, and in many others. Tissue culture studies on these exist, but are scattered. Attempts to study diabetes in tissue culture have repeatedly run up against the fact that if the whole pancreas is excised and cut up, the secretions of the acini quickly attack the islands of Langerhans, and one ends up with a culture either of fibroblasts or of acinar cells. The island cells have not been established in culture. Yet this should not be an insurmountable obstacle. If one followed Banting's original procedure, of first ligating the pancreatic ducts to cause degen-

eration of the acinar cells (the Minkowski operation), any *then* removed the mixed island and scar tissue for placing in tissue culture, it should be possible to establish useful strains of island cells. This was, in fact, apparently successfully done by Russell Richardson in Philadelphia (unpublished). It should be repeated. Mammary gland tissue long resisted satisfactory cultivation because of the large amounts of fatty tissue present, until Lasfargues recently (1957) showed that the fatty tissue could be destroyed by mild digestion with lipase, without reducing the viability of the gland cells. The digestion techniques introduced by Rous and Jones (1916) and perfected by Moscona (1952), in combination with Puck's plating methods (Puck *et al.*, 1955) should open up wide fields indeed for the study of the behavior of gland cells.

While study of endocrine function at the *cellular* level is only just now emerging as an active field, the cultivation of intact or semi-intact endocrine glands has had a much longer history. And here it has already had important clinical results. One of the most striking is in the treatment of postoperative tetany. Occasionally, either because of primary disease or as a secondary result of operations for thyroid disease, the parathyroids may be destroyed. This results in disturbance of calcium metabolism and tetany, which must usually be controlled, if at all, by repeated and long-continued injections of parathyroid extract. If, however, embryonic human parathyroids from autopsy or parathyroid tumors (adenomas) from adults are grown in tissue culture and are then implanted in a tetanic patient, they will serve, so long as they survive, as a continuing source of the required hormone (Kooreman and Gaillard, 1950). Embryonic glands may "take" successfully, particularly in young patients, without special difficulty. Gaillard (1957) reports one patient who has been free of tetanic symptoms for sixteen years following embryonic parathyroid grafts. If, however, adult rather than embryonic glands have been used as a source of tissue cultures, and are then implanted, immunotypic reactions can commonly be expected to result in rejection of the grafts. Yet this, too, can be overcome, and has been in the case of ovarian grafts for treatment of ovarian insufficiency. For if the graft is made as a "two-stage" operation, first removing a small sheet of peritoneal membrane from the intended recipient, placing this on a nutrient substratum and implanting the gland cultures on it, permitting the first graft to take place *in vitro* and only then, when the ovarian tissue is well fused with the peritoneal sheet, reimplanting the membrane with its adherent glands back into the patient, there is no reaction between the patient and his own peritoneum, nor is the gland subsequently rejected. Such grafts have been successfully accomplished, as judged by the patient's complete freedom from symptoms of insuffi-

cency (Gaillard, 1957). In this procedure tissue culture has been important at two stages, first in the propagation and multiplication of the glandular tissue itself, and second in the preoperative conditioning of the host tissue to accept the graft.

c. Nutritional abortion in plants. Although plants do not have endocrine glands, a fundamentally similar tissue insufficiency does exist in many plants whose seeds abort because, although forming normal embryos, their nutritive endosperm fails to develop properly and may be entirely wanting. These are chiefly hybrids (White, 1928; Skirm, 1944), and the success of breeding programs may sometimes depend on the experimenter's ability to overcome this physiological sterility. Such abortion can, in fact, often be by-passed by removing the embryos from the immature seed before they are called upon to demand nutrient from the abortive endosperm and placing them on an artificial nutrient, in tissue culture. Viable plants can then be obtained from them. First used to obtain progeny from "infertile" hybrids of stone fruits (cherries, plums, etc.) by Tukey (1933, 1934), the technique has been fairly widely used for apples (Nickell, 1951), iris (Randolph, 1945), etc. It has been especially useful in accelerating the propagation of range grasses and trees which normally have a very slow germination, due to delaying mechanisms in the seed, but in which the embryos are capable of prompt growth when isolated from these mechanisms (Kent and Brink, 1947; Lammerts, 1942).

These then are a few of the problems to which the methods of tissue culture have been or could be applied. The list is obviously a personal one and far from complete. It is intended to do no more than suggest the breadth of the field and whet the reader's interest to draw up his own lists.

IV. METHODS

A. Nutrient Objectives

What, then, are these methods?

All of these studies, in morphogenesis, in physiology, and in pathology, can be carried out adequately only in surroundings which either do not impair the functions of the cell, or impair them in clearly defined, recognizable, predictable, and controllable ways. Ideally the environment should be defined and controlled in every respect. A major part of this environment is the fluid nutrient which is to bathe the cell, provide its nutrient and its respiratory needs, dissipate its waste products, serve as its protector against change in physical state, temperature, light, acidity, against bioinvaders, etc. This fluid must thus provide

all of the organic and inorganic substances—salts, metal catalysts and coenzymes, energy sources, nitrogenous building blocks, vitamins and hormones, buffering agents, respiratory substrates (including oxygen itself), and a host of other substances. It must provide these in acceptable, preferably optimal, quantities, at acceptable rates. They may be provided in the simplest usable forms, or in more complex form, according to the needs of a particular problem, and one may decide whether a given substance shall be supplied in minimal amounts, in optimal quantities, in excess, or be entirely excluded. The nature of the nutrient supplied will differ with the objectives sought.

In embryonic or juvenile life, in those parts of the body which are not fully organized, or in states of repair and regeneration, cells are dividing rapidly. If one seeks to simulate these conditions, a formula will be sought which will promote rapid cell proliferation. Such a nutrient must be high in available nitrogenous materials, of which the embryo extract introduced by Carrel (1913) is the prototype.

In fully organized portions of the body, in the adult body in general, and in organs in a state of routine function, on the other hand, proliferation is at a minimum. These states can best be maintained in fluids in which nitrogenous materials are available, not so much as building blocks but as homeostatic buffers, and may indeed be of less intrinsic importance than are the inorganic and carbohydrate constituents. Formulas for Warburg studies, such as those of Krebs (1950), and for maintenance of muscle tone in the Schultz-Dale test, may in fact be very simple indeed, although their effectiveness might probably be improved by consideration of knowledge gained from other types of study.

Tissues and organs which have not yet reached a stable degree of organization, either because they are in a juvenile state—and this "juvenile," labile state may normally end at any period in embryonic, adult, or postadult life—or because they are in states of reorganization, may require still a third sort of nutrient formula, in which emphasis will rest on those constituents which effect neither proliferation nor homeostasis, but cellular differentiation. These constituents will be neither the nitrogenous building blocks, nor the energy sources, nor the homeostatic buffers, but rather catalysts of which the vitamins are representative examples. The Fell-Mellanby (1952, 1953, 1955) nutrients mentioned earlier which control differentiation of epithelium and of bone are interesting examples in which vitamin A seems to provide the controlling mechanism in a background of a simple Tyrode solution and serum.

Still a fourth approach may be necessary when the cell under study is not a primary object, but merely a vehicle or tool through which to

approach an understanding of some other member of the system, as is the case when we are growing parasitic organisms or viruses in cells, or using cells as reagents in the screening of pharmacological, cytostatic, or cytotoxic products. Here a judicious balance between homeostasis and proliferation may be our objective.

1. Nutrients

What then are these nutrients?

The first nutrients to be used were simply body fluids: aqueous humor (Arnold, 1887), lymph (Harrison, 1907), blood plasma or serum (Burrows, 1911). Many sorts of cells would survive for days in these, and some would grow. Parallel with the use of these organic complexes, there developed the use of salt solutions (Lewis and Lewis, 1911) modeled, for animal cells, after the inorganic salt content of blood serum, as revealed by crude analysis, or, for plant cells, the soil solution, as found in "good" soils (Knop, 1865, 1884; Gautheret, 1942). The "balanced salt solutions" of Ringer, Tyrode, Locke, Lewis, Gey, Pannett, Earle, Hanks, White, Knop, Gautheret are representative. None of these is, by itself, a complete nutrient. To them is usually added an energy source: dextrose for animal cells (M. R. Lewis, 1922) or sucrose for plants (White, 1934a, 1940). These are supplied either pure or as organic complexes. The most widely used organic complexes are tissue extracts, especially an extract of chicken (Carrel, 1913) or beef (Gey) embryos, extracts of adult tissues (Doljanski and Hoffman, 1943), or bouillons (W. H. Lewis, 1923) for animal cells, and yeast extract (Robbins, 1922), peptone digests (White, 1932; Ziebur *et al.*, 1950), or coconut milk (van Overbeek *et al.*, 1941) for plants. These complexes in turn have been replaced, with greater or less success, by more clearly defined synthetic mixtures of amino acids, vitamins, coenzymes, etc., beginning with the pioneer studies of Baker and Carrel (1926; Carrel and Baker, 1926), continued in the work of White (1946) and culminating in the complex nutrients of Parker (Healy *et al.*, 1955), Evans *et al.* (1956), and the simpler defined nutrients of White (1955) and Waymouth (1956).

While this portion of the tissue culture method is currently extremely active and changing rapidly, so that whatever may be said now is likely quickly to become obsolete, one may perhaps venture to list a few of the nutrients most widely used today.

The "simplest" of all these common nutrients in terms of numbers of ingredients, though of unknown complexity in view of the poorly defined character of some of these ingredients, is the nutrient devised by Fell (Fell and Mellanby, 1955) and widely used in the Strangeways

Laboratory for the study of problems in morphogenesis. It consists of 3 parts chicken plasma and 1 part of chicken embryo extract prepared from equal parts of crushed 10-day chicken embryos and Tyrode solution. The final nutrient contains, per 100 ml.:

NaCl, 100 mg.; KCl, 2.5 mg.; CaCl₂, 2.5 mg.; MgCl₂ · 6H₂O, 1.25 mg.; NaH₂PO₄ · H₂O, 0.625 mg.; NaHCO₃, 12.5 mg.; dextrose, 125 mg.; embryo juice, 12.5 ml.; plasma, 75 ml.; H₂O, 12.5 ml.

Next in complexity among nutrients for animal tissues is that devised by Enders for maintenance of human cells in which to propagate viruses such as that of poliomyelitis (Weller *et al.*, 1952). The procedures for its preparation are rather complex, but the final nutrient is not complicated, consisting of the following, again per 100 ml.:

NaCl, 300 mg.; KCl, 40 mg.; MgSO₄ · 7H₂O, 20 mg.; CaCl₂, 14 mg.; Na₂HPO₄ · 12H₂O, 15.2 mg.; KH₂PO₄, 6 mg.; NaHCO₃, 35 mg.; phenol red, 2 mg.; dextrose, 100 mg.; streptomycin, 5 mg.; penicillin, 5000 units, ox-serum ultrafiltrate, 25 ml.; H₂O, 75 ml.

Nutrients designed to replace the serum, plasma, and embryo extracts with defined substances have been devised. They may be represented by that of Waymouth (1956), developed for maintenance of strain L mouse cells (ML 192/2). It contains, in milligrams per 100 ml.:

NaCl, 600; KCl, 15; Ca(NO₃)₂ · 4H₂O, 20; MgSO₄ · 7H₂O, 20; Na₂HPO₄, 30; KH₂PO₄, 8; NaHCO₃, 224; dextrose, 500; Difco Bacto-Peptone, 500; Armour's bovine albumin fraction V, 50; L-cystine, 0.75; L-tyrosine, 2; glycine, 2.5; L-lysine HCl, 8; L-methionine 2.5; L-threonine, 3.75; DL-valine, 6.25; L-arginine HCl, 3.75; L-histidine HCl, 3.75; L-proline, 2.5; DL-isoleucine, 2.5; β-phenyl-L-alanine, 2.5; L-leucine, 2.5; L-glutamic acid, 7.5; L-aspartic acid, 3; L-tryptophan, 2; phenol red, 5; thiamin HCl, 0.5; Ca pantothenate, 0.05; riboflavin, 0.05; pyridoxine HCl, 0.05; folic acid, 0.01; biotin, 0.01; hypoxanthine, 2.5; glutamine, 5; ascorbic acid, 1.75; cysteine HCl, 7.78; glutathione, 1.5.

A still more complex nutrient is that developed by Parker *et al.* for strain L mouse cells. Parker's nutrient No. 858 (Healy *et al.*, 1955) contains, in milligrams, per 100 ml.:

NaCl, 680; KCl, 40; CaCl₂, 20; MgSO₄ · 7H₂O, 20; NaH₂PO₄ · H₂O, 14; NaHCO₃, 220; Fe(NO₃)₃, 0.01; NaC₂H₃O₂, 5; glucose, 100; L-arginine, 7; L-histidine, 2; L-lysine, 7; L-tyrosine, 4; L-tryptophan, 1; L-phenylalanine, 2.5; L-cystine, 2; L-methionine, 1.5; L-serine, 2.5; L-threonine, 3; L-leucine, 6; L-isoleucine, 2; L-valine, 2.5; L-glutamic acid, 7.5; L-aspartic acid, 3; L-alanine, 2.5; L-proline, 4; L-hydroxyproline, 1; glycine, 5; cysteine HCl, 26; thiamin, 0.001; riboflavin, 0.001; pyridoxine, 0.0025; pyridoxal, 0.0025; niacin, 0.0025; niacinamide, 0.0025;

Ca pantothenate, 0.001; biotin, 0.001; folic acid, 0.001; choline, 0.05; inositol, 0.005; *p*-aminobenzoic acid, 0.005; vitamin A, 0.001; ascorbic acid, 5; calciferol, 0.01; α -tocopherol PO₄, 0.001; menadione, 0.001; Tween 80, 0.05; cholesterol, 0.02; DPN, 0.07; TPN, 0.01; carboxylase, 0.1; glutathione, 1; L-glutamine, 10; phenol red, 2; ethyl alcohol, 1.6; flavine adenine dinucleotide, 0.1; uridine triphosphate, 0.1; adenine deoxyriboside, 1; guanine deoxyriboside, 1; cytosine deoxyriboside, 1; 5-methyldeoxycytidine, 0.01; thymidine, 1; Na glucuronate, 0.42; Na penicillin G, 0.01; dihydrostreptomycin sulfate, 10; *n*-butyl-*p*-hydroxybenzoate, 0.02.

To this is usually added from 1 to 20% of serum.

Finally, to complete the list with a nutrient widely used for plant tissues, we may cite that of Heller (1953), which consists of, in milligrams per 100 ml.:

KCl, 75; NaNO₃, 60; MgSO₄ · 7H₂O, 25; NaH₂PO₄ · H₂O, 12.5; CaCl₂ · 2H₂O, 7.5; FeCl₃ · 6H₂O, 0.1; ZnSO₄ · 7H₂O, 0.1; H₃BO₃, 0.1; MnSO₄ · 4H₂O, 0.01; CuSO₄ · 5H₂O, 0.003; AlCl₃, 0.003; NiCl₂ · 6H₂O, 0.003; KI, 0.001; glucose, 5000.

The very much higher concentration of glucose used in nutrients for plant tissues (Heller 5%; Parker 0.1%; Enders 0.075%) is noteworthy. Much of the osmotic value imparted to plant tissue nutrients by the carbohydrate is replaced, in animal nutrients, by the relatively high level of sodium chloride (Parker, 0.68%; Heller, none).

2. Technical Facilities

The major technical problems of tissue culture are the provision of adequate nutrient media, which we have just discussed, the preparation of cells in a suitable state of physiological tone, the bringing together of these two and their continuance under conditions of complete asepsis, and the study of the materials under some appropriate regime. While it would be out of place to devote much space here to technical details, a brief examination of the physical facilities required may not be amiss.

B. Work Areas

These facilities must include three separate laboratory areas.

1. The Kitchen

First is the preparation or "kitchen" area, where glassware and instruments are cleaned, sterilized, and suitably packaged and stored and nutrients prepared. Glassware may be cleaned in many ways, according to individual preferences. Acids and alkalis, soaps, detergents, oxidizing agents, alcohols and other solvents have all been used. Whatever method

is chosen, the final result *must* be a high order of cleanliness. Cells will usually not adhere to and grow satisfactorily in contact with glass of improper formula, or with the least trace of soiling or unevenness of cleanliness. The final and crucial step in any regime will be the same—thorough rinsing in water purified by at least two steps, one of which will be distillation through Pyrex or quartz. The other step may be a preliminary distillation from tin, or passage through an ion exchange resin. Ion exchange alone, however, cannot be relied upon to give satisfactory results since it does not remove nonionized organic toxins. An ion exchange bed can provide a very rich substratum for bacterial growth and may produce a water free of ions but loaded with pyrogens and other injurious organic materials. Nor can, in most cases, a single glass distillation be considered sufficient. Glassware to be used in contact with cells, especially culture vessels, should be treated, as a final step, either to prolonged soaking in or autoclaving in distilled water. Glassware may also be sterilized by dry heat (140° C. for 3 hours or more). Even with all these precautions glasses of different manufacture may give quite different results.

Once clean, glassware should be sealed against contact with air, especially against contamination by volatile fatty substances such as occur in most city air. Flasks and tubes may be tightly stoppered, or placed in closed containers as is done with pipettes, slides, cover glasses, petri dishes, etc. Or they may be wrapped in a suitable paper such as Patapar, or in metal foil. Foil makes a particularly satisfactory closure for flasks and culture tubes of all kinds.

Stable fluids may be autoclaved, but most organic solutions such as nutrients must be filtered, either through fritted glass or some form of porcelain filter, of which the Selas 0.3 porosity candle is particularly satisfactory (White, 1954). Viscous fluids, such as serums and tissue extracts, may have to be first "scrubbed" by passage through a coarser filter, or treated with hyaluronidase, or both. Once sterile, such fluids will generally be stored in the refrigerator awaiting use. Specially volatile or unstable fluids may have to be kept in the deep freeze at -40° or -70° C. Embryo extract, for example, should be so stored or else lyophilized.

2. *The Culture Chamber*

The second critical work area is the culture room where living materials are handled and cultures prepared. This will generally be a small, separate room, with filtered and preferably "conditioned" air (temperature and humidity control), capable of being closed and kept closed during manual manipulations, dissections, etc. It must be scrupu-

lously clean. In this area all the procedures of dissecting animals or plants to isolate cells, tissues, or organs for culture, the setting up of cultures, the renewal of nutrients, and the removal of cells after cultivation, will be carried out. Of all the areas of tissue culture manipulation, this is the one in which critical errors are most likely to occur.

3. *The Laboratory*

Third, and usually least critical, among work areas will be the laboratory, in which cultures are grown, usually in some sort of incubator [plant cultures may have to be maintained at temperatures *below* usual room temperature, say at 17° C. (Slankis, 1949) which may pose special problems], and are examined and studied. Methods of examination and study may of course be varied and sometimes complex. Tissue cultures are specially apt subjects for examination by modern optical methods. As the living cell spreads out on the glass of the culture vessel, flask, tube, or slide, it presents a thin, flat surface to which phase contrast and interference microscopy can be ideally applied, revealing details of structure and behavior which can be studied in no other way, and which can seldom be studied at all *in vivo*. When these are combined with time-lapse cinematography and the subsequent frame-by-frame analysis of the resulting sequences, they offer an extremely effective means of establishing the reality of phenomena which in ordinary histological sections may appear to be artifacts, for following the movements and transformations of cell inclusions such as mitochondria, etc. The phenomenon of pinocytosis (W. H. Lewis, 1931, 1937), which probably plays an extremely important role in the life of cells, especially in the assimilation of materials to which the cell membrane is ordinarily impermeable, and in the movement of such agents as viruses, would hardly have been recognized by any other means. A further refinement is provided by the method of perfusion (Buchsbaum, 1954), in which the fluid bathing a cell under study may be changed at will without disrupting the sequence of observation, so that the effects of such shifts in environment can be followed step by step.

While electron microscopy cannot, as yet, be applied to the living cell, and consequently cannot be combined with cinephotomicrography, cells grown by tissue-culture methods or transferred to special electron-microscopy grids and then fixed, present beautiful flat areas for study and have given us important information on the fine structure of the cytoplasm, cell membranes, and nucleus and on the effects of viruses (Bang, 1953).

Still other methods, of rapidly increasing importance, are micro-radioautography and radiochromatography, by which the exact location

of substances within the cell can be determined and the steps in metabolic exchanges followed (Markert, 1955, 1957). It appears that chromatographic and radioautographic analysis of cells during culture, and of nutrient fluids before and after prolonged exposure to growing and metabolizing cells, may prove to be extremely important aids in designing nutrients for special types of cells and for promotion of particular cell processes.

C. Culture Methods

The methods used in setting up and maintaining cultures of living cells are as legion as are the problems to be dealt with (see especially Parker, 1950; White, 1943, 1954; Pomerat, 1951; Hanks *et al.*, 1955; Gautheret, 1942).

1. Hanging Drops

The oldest of these methods, if we except Arnold's "cultures" in small dishes, was the hanging drop method. A drop of fluid nutrient, or often a drop of nutrient capable of coagulating into a gel, such as plasma or a fibrinogen solution, is placed on a clean cover glass, and a bit of tissue is placed therein. After clotting (if the drop is a gel), the cover glass, with its culture, is inverted over some sort of well—a depression slide, a pierced slide with a second cover slip floor, a metal, glass, plastic, paper, or even vaseline ring. To facilitate changing nutrient without disturbing the culture, the cover glass is sometimes attached by a capillary film of water to a second, larger cover slip, which is placed in contact with the slide or ring, and from which the culture slip can be floated free merely by adding an excess of water. This is the so-called "Maximov double cover slip method." For phase contrast microscopy still greater optical flatness may be obtained by causing the drop to make contact with both an upper and a lower cover glass separated by a glass, metal, or plastic shim. A useful modification is to place several drops in rows on a large, rectangular cover glass separated from a second in the same manner. Or the culture drops can be placed on the lower side of the cover of a small petri dish. In any case, cells will migrate out from the inoculated fragment into the surrounding nutrient where they can be observed. This method may be usefully expanded by making cultures in a variety of perfusion chambers in which nutrient can be continuously renewed or replaced at will. One of the best of these is that designed by Buchsbaum (1954).

The hanging drop method is best suited for detailed observation of relatively small tissue fragments, and especially for relatively short-term cultures, at high magnification. Most of the excellent cinematographic

records of living cells have been made with some form of hanging or sitting drop culture. But the volume of fluid available in a drop is small in proportion to its area, exposed to the glass on the one side and to the air on the other. Chemical changes are therefore likely to be rapid, and one has little control over the composition of the nutrient. And renewing nutrient, except in complicated perfusion slides, is tedious and risks loss or contamination. The method is therefore poorly adapted for prolonged studies, for handling large masses of cells, for dealing with metabolic processes, etc. For these, other methods are better.

2. Flasks, Bottles, Petri Dishes

For many purposes cultures in flasks of some sort may be much more satisfactory. The oldest of these, and still widely used, is the Carrel flask. In its most common form this is circular, about 3.5 cm. in diameter by 1 cm. thick, with the upper and lower faces blown flat and thin enough to permit observation of cultures with high-dry objectives. There is a narrow neck placed in the circular margin and at a shallow angle to the face so that when the flask rests on one face, nutrient fluid will not run into the neck. Cultures are made on the inner surface of this lower face, where, during cultivation, they are covered by a thin layer (1-2 mm.) of nutrient fluid. Cultures may be made in a plasma clot or directly on the glass. Cultures are observed either on an inverted microscope or by inverting the flask on the stage of an ordinary microscope. In this latter case the microscope must be tilted to prevent fluid running into the neck of the flask. This has the disadvantage that the fluid is drained away from the culture during observation, so that a culture cannot be observed for more than a few minutes without drying out. The pentagonal T-flask designed by Earle and Highhouse (1954) is a modification of the Carrel flask, designed to facilitate collection of cells and the preparation of subcultures.

For routine maintenance of cultures which do not require critical observation, as in the cultivation of viruses for the preparation of vaccines, small, flat, hard-glass medicine bottles may be used quite successfully. They are usually not, however, optically uniform enough for close study.

Small Erlenmeyer flasks have also been used for like purposes (de Bruyn, 1955). These cannot be inverted and can be studied *only* on an inverted microscope. Small petri dishes (Warren *et al.*, 1955) with ground seals have also proved very satisfactory for some purposes, especially for the preparation of plaques. Here a "nurse" layer of one sort of cell is spread evenly over the bottom of the dish, usually on a plasma or agar substratum. A dilute suspension of cells of another sort.

distinguishable in some way from the nurse cells, is then washed over the surface of the first so that the cells are widely separated. The colonies which arise from such separate cells are discrete and can be picked off, permitting the isolation of clones of single-cell origin (Puck *et al.*, 1955).

3. Test Tubes

Carrel flasks and T-flasks are expensive, somewhat cumbersome, and difficult to clean. Erlenmeyer flasks, petri dishes, and medicine bottles have limited usefulness. For many purposes test tubes may provide a valuable substitute. An ordinary 16 × 150 mm. lipless Pyrex test tube is thin enough so that a culture spread over its interior face can be observed with the lower-power objectives of the microscope. If rested in a sloping rack, it can be charged with 1–2 ml. of nutrient without wetting the stopper. On a rigid rack, cells inoculated into such a tube will settle onto the lower side of the tube and will grow at that level in the tube where the depth of fluid is best suited to their requirements (Syverton and Scherer, 1954). Excellent cultures for the propagation of viruses are often prepared in this way. An added refinement of great usefulness is provided by rotating the tubes around their own axes in a drum kept at the desired slope (Gey, 1933; Gey and Gey, 1936). In such tubes the culture may cover the entire inner face of the tube. Cultures are much more easily manipulated and removed for subculturing in such tubes than from flasks. The tubes are cheap and easy to clean with standard equipment. While the walls of such a tube are neither flat enough nor thin enough for high-magnification study, this objection has been met by special tubes with flat faces. It can also be met by inserting strips of cover glass in the tubes (Pomerat, 1952); cells will grow on these glasses, which can be removed at intervals and mounted for high-power observation for short periods.

4. Shaker Flasks

Since cells grown on static surfaces cannot obtain adequate oxygenation except under a very shallow layer of nutrient, the use of hanging drops, flasks, petri dishes, or test tubes is limited to small volumes of fluid and small total mass of cells. This is somewhat greater with roller tubes. In order, however, to permit the use of large volumes, some modification of method must be made. This has been provided in the shaker flasks now coming into use (Earle *et al.*, 1954). A relatively large volume of nutrient is introduced into an Erlenmeyer flask, or better still a balloon flask, and inoculated with living cells. The flask is then placed on a mechanical shaker, which imparts a rapid oscillation to the fluid, usually about 400 r.p.m. At such speeds the fluid is well aerated and

cells do not settle onto the glass, but multiply free in the fluid or as small spherical "pearls." Fluid can be drawn off at will, either containing suspended cells or after permitting the cells to settle out, and can thus be used either for chemical studies or as a source of inoculum for new cultures. Under properly adjusted conditions, cells will multiply with great rapidity and to very large total volumes (Earle *et al.*, 1954). The method is thus particularly well suited for obtaining the large amounts of cellular material needed for chemical and metabolic studies.

5. Watch Glasses

The relatively small volume of the hanging drop, the necessarily thin layer of fluid in a static flask, and the constant agitation of the shaker flask, are all unsuited for the unfolding of the formative morphogenetic processes of relatively massive organs such as bones, eyes, ovaries, lung membranes, etc. For these still another method had to be devised. This is the watch-glass method. In its simplest form, a soft clot is prepared in an ordinary chemical watch glass of about 3-cm. diameter. This is placed in a petri dish and surrounded by moist cotton to maintain humidity. The organ to be studied is placed on this clot (Fell, 1928, 1951). The clot may be replaced by a grid of glass rods (Martinovitch, 1954) or a table of paper or wire mesh (Trowell, 1954). A layer of lens paper (Trowell, 1954) or of cellulose acetate net (Shaffer, 1956) may be interposed between the clot or table and the culture, to facilitate handling. In general, cultures of this sort should be disturbed as little as possible. If treated properly, isolated bone can be followed in its development (Fell and Mellanby, 1953), ovaries can be grown to the maturation of oöcytes, the organization of feather follicles can be followed, etc. This method has proved particularly suitable for the cultivation of excised glands to be used for subsequent therapeutic implantation, as in the treatment of postoperative tetany with parathyroid grafts mentioned above (Kooreman and Gaillard, 1950; Gaillard, 1957).

6. Three-dimensional Matrices

None of these methods duplicates at all closely the massive alveolar structures which exist within an animal body where most cells normally grow. This has long been recognized. A closer approximation to these conditions may be arrived at by permitting a culture to grow into some sort of spongy matrix saturated with nutrient fluid. This was, in fact, the case with Arnold's cultures in 1887, made in a matrix of elder pith saturated with aqueous humor. Cellulose sponge makes a suitable matrix (Leighton, 1951). Saturated with a serum-embryo extract nutrient, such sponge has been used to permit the establishment of three-dimensional

colonies, rather closely simulating conditions in the body. The disadvantages are two (at least). No substitute for a circulating capillary bed has yet been devised, so that such cultures must be kept to small volume if central necrosis is to be avoided. And the interior of such a mass cannot be examined except by fixing, sectioning, and staining, as one would a piece of body tissue. Nevertheless, this method is giving interesting results, for example in studying the invasion of a culture of normal cells by a second inoculation of a strain of tumor cells (Leighton and Klein, 1954).

7. Special Methods

Two of the tissue culture methods which are today being most actively developed represent beautifully two opposite poles of its potentialities. One has already been mentioned; the organ culture method developed at the Strangeways Laboratory in England. The second, originally developed at the Rockefeller Institute by Rous and Jones (1916) and perfected by Moscona (1952) at the Strangeways, is the preparation of cell suspensions by mild digestion with trypsin. These two methods have in turn, been fused in exciting fashion by Wolff in Paris, Grobstein in Washington, and others.

When an organ is removed from the body, its normal circulatory supply is disrupted. Without adequate metabolic exchange, and particularly without adequate access to oxygen, it will, of course, soon necrose. Circulation can be replaced by the complex systems of Carrel and Lindbergh (1938) and of Thomas (1949), but these are scarcely tissue cultures and have in any case proved, in practice, too complex and uncertain for general use. Exchange can be taken care of by simple diffusion if the organ segment is kept sufficiently small. This can be done in the watch-glass methods already discussed, and information can be gained on many metabolic questions as, for example, the phosphate metabolism in a developing bone (Fell and Robison, 1929).

But it is also possible to take an organ, and, by judicious digestion with trypsin, to separate it into its constituent cells without destroying their viability. They can then be planted in nutritive substrate and allowed to reaggregate, forming new organs of the same, or more usually, of somewhat simplified type (Moscona, 1956). Ovarian or testicular tissue, for example, will not grow organotypically unless surrounded by an epithelial capsule, and an ovary from which the capsule has been stripped away before it is placed on the clot of an ordinary watch-glass culture, will degenerate quite rapidly (Gaillard, 1957). But if the ovary is cut up into small fragments, and these are mixed in a watch glass or depression slide in a nutrient, random epithelial cells from the interior

will take up the function of capsules. Indeed, any epithelium can serve this function, and kidney cells may build a capsule for ovary if fragments of the two organs are mixed (Wolff, 1954; Wolff and Weniger, 1954; Wolff and Bresch, 1955). This can be carried still further by digesting the organ, such as an ovary, with trypsin. If the disaggregated cells are then planted, not as a capsuleless organ but as a mixture of loose cells, some oöcytic cells will take up positions on the exterior of the reaggregating mass, will take over the function of capsular epithelium and permit the re-establishment of an organ which will mature without central necrosis (Wolff, 1957).

This approach has been carried still further in most spectacular fashion by Wolff and by Moscona. If one disaggregates, either by mincing (Wolff) or by trypsinization (Moscona), corresponding tissues of quite unrelated animals, such, for example as the mouse and the duck, and mixes them, they will reaggregate into chimeric organs in which the typical organ structure is built up by cells from the two species working in apparently complete harmony (Wolff, 1954; Wolff and Bresch, 1955; Wolff and Weniger, 1954). Thus chimeric kidneys, lung, bone, ovary, etc., have been synthesized, the cells from mouse and duck being clearly distinguishable by their size and optical qualities. The species incompatibilities which normally prevent "grafting" between members of mature and intact organisms, simply do not exist at the cellular, embryonic, and disorganized levels. They are a function of the organism, not of its constituent cells. Much can be learned about cellular interactions and the control of organogenesis by such means.

V. CONCLUSIONS

This, then, is in outline the method which is loosely called "tissue culture." It is a versatile method, capable of application to the study of a host of problems concerned with the nature of life's lowest common denominator, the cell. It is a method of growing importance in the biology of today. What has been said above is no more than a sketch, an outline which the reader can fill in and pad out wherever his professional inclinations may lead. In expanding this picture he may well refer particularly to Volume 58 of the *Annals* of the New York Academy of Sciences (pp. 972-1325) covering the 1954 Symposium on Tissue Culture Technique in Pharmacology; Volume 67 of the *Annals* of the New York Academy of Sciences (pp. 309-446) on "Viruses in Search of Disease"; Volume 19 of the *Journal* of the National Cancer Institute (pp. 467-843) covering the 1956 International Decennial Review Conference on Tissue Culture at Woodstock, Vermont; and "A Bibliography of the Research in Tissue Culture" (to 1950) by Margaret R. Murray and Gertrude

Kopech, published (1953) by Academic Press. A supplement to this bibliography, to cover the years 1950-1955, is now in preparation. The bibliography furnished here (see References below) lists only papers cited in this chapter.

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CHAPTER 9

Fertilization

By J. RUNNSTRÖM, B. E. HAGSTRÖM, and
P. PERLMANN

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I. GENERAL ASPECTS OF FERTILIZATION

A. Preliminary Characterization

Fertilization involves the fusion of two gametes of opposite sex. This is called *syngamy*. The fusion is, in general, permanent, involving the formation of a zygote. The fusion between the gametes may also be temporary. In the former case, one speaks about copulation, in the latter about conjugation.

It is of particular importance that in fertilization the haploid nuclei of the gametes (male and female pronuclei) are brought together. A more or less intimate fusion of the gamete nuclei takes place (karyogamy). In this way the diploid syncaryon is formed. This process is the essential basis for biparental inheritance.

The gametes may be of equal size and shape. Fertilization is then *isogamous* (cf. Fig. 1). Despite the exterior similarity between the gametes, they are sexually different; this involves certain differences in biochemical character and sometimes also in physiological behavior.

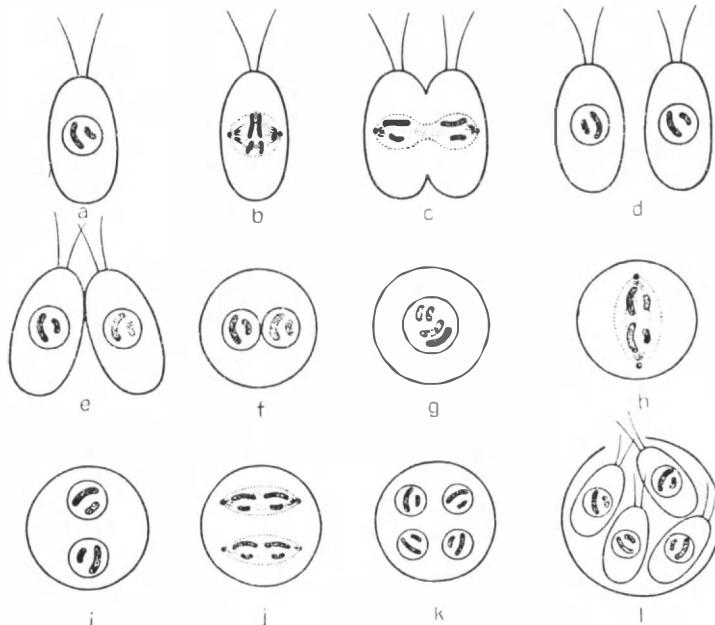


FIG. 1. Fission (b-d), isogamous fertilization (e-g), zygotic meiosis (g-k), and spore formation (k, l) in a flagellate, *Chlamydomonas* (a). After Hartmann (1956a).

One speaks about *heterogamous* fertilization when the gametes present differences also with respect to size and structure. Isogamous and heterogamous conditions may be found in closely related organisms, even within one and the same genus (e.g., *Chlamydomonas*). Nevertheless, the heterogamous condition is on the whole correlated with higher differentiation of the organisms (cf. Figs. 2 and 3).

The fusion of the gametes may also bring together cytoplasmic components, which are of importance from the point of view of heredity. In isogamous copulation, the contribution of the two gametes is equal

with respect to cytoplasmic components. In heterogamous copulation, the contribution of the male gamete is more restricted, but may still involve particulate components, mitochondria or plastids. The fate of these components is variable. The contribution of the female gametes to the cyto-

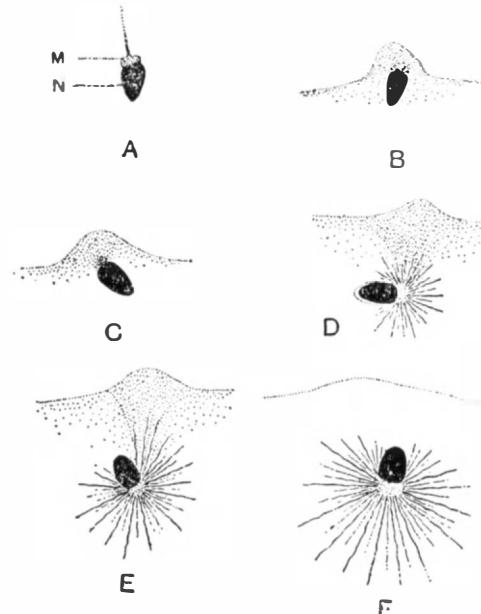


FIG. 2. Fertilization of the sea urchin egg. A. sperm head before attachment. B-F. Engulfment and rotation of the sperm head; development of the sperm aster centered around the middle piece (*M*) which contains the centrosome.



FIG. 3. Approach of the male and female nucleus; *C* = entrance cone. Fig. 2 and 3 after Wilson (1895), Fig. XI.

plasm predominates. In many organisms, such as the metazoa, a centrosome is contributed by the male gamete (cf. Figs. 2 and 11).

Organisms in which one individual forms both male and female

gametes are called monoecious; whereas in dioecious organisms, each individual is either male or female. The relative character of sexuality has been demonstrated in algae and fungi (cf. Hartmann, 1956a). This means that different gametes may have different potentialities with respect to male or female character. Let us assume that gametes *A*, within a certain species or variety, behave as male toward gametes *B* and syngamy thus is realized. Gametes *B* may, however, behave as male toward gametes *C*. In such case, the sexual reaction between *A* and *C* must be still stronger than between *A* and *B* and between *B* and *C*, respectively.

Autogamy is a special form of fertilization in which two daughter nuclei fuse within the same cell (cf. Sonneborn, 1947; Grell, 1956). The two fusing nuclei have here identical genetic character.

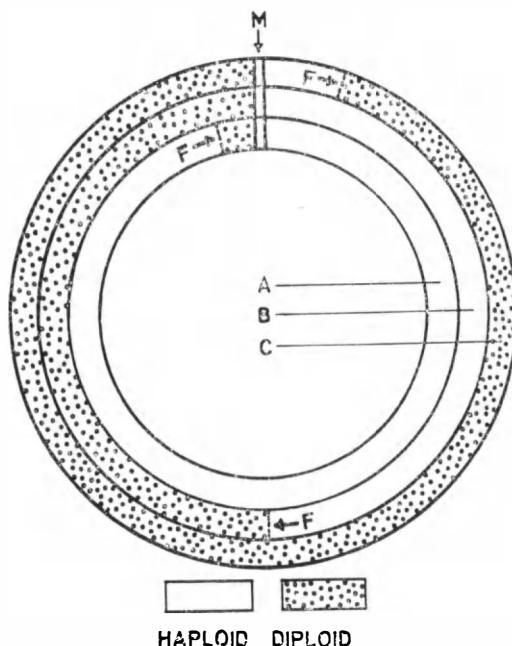


FIG. 4. Diagram indicating the place of fertilization, *F*, in the life cycles of different organisms; *M* = meiosis. Type A: the diploid phase is limited to the zygote (cf. Fig. 1); haploid phase dominates. Type B: alternation of haploid and diploid phase; from the former the gametes are formed; fertilization initiates the diploid phase; at meiosis spores are formed from which the haploid phase starts. Type C: meiosis leads to the formation of the gametes; their fusion (*F*) gives rise to the predominating diploid phase (cf. text). Modified from Raper (1954).

B. The Place of Meiosis and Fertilization in Life Cycles

The haploid state is brought about through two meiotic divisions. Meiosis is thus a necessary prerequisite for fertilization. In most organ-

isms with natural parthenogenesis, the meiotic divisions are suppressed (cf. White, 1954, p. 339).

The time relations between meiosis and fertilization may vary within the life cycles of different organisms. In type A (Fig. 4), the reduction (M) is zygotic, i.e., the first divisions occurring after syngamy (F) are meiotic in character. The haploid state thus prevails during many cell generations until finally gametes arise. Zygotic reduction prevails in most green algae, certain fungi (Phycomycetes and more primitive Ascomycetes), and in certain Protozoa (coccidians and gregarines).

In type B (Fig. 4), the haploid spores arising from the two meiotic divisions (M) grow into multicellular plants (gametophytes) from which the gametes are eventually formed. The fusion of male and female gametes gives rise to multicellular plants (sporophytes) with diploid cells from which eventually the haploid spores are formed. This type is known from red and brown algae; but also a number of green algae belong to type B. In certain brown algae, there is a tendency toward reduction of the gametophyte generation. This culminates in the genus *Fucus*, which belongs to type C (Fig. 4), i.e., the plants are diploid and only the gametes represent the haploid stage.

An alternation between a haploid and a diploid generation prevails also in the higher plants (cormophytes). In the phanerogams, the gametophyte is strongly reduced and remains enclosed in the body of the diploid sporophyte.

The Metazoa belong to type C (Fig. 4). The diploid state predominates; only the gametes (including the polar bodies) are haploid. Among Protozoa, the Heliozoa, Infusoria, and certain amebas belong to the same "diplont" type.

A more detailed survey and discussion with respect to haploid and diploid states in organisms is found in Hartmann (1956a, pp. 1-16). Concerning the manifold conditions prevailing in Fungi, the reader is referred to Raper (1954).

C. The Biological Significance of Fertilization

Fertilization brings about new genetic recombinations, which widen the range of variation within an interbreeding population belonging to one variety or species. This gives material for natural selection. In many cases interbreeding also gives increased vigor to the progeny (heterosis). The role of recombination (amphimixis) was stressed by Weismann as early as 1891 and cannot be excluded from a discussion of the biological significance of fertilization. One may object that autogamy does not entail any recombination but replaces syngamous fertilization. It has been reported for a certain strain of *Paramecium aurelia* that suppression of autogamy leads to the extinction of the strain (Sonneborn, 1947). In *Paramecium*, the advantage of autogamy seems to be comprehensible.

As a rule, it leads to the elimination of the macronucleus and its replacement by a new one which originates from the division of a micronucleus. While the latter forms a reserve of genetic material in the cell, the macronucleus exerts a functional role in the cellular metabolism. Rejuvenation is, no doubt, a rather vague term for the characterization of the role of autogamy, but it may be possible to translate it into more precise terms, such as extent of specific synthetic processes. A rejuvenation in this sense may be a trait in fertilization processes in general. In Metazoa, the establishment of the germ line seems to be an additional method of avoiding wear and tear on exacting mechanisms. These long-standing attempts to explain the biological significance of fertilization seem to be reversed by the fact that certain organisms are able to get along without sexual processes.

In the diatom group, Centrales, certain species in which sexual processes rarely occur have been constant since Miocene times (cf. Patrick, 1954). The constancy may be advantageous under certain conditions, but the greater flexibility adduced by recombination seems more advantageous under other more variable conditions. Likewise "rejuvenation" may be more or less urgent according to the conditions.

D. Activation

As indicated in the preceding paragraph, reproduction may be independent of fertilization. On the other hand, fertilization is an obligatory link in the reproduction of many organisms, e.g., in most metazoans, archegoniates, and phanerogams. In certain algae, gametes may be able to develop parthenogenetically if they fail to undergo syngamy (cf. Hartmann, 1956a). In Metazoa and higher plants, fertilization brings about an "activation" of the gametes. This means that they are transformed from a state with largely blocked capacity of cell division and differentiation to a state in which this capacity is released. The main part of this chapter will be devoted to the phenomenon of activation, which constitutes an important step in the events of embryonic development. Activation is not restricted to multicellular organisms. Certain activation phenomena may be recognized even in unicellular organisms, as will be detailed further below.

E. Species Specificity in Fertilization

Species specificity is an inherent characteristic of fertilization. This specificity is not absolute, but allows a certain degree of cross-fertilization between related species. The specificity leads to the assumption that specific molecular patterns are involved in fertilization. Moreover, male and female gametes should be complementary in this respect. The

complementariness exhibited in fertilization or mating reaction must depend on a complementary structure of macromolecular complexes in the surface of the reacting cells. Positive and negative electrical charges may be so located that they fit into each other. *A priori* it is possible that structures of identical pattern could attract each other. Let us assume that the pattern consists in a certain location of negative charges. The surfaces could then become connected by, for instance, calcium ions, which are known to play an important role in fertilization; concerning similar mechanisms in virus attachment, cf. Puck *et al.* (1951). Possibly both of the types of attraction discussed are instrumental in fertilization. Moreover, the interaction between gametes may involve several steps which present different degrees of specificity. On the whole, the union of the gametes at fertilization should be dependent on the degree of complementariness prevailing between the surfaces of the two gametes. The role of specificity in fertilization was emphasized by F. R. Lillie (1919). He assumes the presence of species-specific substances in the surfaces of egg and spermatozoon. At fertilization, these interact in a process which presents similarities to antigen-antibody reactions. This view has been advocated also by Tyler (1948). In certain organisms, the complementarity in fertilization is not directly determined by sex factors: for example, in *Paramecium*, the gametocyte is monoecious, i.e., both male and female. In several species, "mating types" have been demonstrated. Only individuals belonging to different mating types are able to unite in the mating reaction. The mating-type factors are here responsible for the complementariness in surface structure.

In the intestinal bacterium *Escherichia coli*, strain K12, two different mating types have been distinguished, F^+ and F^- . The F^+ is monoecious and conjugation may occur within the F^+ type. The tendency for conjugation is, however, much stronger between the different types, F^+ and F^- (cf. Lederberg and Lederberg, 1956). Also among Fungi mating types have been distinguished in many species (cf. Raper, 1954).

In many cases, genetic differences do not exist between the gametes. Numerous examples are found among Flagellata, Fungi, and Algae. The already mentioned F^+ of *Escherichia coli* also belongs here. The differentiation, including complementariness of the gametes, must be brought about in these cases by exterior circumstances. As Hartmann (1956a) points out, the factors modifying the nuclei during gamete formation may be located within the organism itself. Regional differences in the organization of the mother cell of the gametes (gametocyte) may influence the nuclei in the direction of male or female differentiation. This explanation may be applicable to the remarkable conditions described by Cleveland (1949) for representatives of the hypermastigote

flagellates, e.g., *Trichonympha*. The gametocyte is haploid; the chromosomes of the male and female gametes are recognizable by their different staining capacity already in pro- and metaphase of the gametocyte division. The species is heterogamous, the two gametes being distinguished both by size and certain morphological differences. Hartmann (1956a) interprets certain of the data reported by Cleveland (1949) as being the expression of relative sexuality. Two females, but also two males with different degrees of sexual differentiation, may copulate.

Incompatibility leading to self-sterility in monoecious forms is known also among higher animals and plants. The well-known self-sterility in the ascidian *Ciona intestinalis* is, however, not an incompatibility of the gametes: the resistance to the penetration of the spermatozoa resides in a layer of cells surrounding the oöcyte. After removal of this layer, syngamy is possible between gametes from the same animal (Morgan, 1939).

In higher plants, the growth of the pollen tube is chiefly implicated in cases of incompatibility within a species (cf. Linskens, 1955).

F. Different Steps in Fertilization

In fertilization, two main steps may be distinguished: (1) coordination (cf. Raper, 1957) and (2) syngamy, which involves plasmo- and karyogamy (cf. Sharp, 1934, p. 231).

The coordinating mechanisms serve to bring the gametes together. These mechanisms are extremely varied in different organisms according to the conditions of life and structural development. The case most completely worked out refers to two species of the phycomycete *Achlya*, in which seven different hormones have been distinguished. In a purposeful way, they regulate the interaction between a male and a female plant until finally syngamy is established (Raper, 1954, 1957). In the water mold *Allomyces* a thermostable hormone attracts male gametes to female gametes. This hormone has been highly purified, its elementary composition and certain of its chemical properties have been tentatively determined (Machlis 1958a, b). Some few other coordinating mechanisms will be discussed below.

The process of syngamy is less varied, but important differences may prevail, e.g., between isogamous and heterogamous forms. The first step may be a looser contact of the gametes, which then is transformed into an effective attachment and ensuing fusion (copulation or conjugation).

II. FERTILIZATION IN SELECTED CASES

After this cursory survey, the fertilization process will be described and discussed in detail for a few types which illustrate different aspects of the fertilization problem.

A. Fertilization in Bacteria (Particularly in *Escherichia coli* K12)

Tatum and Lederberg (1947) demonstrated recombinations of genes after mixture of two mutant substrains of *Escherichia coli* strain K12. It was inferred that some kind of sexual process had occurred.

One of the substrains, Y10, required the presence of threonine, leucine, and thiamin in the medium, whereas the other substrain 58-161 required the presence of biotin and methionine. Y10 could thus be characterized as T⁻ L⁻ B₁⁻ B⁺ M⁺, whereas the substrain 58-161 would correspond to T⁺ L⁺ B₁⁺ B⁻ M⁻. The letters correspond to the genes aligned on a chromosome. After fertilization the zygote would have the formula indicated in Fig. 5. The broken line indicates a recombination by which one chromosome would contain an alignment of plus genes only, the other one, minus genes only. The two chromosomes separate at meiosis and two different clones arise. Until this point the culture medium was supplemented by the metabolites required by the mutant substrains. The

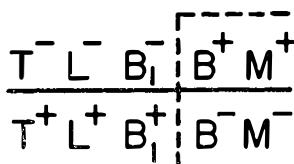


FIG. 5. *Escherichia coli* K12. Recombination of genes giving a prototroph clone. From Lederberg and Tatum (1954).

supplemented medium is now washed away and replaced by a minimum medium in which only the "prototrophs" T⁺ L⁺ B₁⁺ B⁺ M⁺ survive, whereas the auxotrophs, i.e., individuals requiring a supplemented culture medium, die. The frequency of prototrophs (1:10⁷ cells) was too great to be explained by a back-mutation of the minus mutants (expectation on the basis of back mutations: 1 prototroph per 10¹² cells). Several observations indicated that a direct contact between the cells of the different substrains is necessary in order to obtain the recombinants (cf. Lederberg and Tatum, 1954).

As already mentioned above, one mating type, F⁺, is self-fertile. Another mating type, F⁻ is self-sterile, but compatible with F⁺. The F⁺ quality has proved to be contagious, that is, it may be transferred to a population of bacteria of F⁻ mating type.

Mutants of the F⁺ strain which gave a much higher frequency of recombinants than the original F⁺ were isolated. These so-called Hfr were not contagious (cf. Wollman *et al.*, 1956).

Experiments with Hfr × F⁻ indicated that the fertilization had the character of a conjugation in which chromosomal material is transferred

one way, i.e., from *Hfr* (donor strain) to *F*⁻ (receptor strain). This result was obtained by use of appropriate marker genes, giving a selective survival of certain recombinants.

Only one chromosomal segment is involved in this transfer of genetic material from *Hfr* to *F*⁻ of the donor. The farther a gene is located from a certain zero (0) point (cf. Fig. 6), the less probable is its transfer. From a certain point which may represent a break (R), usually no transfer occurs. In certain experiments, the conjugation was interrupted after different intervals of time by treatment in a Waring Blender. The marker genes located closer to 0 have a greater chance of being found among the recombinants than those located at a greater distance from this point. A typical sequence of recognizable genes in the chromosome segment O-R of the donor was: T⁺ L⁺ Az^S T₁^S Lac₁⁺ . . . Gal_b⁺. Of these T⁺ (threonine) is located near 0, whereas Gal_b⁺ (galactose) is adjacent to R (cf. Fig. 6). The corresponding chromosome region of the receptor carried the alleles T⁻ L⁻ A^r T^r Lac⁻ . . . Gal_b⁻. The Blender technique

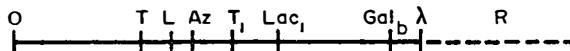


FIG. 6. *Escherichia coli* K12. Linkage group transferred at high frequency in crosses *Hfr*⁺ \times *F*⁻; T, threonine; L, leucine; Az, resistance to azide; T₁, to bacteriophage T₁; Lac₁, fermentation of lactose; Gal_b, fermentation of galactose; λ, lysogeny; R, break on chromosome. Superscripts + and - are used in the text to indicate the presence or absence of the capacity to synthesize or ferment the substances in question. The superscripts r and s refer to resistance or sensitivity to drugs added to the medium. From Wollman *et al.* (1956).

demonstrated that at conjugation the T⁺ and L⁺ (leucine) genes were the first to be transferred from the donor to the recipient individuals. Their transfer took about 8–8½ minutes, whereas the transfer of Gal_b took as much as 25 minutes. The T⁺L⁺ were recognized after transfer to a minimum medium as surviving recombinants with S^r resistance against streptomycin, a gene located beyond Gal_b. These recombinants gave also information about the time of transfer of the nonselected genes Az^S, T₁^S and Lac₁⁺, i.e., 9, 11, and 18 minutes. The times of transfer correspond to the distances between the genes in the transferred chromosome segment. The donor contained the gene of S^s as allele of S^r. The presence of S^s leads to the elimination of the donor cells in the streptomycin containing medium.

Similar results to those reported were obtained when the donors were eliminated, after different intervals of time, with a phage to which it was sensitive. The locus λ, a lysogeny gene (cf. Fig. 6), also played a role in these studies (Lederberg and Lederberg, 1953). When it is

introduced from *Hfr* cells into λ -negative F^- cells, lysis occurs as a consequence of the transformation of λ into phage (cf. Wollman *et al.*, 1956). The lysis was observed, however, in only about 50 per cent of the zygotes. This is a further indication of a partial transfer. As a consequence of its distance from the zero point the λ -gene is not transferred in all events of conjugation.

The conjugation has been studied in both the phase contrast and the electron microscopes. Direct observation does not, however, reveal the details about the chromosome mechanism which have been recognized by the genetical analysis.

The contagious male character F^+ seems to be represented by extra-nuclear particles which spread to F^- individuals converting them into F^+ individuals. However, the F^+ factor occurs also in a state when it is bound to some chromosomal locus. This is the case in the *Hfr* mutants which explains why the *Hfr* character is not contagious (Lederberg, 1958).

No doubt a certain specificity prevails in the sexual phenomena in the bacteria but cross fertilizations have also been carried out (for further details concerning the subject of this section, the survey by Cavalli-Sforza, 1957, should be consulted).

Wollman *et al.* (1956) propose to call the zygote formed in *Escherichia coli* a merozygote because the genetic transfer from the donor strain is not complete. Moreover, they designate this type of genetic transfer as meromixis. Under this designation they also wish to include transformation (cf. Hotchkiss, 1955) and transduction (cf. Lederberg, 1955), two other forms of partial genetic transfer known among bacteria.

B. Fertilization in Green Algae (Particularly in *Chlamydomonas*)

The propagation of the unicellular algae of the genus *Chlamydomonas* is brought about by fission of the haploid form (cf. Fig. 1). Gametes arise particularly under certain conditions of the medium, e.g., at low nitrogen content (Sager and Granick, 1954). The gametes are in general of equal size and appearance (isogamy) and apparently do not differ from the vegetative form. On mixing individuals of different sexes from dioecious clones, a "group formation" may occur. This latter leads to copulation between individuals of opposite sex (cf. Hartmann, 1956a, b).

The group formation is caused by hormones or "gamones" produced by the gametes and, in *Chlamydomonas eugametos*, these gamones can be demonstrated in filtrates of the culture medium. In some other species, it proved to be more difficult to get active filtrates. Filtrates from female individuals cause a group formation of male individuals, and vice versa.

The female individuals become sexually mature and gynogamones (female gamones) are formed in the absence of light. The male individuals reach their sexual maturity only after having been illuminated in the culture medium.

The male gametes do not remain responsive to the gynogamone in darkness. It seems that, in male individuals, photosynthesis is directly necessary for the formation of androgamones (male gamones) as well as for maintenance of responsiveness to the gynogamone.

The gamones of the two species *Chlamydomonas eugametos* and *C. reinhardi* have proved to be species specific. There is no cross-aggregation, neither is there any cross-fertilization.

Förster *et al.* (1956) have determined the chemical and physical properties of the female gamone secreted in *Chlamydomonas eugametos*—the female gamone being more resistant and easier to obtain than the male gamone. The female gamone was found to be a glycoprotein of spherical form and of high molecular weight, about 10^8 .

The pluricellular threadlike green alga *Chaetomorpha aerea* is dioecious but isogamous. Its gametes present a typical group formation, but no release of gamones to culture medium could be demonstrated (Köhler, 1956). Probably the gamones remain attached to the flagella, particularly to their terminal regions. Electron microscopic observations gave indications of the presence of certain blisters in this region. The gamones may primarily be components of the surface of the flagella. In some species, they may be produced in such quantity that they are given off to the medium, whereas in other species they may be formed less abundantly or may be more firmly attached to the surface of the flagella.

The group formation is, in the first place, a coordinating mechanism which brings the sexually different gametes together. The mechanism of the cardinal event, the syngamy, is, however, unknown. Do the "gamones" play a direct role also in the fusion of the cell bodies, or is the surface system here a different one?

In *Chlamydomonas*, as in many other green algae, the change from diplo- to haplophase occurs in the zygote (cf. Figs. 1 and 4).

C. Fertilization in Infusoria (Particularly in Paramecium)

The fertilization process has been most extensively studied in *Paramecium aurelia*, which will therefore be chosen as a representative of the ciliate infusoria (cf. reviews by Sonneborn, 1947; Kimball, 1943; Metz, 1954; and Beale, 1954). In this organism also, the propagation is mainly brought about by fission. The fertilization has the character of a conjugation between individuals belonging to plus and minus mating types within the same variety of the species. Conjugation can occur

between plus and minus individuals belonging to different varieties only to a limited extent. Autogamy as well as conjugation plays an important role. The conjugation is initiated by an aggregation of a great number of animals. Gradually the aggregates break up and now mating pairs and single individuals separate from the masses. The aggregation is the result of the action of the mating substances. These cannot be demonstrated, however, in the culture medium. The aggregation is brought about by occasional contact between the animals of different mating types. The mating substances cannot be removed from the surface of the animals by washing. Evidently they are components of the cell surface.

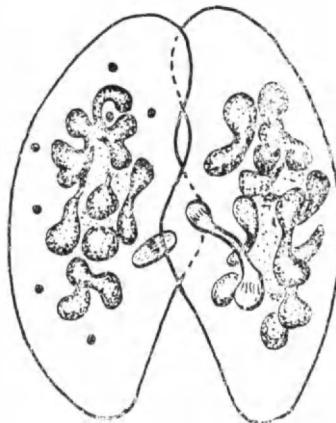


FIG. 7. Conjugating *Paramecium aurelia* in a holdfast and paroral union. The two nuclei of the gametocytes have undergone meiotic divisions which result in the formation of eight haploid nuclei of which seven degenerate, cf. left-hand individual. The eighth nucleus divides into two, one is the male migrating pronucleus, the other the remaining female pronucleus. In the right-hand individual, this division is represented in a more advanced stage. The macronuclei are undergoing fragmentation. From the diploid zygote nucleus formed by the union of the male and female pronuclei, two micro- and two macronuclei develop. At first fission after conjugation, each individual gets two micronuclei and one macronucleus. From Grell (1956).

The specific reaction between the mating substances of opposite types seems to start a chain of reactions. The two individuals of conjugated pairs which appear when the first-formed aggregates dissolve are held together by their anterior parts. This is the "holdfast" union (cf. Fig. 7) which is mediated by special substances. These do not exhibit mating-type specificity. In the stage of holdfast union, the conjugating animals can still be separated without injury.

The formation of the holdfast substances is probably a consequence

of the specific reaction of the mating substances. According to Metz (1954) however, the reaction of the mating substances is not an obligatory link but can be replaced by other factors. "Killer" fluid from animals belonging to a certain stock of one variety of *P. aurelia* was found to bring about activation in a "sensitive" stock of another variety. If conjugation occurred, paired animals in holdfast union appeared without previous general aggregation.

Another important change following conjugation and autogamy is the loss of mating reactivity. This may occur soon after the aggregation. From this, Metz (1954) concludes that the loss of mating reactivity is independent of activation phenomena other than the mating reaction. According to the view of Sonneborn (1947), the disappearance of the mating reactivity means a decrease in concentration of the mating substances. A regeneration of these substances occurs after a certain interval of time which varies according to circumstances and the species concerned.

The paroral cones are formed only after formation of the holdfast union of the conjugants (cf. Fig. 7). By their fusion a still stronger union of the conjugants is established.

Already before the formation of the paroral cones, the meiotic processes are started within the mating cells. In *P. aurelia*, two micronuclei are present in each individual. At the onset of meiosis each of these is divided by two consecutive meiotic divisions into four nuclei. Of the eight nuclei thus present, seven break down. In each of the mates, the remaining nucleus divides into two (third prezygotic division) and there follows an exchange of nuclei between the mates. This occurs by way of a fine bridge formed in the paroral region (cf. Fig. 7). The diploid zygote nucleus is the product of fusion between one remaining nucleus and one received from the other mate.

During the second half of the mating period, the macronucleus fragments (Fig. 7) and is then completely broken down after separation of the conjugants. The zygote nucleus divides twice so that four nuclei are formed; of these, two are transformed into new macronuclei and two remain micronuclei. The separation of the mates occurs before the second division (for further details cf. Sonneborn, 1947).

The preceding description demonstrates an important connection between surface changes and the initiation of complicated nuclear events.

It has been proved that even individuals killed by certain reagents are still capable of inducing mating reactions with living animals of opposite mating type (Metz, 1947; Metz and Foley, 1949). For these studies, the strongly reactive animals of the two mating types of variety

4 were used. Animals of one or the other of these types were killed by treatment with about 1% formaldehyde. Mixing of killed and living animals of the different types caused a strong aggregation. From this, single animals and mating pairs were gradually released. The latter did not enter into a real conjugation. Only the holdfast union was established, and this was soon broken. The animals which entered into holdfast union belonged to the same mating group; but following the contact with killed animals a partial reversal of mating type occurred. The phenomenon observed was called pseudoselfing—"selfing" because pairing occurred between individuals belonging initially to the same mating type, but "pseudo" because no real conjugation ensued. Nevertheless a nuclear reorganization was induced in the pseudoselfing individuals. This resembles what occurs in autogamy, i.e., meiotic divisions take place. The nuclei which arise in the third prezygotic division reunite to form a diploid zygote nucleus. The macronucleus undergoes fragmentation. As following complete conjugation, two new macronuclei and two micronuclei are formed from the zygote nucleus before the fission starts.

Nuclear reorganization was also observed in single individuals which separated from the initial aggregation. This is obviously a consequence of the contact with killed animals of opposite mating type.

This experiment demonstrates that a reaction between cell surfaces may induce significant changes in the interior of the animal even if the individuals belonging to one of the mating types involved are killed. No essential change of the surface pattern seems to ensue subsequent to killing the animal.

Metz and Foley (1949) studied also a stock of *Paramecium aurelia* in which the animals had no ability to form conjugation pairs. Nevertheless, individuals belonging to this so-called CM stock gave a strong mating reaction with CM or normal animals of opposite mating type, but after dissolution of the aggregates no further changes occurred in the CM animals. There is a block in these animals which interrupts the chain of reactions started in normal animals by the mating reaction. On the other hand, living or killed CM animals are able to induce "pseudoselfing" in normal animals belonging to the opposite mating type. Killed normal animals initiate the mating reaction in CM animals but, in this case also, they undergo no further change. The nuclear reorganization is brought about by autogamy in CM animals.

In conjugation, the series of events probably reflects a branching chain of reactions: $a \rightarrow b \rightarrow c \rightarrow d \dots$, where a may represent the mating reaction which seems to start the events. In autogamy, however, the mating reaction does not operate. The first link in the series may now be represented by c , and this initiates the subsequent chain of

reaction, *d*, etc. The CM character may reflect a block in *b*, which cannot be surpassed even if the mating reaction *a* is operating. In consequence of this, *a* fails to start the sequence of reactions. However, if by internal conditions *c* becomes activated, the somewhat abbreviated and modified chain of events gets started which corresponds to autogamy.

Conditions analogous to those prevailing in *Paramecium aurelia* were found in other ciliates (cf. Metz, 1954). In *Euploites patella* no mass aggregation was found. The conjugation is induced by a soluble substance, the presence of which can be demonstrated in the culture fluid. The substance secreted by one mating type induces conjugation in other mating types which do not produce the same substance (or the same pair of substances when heterozygotic animals are concerned). As in the green algae, the specific substances involved in the primary step of interaction between gametes may thus be either more firmly fixed components of the surface of the cells or soluble agents which are released to the culture medium.

According to the extended tests carried out by Metz (1954), the mating substances in *Paramecium aurelia* have a protein nature. The formation of the mating-type substances is probably under nuclear control, but the lability which is common in the mating-type determinants cannot yet be explained in a satisfactory way (cf. Kimball, 1943; Sonneborn 1947). Clarification may be expected from Nanney's (1957) more recent work on *Tetrahymena*.

D. Fertilization in Metazoa (Particularly the Sea Urchin)

1. Time Relations between Maturation of the Egg and Acceptance of the Spermatozoon

After the oögonial divisions have taken place, the oöcytes enter a period of growth. Sooner or later following conclusion of this period, a block is established which inhibits further division of the cell and probably creates a certain inertness, if not a complete standstill of the anabolic processes. In some animals, the block inhibits the meiosis (*a*); in others, this process may proceed until the metaphase of the first (*b*) or to that of the second meiotic division (*c*). To the first (*a*) of these types belong nematodes, for example, *Ascaris*, various annelids, such as *Thalassema* and *Nereis*, mollusks and crustaceans. To the second type (*b*) belong nemertines, e.g., *Cerebratulus*; annelids, e.g., *Chaetopterus*; and the insects. Type (*c*) is characteristic of chordates. Finally there is a type (*d*) in which both meiotic divisions must be accomplished before the egg is able to accept a fertilizing spermatozoon. To this group belong the sea urchins and certain representatives of Coelenterata. For

literature and further details, see Wilson (1925), Just (1939), Rothschild (1956).

In cases *a-c* the reception of the spermatozoon induces the accomplishment of the meiosis as well as the cleavage of the egg. In case (*d*), the completion of meiosis is independent of fertilization.

2. Time-Graded Changes in Oöcytes, Eggs, and Spermatozoa

In sea urchins, spermatozoa are able to penetrate into the primary oöcytes. There is no barrier against the penetration of more than one spermatozoon (polyspermy), but no swelling of the sperm head and no aster formation occurs. Thus no effective fertilization ensues. The breakdown of the nucleus (the germinal vesicle) of the primary oöcyte and the release of its contents seems to be a prerequisite for the evolvement of the nucleus and the aster of the spermatozoon. Delage (1901) separated the primary oöcytes of starfish into two fragments, one containing the germinal vesicle, the other devoid of nuclear material. Only in the first-mentioned fragment did an effective fertilization occur. If the separation was carried out after the dissolution of the membrane of the germinal vesicle, an effective fertilization was possible also in the non-nucleated part of the cytoplasm. Similar conditions were also found in mollusk and annelid eggs (Delage, 1901; Costello, 1940). In these and in starfish eggs, the protection against polyspermy is built up as early as the oöcyte stage. Conversely, in the sea urchin egg, the protecting mechanism is built up only after the completion of the meiotic divisions. During these divisions, several spermatozoa can penetrate the egg. The nuclei immediately form mitotic figures of the same stage as that exhibited in the meiotic division. Such polyspermic eggs remain blocked in this stage until they finally cytolize (Brachet, 1922). Similar observations have been made on amphibian eggs, *Hyla* and *Triturus*, when they are inseminated in the stage of the first maturation spindle (Bataillon, 1929a, b; Bataillon and Su, 1934). Even after completion of the meiotic divisions, more than one spermatozoon may penetrate into a sea urchin egg. This corresponds to a stage of cytoplasmic underripeness in which the mechanism protecting against polyspermy is not yet complete, (Runnström and Monné, 1945). The structure of the cortical layer is an important factor in the protection against polyspermy. In sea urchins, this layer undergoes considerable changes in connection with the meiotic divisions. Nothing demonstrates this more clearly than a comparison between the position of the so-called cortical granules in the oöcytes and in the mature egg. As was first shown by Monné and Härde (1951), these granules, which in the fixed and sectioned material are stained by pyronine, are scattered in the interior of the cytoplasm, whereas in the

mature egg they are distributed in a uniform layer in the egg cortex (cf. Fig. 8). This rearrangement can only occur through considerable displacements in the cytoplasm. Also, in the starfish egg, cortical granules are recognizable, but they enter the cortical layer as early as in the primary oöcyte, i.e., prior to the meiotic divisions (Runnström, 1944). In a mammal, the hamster, a layer of cortical granules is also found in the primary oöcyte as in the starfish (Austin, 1956). These data suggest that the difference between sea urchin eggs on one hand and the types *a-c* on the other (referred to in Section II, D, 1) resides in a different rate of establishment of the cortical structure. The earlier this

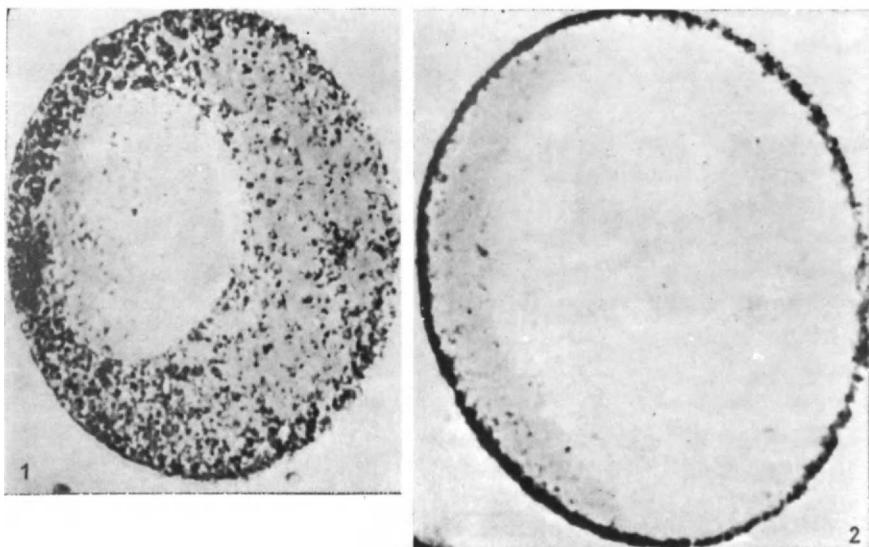


FIG. 8. Oöcyte (1) and mature unfertilized egg (2) of *Arabacia lixula*. In the oöcyte, the prospective cortical granules are irregularly scattered, whereas in the mature egg they are present only within the cortex. Nucleic acids extracted, granules stained with pyronine. After Monné and Härde (1951) Plate II, Figs. 1 and 2.

is established, the earlier also the protection against polyspermy develops. Without this protection effective fertilization is not possible. So-called physiological polyspermy prevails in certain metazoa—Insects, selachians, urodeles, reptiles, and birds. The eggs are rich in yolk in all these animals. Despite the polyspermy, the syncaryon is formed by the fusion of the female pronucleus with one male pronucleus, whereas the other sperm nuclei gradually vanish. The mechanism of exclusion differs from that operating in monospermic eggs, but the end result is the same (cf. Fankhauser, 1948).

In work with sea urchins, the impression is gained that the state of the egg cortex is slowly but continuously changing. In the underripe egg, the fertilization is comparatively slow and the cortical changes are incomplete. In sea water, the eggs undergo a maturation which involves an increase in the rate of fertilization and improvement with respect to the cortical changes (B. Hagström, 1955). Finally, in the overripe state, fertilization becomes abnormal, with failure of formation of the fertilization membrane, etc. The state of underripeness may be removed by treatment of the eggs, e.g., with diluted serum, 0.1–0.2% serum albumin, 0.025–0.1 M amino acids, and 10^{-5} M sodium periodate (cf. Runnström, 1949, 1954; Wicklund, 1954). These treatments bring about a decrease in the rigidity of the egg cortex (Wicklund *et al.*, 1953). It is evident that calcium enhances the state of overripeness. The period of fertilizability of the sea urchin egg is prolonged by exposure to calcium-free sea water (Lindahl, 1936). Mammal eggs undergo an aging after ovulation in which the rate of reaction of the egg to contact with the spermatozoon becomes slower (cf. Austin and Bishop, 1957).

Sea urchin spermatozoa also undergo a maturation which renders them more agglutinable by "egg water," i.e., sea water which has been in contact with eggs (Goldfarb, 1929). Sea urchin spermatozoa undergo rapid aging when suspended in sea water. The more diluted the sperm, the more rapid is the aging (F. R. Lillie, 1919). Recently this phenomenon has been studied by Rybak (1956). Addition of amino acids or serum albumin to the sea water prolongs considerably the fertilizing period of the spermatozoa (Tyler, 1950; Vasseur *et al.*, 1950). Important work on the physiology of mammal semen (Mann, 1954) can only be mentioned here.

In the sea urchins, seasonal variations prevail with respect to the state of eggs and spermatozoa. Early in the breeding season the state of cytoplasmic underripeness of the eggs is more frequent than at the height of the season. Moreover, the agglutinability of the spermatozoa by egg water is not pronounced in the earlier part of the breeding season. In observations on *Strongylocentrotus droebachiensis*, this was shown to depend on the sperm rather than on the egg water (Vasseur, 1949).

3. Coordinating Factors in Metazoa

Even where the fertilization is external as in sea urchins, the genital products are not released in a haphazard way. It has been observed how the spawning of a male sea urchin induces the spawning of other males and females (Fox, 1924). Galtsoff (1940) showed that under natural conditions spawning in oysters is initiated by the males. Wada (1954),

on the other hand, found that ovarian suspensions induce spawning in tridacnic clams, whereas sperm or testicular tissue are ineffective.

Internal fertilization should secure the highest chance for male and female gametes to meet. Austin and Bishop (1957) remark that despite "the large number of spermatozoa ejaculated in mammals surprisingly few reach the site of fertilization." Evidently a strong selection is exerted during their passage in the female ducts. For further data concerning coordinating factors in mammals the reader is also referred to the monograph by Chang and Pincus (1951).

The spawning-inducing agents mentioned above act directly or via nervous mechanism on muscles which regulate the release of the gametes. Other agents mediate a direct interaction between male and female gametes and may play an important role in the coordination of the gametes.

Hartmann and his colleagues have aptly proposed calling such interacting substances andro- or gynogamones according to their origin from male or female gametes (cf. Hartmann and Schartau, 1939; Hartmann, 1956a). Our knowledge about the gamones, their chemical nature and biological action is still very incomplete. Therefore the strict systematization of the gamones proposed by the German workers will not be followed in the following presentation.

Sea water in which sea urchin eggs are suspended ("egg water") was shown to contain an agent causing a temporary agglutination of the spermatozoa. F. R. Lillie (1913, 1914) undertook a thorough study of this phenomenon both in sea urchins and in *Nereis* (cf. Lillie, 1919; Lillie and Just, 1924). Lillie considered that the agglutinin is secreted from the egg. It may be stored in the jelly layer which surrounds the egg, but its secretion continues even after removal of the jelly from the egg surface. The secretion of the agglutinin commences at maturation and stops at fertilization of the egg. Lillie proposed calling the agglutinating substance "fertilizin" because he considered that this substance plays an essential role in fertilization. According to Lillie, the fertilizin is a diffusible component of the egg surface and fertilization involves a species-specific reaction between this component and a complementary factor present in the surface of the spermatozoon or (cf. Section I, e.). The egg contains another factor, antifertilizin. The latter reacts at fertilization with the fertilizin and saturates the groups which had not reacted with the sperm factor. In this way, the fertilizin of the egg surface is bound. After the completion of this process, no other spermatozoon is able to react with the egg surface. In Lillie's concept, "fertilizin" is the central factor in the activation system. Loeb (1914, 1915) stated, however, that after removal of the jelly layer no sperm agglutination occurs. Nevertheless the egg may be fertilized, whereas according to Lillie's

original idea the fertilizability disappears with the discontinuance of fertilizin secretion. Today the view is held that the sperm-agglutinating factor is released from the jelly surrounding the egg. (Hartmann and Schartau, 1939; Tyler, 1940, 1941a; Vasseur, 1952b).

The jelly layer of the sea urchin egg can be removed by treating the eggs with moderately acidified sea water. The jelly is therefore accessible to a chemical analysis. It has proved to be acid in character and behaves electrophoretically as a uniform substance. In the ultracentrifuge, a well-defined main component and a more rapidly migrating minor component were observed. The molecules or micelles are asymmetric, which also accounts for the strong birefringence of flow (cf. Runnström *et al.*, 1942; Runnström, 1949; Tyler, 1949, 1955). Vasseur (1947, 1948) demonstrated that 80% of the jelly substance consists of a polysaccharide

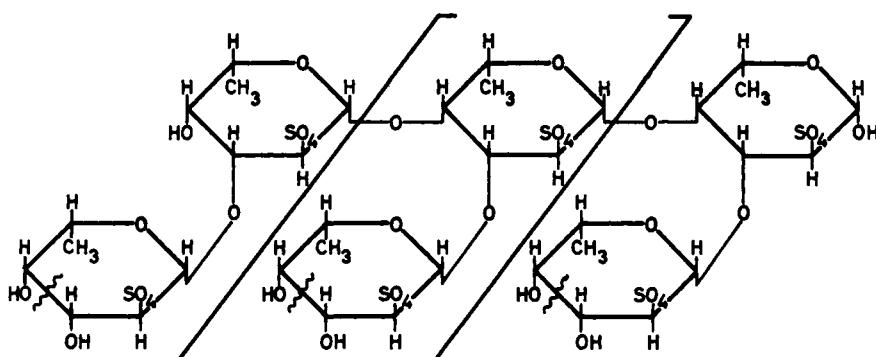


FIG. 9. Tentative formula of the main polysaccharide component of the jelly coat of *Echinocardium cordatum*. From Vasseur (1952a).

esterified with sulfuric acid; 20% of the jelly substance consists of amino acids, probably in polypeptide linkage. Vasseur and Tyler have presented data concerning the building blocks of the acid polysaccharide as well as of the peptide moieties of the jelly. Figure 9 gives a tentative formula of the polysaccharide chain contained in the jelly of *Echinocardium cordatum*. According to this formula, there is a backbone of fucose residues. From this, other fucose residues branch off in a comblike way. Each fucose residue is esterified with sulfuric acid in the 2-position. This esterification accounts for the acid character of the jelly. The wavy lines of Fig. 9 indicate the places accessible to oxidation with sodium periodate at pH 3.5. The jelly layer does not contain hexoseamine. The jelly substance exerts a heparinlike action in a blood-clotting system (Immers and Vasseur, 1949).

On basis of the physicochemical data, Tyler regards the jelly as a

uniform substance and identifies it with fertilizin, i.e., the species-specific factor complementary to a factor in the surface of the spermatozoon which is called "antifertilizin."

According to Tyler, the "fertilizin" (jelly substance) should be an essential factor for fertilization. He was led to this view by experiments which seemingly demonstrated that the presence of the jelly coat had a favorable effect on fertilization (cf. Tyler, 1948).

Recently, Hagström (1956a, b, d, e) has emphasized the fact that the sea urchin egg is fertilizable after removal of the jelly layer. Moreover, he stated that the rate of fertilization is even higher in the absence than in the presence of the jelly coat. The contrary results obtained by Tyler were due to the use of sea water of a sub-optimal pH when removing the jelly from the eggs. In this way the eggs were damaged. Hagström's data will be considered in more detail in a following section (II, D, 4, c). Let us, however, refer to his conclusions already in this place. The jelly layer does not improve fertilization, but acts as a sieve on the spermatozoa. Through an elimination process, the number of spermatozoa capable of effective attachment to the cytoplasmic surface of the egg is strongly reduced. Certain experiments indicate that 15–20% of the spermatozoa are still capable of fertilizing the egg even after contact with the jelly layer. This layer is subjected to great variations with respect to swelling and other properties which may also cause variations in the sieve action of the layer. As already mentioned above in this section, only a reduced number of the ejaculated spermatozoa reach the site of the egg in mammals. In the sea urchin egg, the jelly layer provides a similar reducing mechanism which cannot fail to be an important factor in the protection against polyspermy. The agglutination of the spermatozoa by egg water—jelly substance in solution—is probably only another aspect of the sieve action of the jelly. Lillie and Tyler consider that spermatozoa which have reacted with fertilizin are no longer capable of fertilizing. This would mean that they are eliminated. However, more experimental work is needed to ascertain the possible significance for fertilization of the sperm-agglutinating effect of the jelly substance.

The induction in egg water of the so-called acrosome reaction (Dan, 1956) could also contribute to the sperm elimination. Contact with egg water seems to induce a change in the acrosome region of the spermatozoon which results in the projection of a stalklike filament from the anterior part of the spermatozoon (Fig. 11).

Motomura (1950a) showed that a sperm-agglutinating factor can be extracted by short boiling of unfertilized eggs devoid of their jelly layer. This factor has been called "cytostabilin." It was demonstrated in two

species of *Strongylocentrotus*. Moreover, Motomura's results were confirmed by Hagström (1956c) for *Paracentrotus lividus*, *Arbacia lixula*, and *Psammechinus miliaris*, and *P. microtuberculatus*. Cytofertilizin is released from jelly-free aging eggs, kept for 12–16 hours in sea water. Its secretion increased until 24–30 hours when the eggs began to cytolyze (Hagström, 1956c). According to Motomura (1950a, c, 1953) a secretion of cytofertilizin occurs at fertilization so that the factor is present in the perivitelline space. The properties and location of the cytofertilizin are different from those of the agglutinating factor of the jelly. The cytofertilizin has been mentioned in this context although it is in no way certain that it should be referred to as a coordinating factor.

F. R. Lillie distinguished between two properties of the "fertilizin," one sperm-agglutinating and one activating the motility of the spermatozoa. He left it undecided whether these are two common properties of one factor or if they are due to two distinct factors. Further work has shown that a high-molecular component of egg water or jelly solution is able to increase the sperm motility; but a low-molecular component which dialyzes through a cellophane bag also has this effect (cf. Cornman, 1941; Vasseur and Hagström, 1946). The dialyzable factor may simply be a depolymerization product of the nondialyzable factor. The agglutinating, but not the motility-activating factor is destroyed by heating the jelly solution at 90–100° C. for 1–2 hours (Vasseur and Wicklund, 1953). The sperm agglutination is species specific whereas the sperm activation is not (Hagström, 1956d). Further work will probably reveal still unknown agents in the jelly coat (cf. Lybing and Hagström, 1957).

It has been considered that coordinating agents are released also from the spermatozoa. Moreover, the seminal fluid certainly contains substances which do not necessarily originate in the spermatozoa. Hultin and Hagström (1955) demonstrated that substances present in the seminal fluid may influence the rate of fertilization in the sea urchin. One of these substances seems to have lipid character.

Hartmann and Schartau (1939) claimed that sea urchin spermatozoa carry a factor which dissolves the jelly layer of the sea urchin egg; but no conclusive evidence has been presented so far for the presence in the sea urchin spermatozoon of an enzyme which would split the jelly substance. Hartmann (1956a) described how spermatozoa may attack fragments of the jelly layer and bring it to dissolution. Certain substances chemically related to the jelly may act as inhibitors of this dissolution (Runnström and Hagström, 1955). These observations indicate, but do not prove, that enzymatic activity is involved.

In certain mollusks, on the other hand, the spermatozoa carry a sub-

stance which is capable of dissolving the vitelline membrane of the egg. This was first shown by Tyler (1939) for the gastropod *Megathura crenulata*. These results have been confirmed and extended to the pelecypod *Mytilus* by Berg (1950). The "lysine" has protein character and is probably an enzyme. McClean and Rowlands (1942) discovered that mammalian spermatozoa are carriers of a hyaluronidase. This enzyme dissolves the cement substances of the follicle cells which surround the eggs and may in this way facilitate the passage of the spermatozoa to the egg (cf. Swyer, 1951).

A further coordinating factor may easily be extracted from a suspension of sea urchin sperm by such treatment as lowering of the pH (Tyler and O'Melveny, 1941). Great interest was attached to this factor because it precipitated the jelly layer and suppressed the sperm-agglutinating action of jelly in solution. The factor was therefore called "antifertilizin" and was considered to be closely related to the antifertilizin from the egg. It would represent the complementary factor in the spermatozoon which reacts specifically with fertilizin. However, the precipitating and agglutination inhibiting action was found to be due to basic proteins and deoxyribonucleoproteins which are easily extracted from the spermatozoa (Hultin, 1947, 1949b). The precipitation is of a nonspecific character, as stated also by Hartmann and Schartau (1939). It is possible that here an unspecific reaction covers a specific one. It may also be that the complementary sperm factor is not soluble. Its existence is perhaps supported by an experiment carried out by Hultin *et al.* (1952). It was shown that spermatozoa of *Echinocardium cordatum* are able to remove homologous jelly substance from a solution, whereas spermatozoa of another irregular sea urchin *Briissopsis lyrifera* were inactive in this respect. This is probably an expression of the species specificity of the reaction, cf. also Monroy *et al.* (1954).

4. Plasmogamy

a. Attachment of the spermatozoon to the egg surface. The number of spermatozoa which come into contact with egg surface depends on the relative quantities of spermatozoa and eggs. Several spermatozoa may be seen to have contact with the egg surface in sea urchins, but, under a wide range of sperm densities, only one spermatozoon becomes effectively attached and thus brings about fertilization.

It seems that the contact between egg and spermatozoon releases the acrosome reaction which has been briefly mentioned above. In the acrosome reaction, the acrosomal globule is released (Figs. 10 and 11) (Afzelius, 1955; Afzelius and Murray, 1957). As seen in Fig. 11, the end of the filament is in contact with the egg surface, which is provided

with numerous papillae (Cheney and Lansing, 1955). If several spermatozoa have contacted the egg surface, they all undergo the acrosome reaction. The contact between the egg surface and acrosome filament must first be loose, so that the spermatozoon is still able to rotate around its axis, often with a spirally contracted tail. The next step is an attachment. Even if this is effective, i.e., leads to fertilization, the spermatozoon is rather loosely attached for 15–20 seconds. This probably means that it is still attached only by its acrosome filament (Runnström, unpublished). According to Dan (1956) and Afzelius and Murray (1957), the attached acrosome filament contracts; in this way the border of the concavity visible in Fig. 11 will press itself against the egg surface. Soon after, the elevating membrane sweeps over the sperm head.

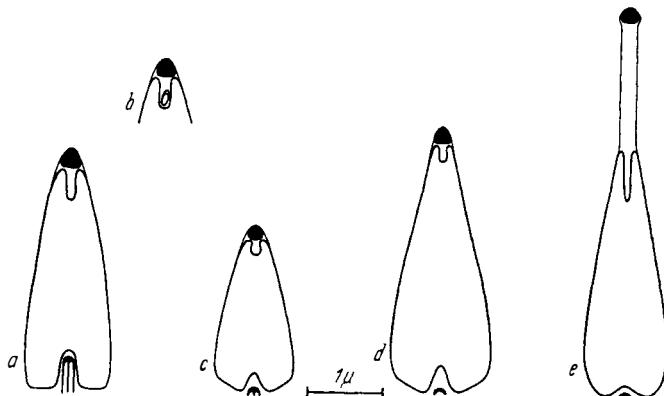


FIG. 10. Outlines of the heads of sea urchin spermatozoa. a, b, *Strongylocentrotus droebachiensis*. c, *Psammechinus miliaris*. d, *Echinus esculentus*. e, *Echinocardium cordatum*. From Afzelius (1955).

In many forms the acrosome reaction is still more obvious than in sea urchins. However, further details must be omitted and only a reference made to Colwin and Colwin's (1955, 1956) and Dan's (1956) numerous observations on starfishes, holothurians, and mollusks. It is of great interest that, in *Mytilus*, Wada *et al.* (1956) have very probably located the sperm lysin (cf. Section II, D, 3) in the acrosome. It has also been subject to study in mammals (cf. Nath, 1956; and Austin and Bishop, 1957). Bowen (1924) suggested that the acrosome is responsible for the activation of the egg. This view is amply supported by observations made by Bataillon (1910) and Reverberi (1935). They carried out cross-fertilization respectively between amphibian and ascidian species. In many cases, the eggs were activated by mere contact between the spermatozoa and the foreign eggs. The active part of the spermatozoon

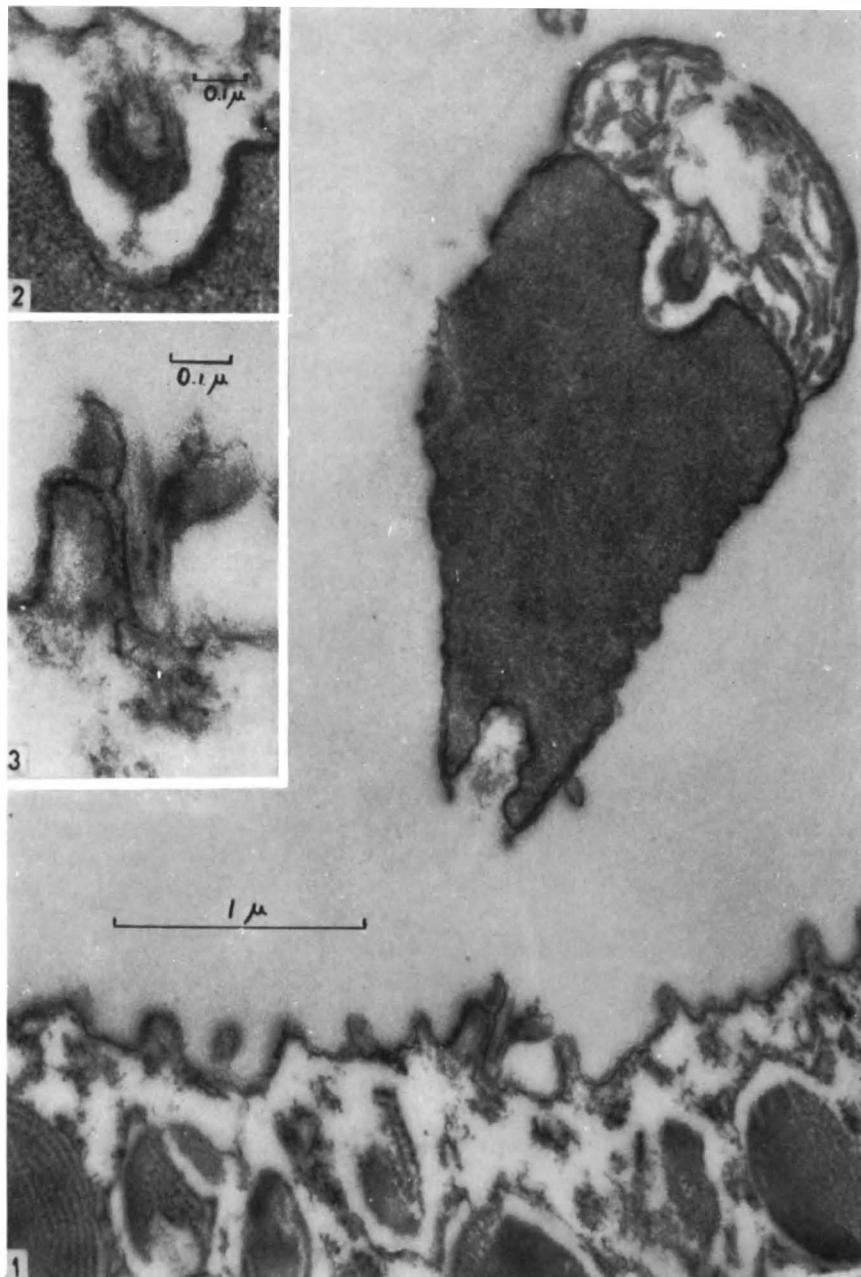


FIG. 11. *Strongylocentrotus droebachiensis*, spermatozoon. 1. The acrosomal globule expelled. The acrosomal filament fixes the spermatozoon to the egg surface. The lower inset (3) shows the fixation of the filament to the egg surface; the upper inset (2), the centrosome with its condensed part, the centriole at higher magnification. From Afzelius and Murray (1957).

must here have been the acrosome region. More recently, Runnström (1957a) pretreated eggs of *Paracentrotus lividus* with different concentrations of the oxidation-reduction indicator porphyrexid, e.g., $10^{-4} M$. After washing, the eggs were inseminated. As a consequence of a toughening of the vitelline membrane, the spermatozoa were not always capable of penetrating this membrane but the acrosome part became attached to it, as shown in Fig. 12. Nevertheless the egg was activated with

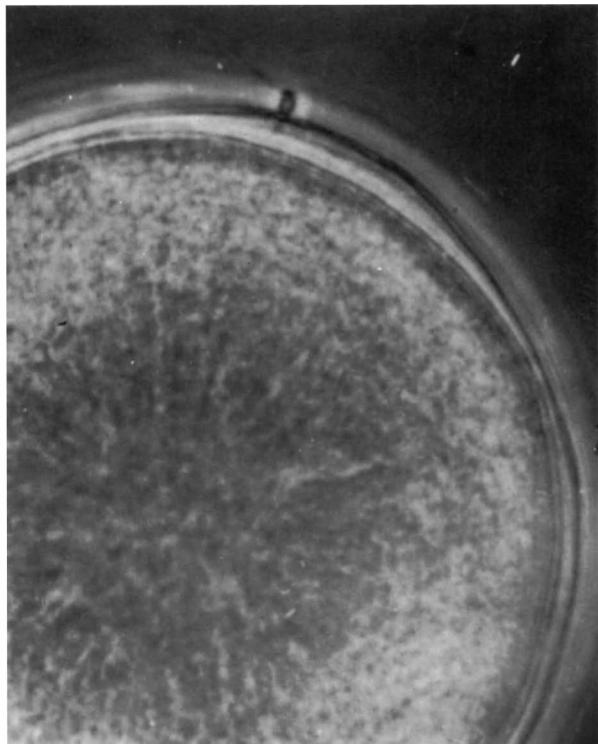


FIG. 12. *Paracentrotus lividus* egg pretreated for 17 hours with $7.4 \times 10^{-5} M$ porphyrindine in sea water. At fertilization in normal sea water, the spermatozoon is trapped in the membrane but nevertheless causes cortical changes and monaster formation. From Runnström (1957a).

ensuing cortical changes. In some cases, only a small region of the egg was activated or the activation spread over a great part of the egg surface without, however, covering the whole egg. It is possible that the different steps of activation may depend on different degrees of attachment of the acrosome filament. The fact that the spermatozoa were lifted up with the membrane elevation indicates that the spermatozoon in these cases never reached the stage of attachment by the border of its anterior concavity visible in Fig. 11. The observations again suggest a

transfer of certain activation-inducing substances by mediation of the acrosome filament. Such phenomena may be designated as pseudogamy.

b. Kinetics of sperm-egg attachment. Rothschild and Swann (1949, 1951) assume that the sperm-egg contact can be treated in the same way as collisions between gas molecules. They put Z , the number of collisions per time unit, equal to the product of egg surface, number of spermatozoa, and mean speed of spermatozoa. The question arises as to how many collisions are necessary to obtain fertilization. The authors carried out a number of determinations of the rate of effective attachment, i.e., fertilization at different times after insemination. This is

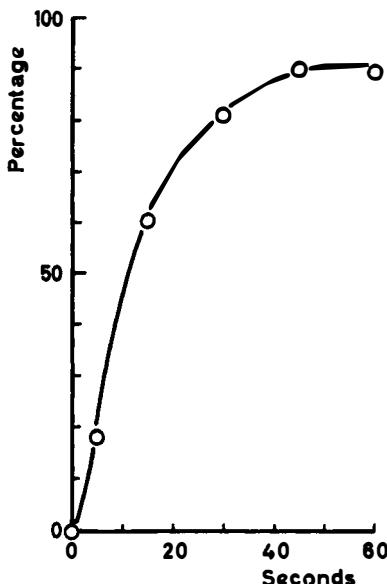


FIG. 13. *Psammechinus miliaris*. Curve showing the percentage of fertilized eggs at different intervals after insemination; sperm density $\sim 4 \times 10^5$ per milliliter. From Rothschild and Swann (1951).

brought about by immersing samples of the sperm-egg mixture in hypotonic sea water at different intervals, e.g., 5, 15, 30, 45, etc., seconds after insemination. In this way, fertilization was interrupted. The medium was thereafter made isotonic by addition of hypertonic sea water and estimation could be made of the proportion of fertilized eggs at each moment of interruption. Graphically represented, the data take the form of Fig. 13. If the logarithms of the proportion of fertilized eggs are plotted as a function of the times of egg-sperm interaction, the values may be fitted into a straight line; the slope of this line represents the rate constant, α , of fertilization. From the number of "collisions" at a

certain sperm density, n , prevailing in the experiment, and the α value, the probability of an effective attachment, p , can be calculated, $1/p$ is the number of collisions necessary to get one effective attachment. In seventeen experiments, where the number of spermatozoa varied from 7.4×10^4 to 9.6×10^6 per milliliter, p decreased from 0.226 to about 0.017. Thus at one extreme, 4–5, and at the other, 100 collisions are necessary to get fertilization. This is not what would be expected on the basis of the collision theory. The authors assume that the deviation from the theory may be due to sperm-sperm interactions of a physical nature. This assumption is contrary to evidence available to the present writers. Eggs were first inseminated with homologous sperm and the rate of fertilization recorded. A second experiment was made with the same egg material and the same homologous sperm suspension, but foreign sperm was added to the latter. No lowering of the fertilization rate was found to occur although the mechanical sperm-sperm interaction must have increased (Allen, Hagström, unpublished).

E. Hultin (1956) prefers to describe the course of fertilization experiments by a statistical method involving no assumptions. He uses probit analysis in order to evaluate the 50% fertilization time (FT_{50}) and its statistics for a group of eggs (cf. Hultin and Hagström, 1956). Hultin developed a saturation theory for the influence of sperm density on the fertilization rate ($1/FT_{50}$). The mathematical treatment is similar to that used by Michaelis and Menten for the influence of substrate concentration upon enzyme reaction rates or to the adsorption theory of Langmuir. The characteristic value in this treatment is $n/2$, the sperm concentration which gives half the fertilization rate ($1/FT_{50}$) prevailing at saturation ($1/\lim FT_{50}$).

Chemical interaction between substances secreted from the egg and substances present in the sperm fluid may influence the fertilization rate (cf. Hultin and Hagström, 1955) and thus α in the calculations of Rothschild and Swann or the constant $n/2$ in Hultin's equation.

It is interesting that a kinetic approach rather similar to the one referred to above has been made for the conjugation in *Escherichia coli* K12 (cf. Wollman *et al.*, 1956). At intervals after mixing of two different mating types in liquid medium, aliquot samples were removed, diluted, and plated for recombinants (cf. above, Section II A).

c. Factors bearing upon the rate of fertilization. Irrespective of any theoretical treatment, the method of determination of the rate of fertilization is extremely useful for testing the effect of various conditions on fertilization. In principle, it is possible to distinguish between the role played by effects on eggs and on sperm. In the first case, differently treated eggs may be inseminated by the same sperm suspension. In the

second case, differently treated spermatozoa are used to inseminate different test suspensions of the same egg material. In certain cases, the respective gametes may be pretreated with the agent to be tested and the insemination may be carried out in normal medium. The determination of the fertilization rate can be carried out routinely, in particular since B. E. and B. Hagström (1954a) introduced lauryl sulfate as a means of interrupting fertilization. At different intervals of time (e.g., 5, 10, 15, 20, etc., seconds) after insemination, samples of the egg suspension are transferred to 0.001% lauryl sulfate in sea water. This instantaneously interrupts the activity of the free spermatozoa. If, however, a spermatozoon has been effectively attached to the surface of an egg prior to the transfer, fertilization follows its normal course despite the presence of lauryl sulfate. This is removed after 5-10 minutes to insure normal development. Under the conditions of the test, fertilization has an all-or-none character. This justifies the designation "rate of fertilization."

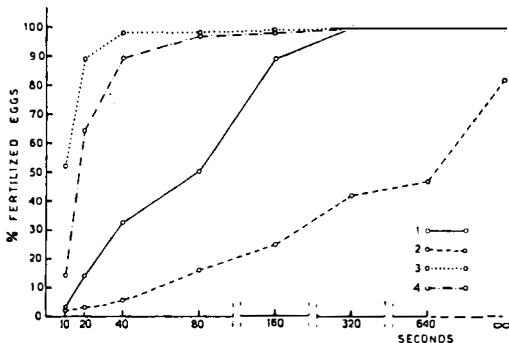


FIG. 14. Eggs of *Psammechinus miliaris*. Rate of fertilization according to the lauryl sulfate method (cf. text). Sperm concentration: 10^6 per milliliter. Curve 1, eggs with intact jelly layers inseminated with *Psammechinus* sperm; curve 2, eggs with intact jelly layers inseminated with *Paracentrotus* sperm; curve 3, jelly-free eggs inseminated with *Psammechinus* sperm; curve 4, jelly-free eggs inseminated with *Paracentrotus* sperm. From Hagström (1956d).

Hagström (1956a, b, d, e) used fertilization-rate determinations as a means of characterizing the role of the jelly coat in fertilization. For this purpose, the jelly coats were removed from the eggs by treatment with acidified sea water. Care was taken that the pH did not sink below 5.5 (Vasseur, 1948). Figure 14 gives some data for *Paracentrotus lividus* and *Psammechinus microtuberculatus* eggs inseminated with homologous or heterologous sperm. It is at once evident that removal of the jelly coat considerably enhances the rate of fertilization. If, on the other hand, the jellyless eggs were exposed to a solution of jelly substance in

sea water, the rate of fertilization was decreased almost to the rate prevailing in eggs with jelly layer (Hagström, 1956a). If the jelly layers were removed mechanically by filtering the eggs under weak pressure through appropriately meshed bolting silk, the result was the same as when they were removed by acidified sea water (Hagström and Markman, 1957).

These data give the quantitative basis for a conclusion already mentioned above (Section II, D, 3), namely, that the jelly layer exerts a sievelike selecting effect on the spermatozoa.

It has been maintained that in experiments such as those described above, the jelly substance (fertilizin) will remain as a thin layer on the egg surface without which fertilization does not occur. This layer would serve as the sperm receptor (Tyler, 1941a, 1948; Tyler and Brookbank, 1956).

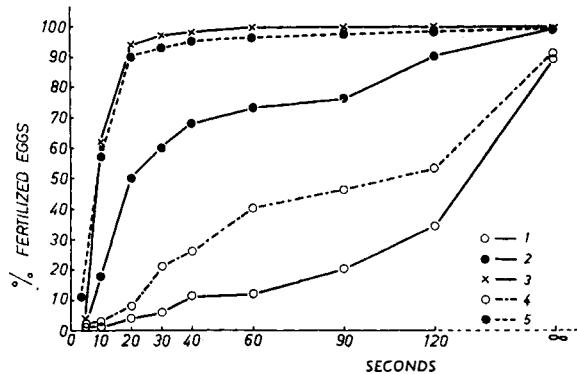


FIG. 15. *Paracentrotus lividus*, rate of fertilization according to the lauryl sulfate method. Curve 1, eggs with jelly coat; curve 2, jelly-free eggs; curve 3, jelly-free eggs, 0.013% albumin added at insemination; curve 4, 2 ml. jelly-precipitating extract from eggs added at insemination to 98 ml. suspension of eggs with jelly layer; curve 5, 2 ml. of the same extract added at insemination to 98 ml. suspension of eggs deprived of their jelly layers. From B. E. Hagström and B. Hagström (1955).

Hagström and Hagström (1955) obtained an egg extract by spinning down the microscopic particles of a homogenate of unfertilized *Paracentrotus* egg. The supernatant contains a jelly-precipitating factor (anti-fertilizin). Despite a slight precipitation of the jelly by addition of the egg extract, the rate of fertilization was somewhat higher than in the control (cf. Fig. 15, curves 1 and 4, whereas curve 2 again demonstrates the enhanced fertilization following removal of the jelly layer). An addition of jelly-precipitating factor to jelly-free eggs brought about a further increase in fertilization rate (cf. curve 5), although any remaining "fertilizin" must have been removed in this experiment.

Certain mucopolysaccharides, e.g., heparin, chondroitin sulfate, dextran sulfate, have an inhibitory effect on the rate of fertilization in sea urchins (Runnström and Wicklund, 1950; Harding, 1951). The most efficient agent so far in decreasing the rate of fertilization is an inhibitor present in an extract from *Fucus vesiculosus*. This inhibitor was considerably purified by Vasseur. It is not identical with fucoidin, a fucose-containing acid polysaccharide present in the alga. The inhibitor seems to contain a polysaccharide esterified with sulfuric acid and a phenollike group. The inhibitor has not been proved to be a uniform substance. It is a powerful inhibitor of hyaluronidase and ribonuclease. The inhibitory effect of the hyaluronidase action and that on fertilization showed a remarkable parallelism (cf. the report by Esping, 1957a, b).

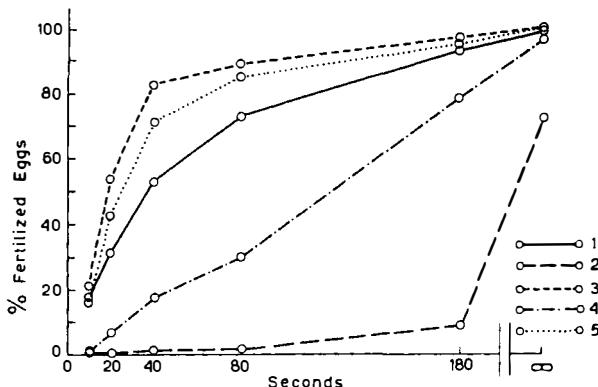


FIG. 16. *Psammechinus miliaris*, rate of fertilization. Curve 1, control; curve 2, insemination in the presence of 0.018% fertilization inhibitor from *Fucus vesiculosus*; curve 3, eggs pretreated with 2×10^{-6} M sodium periodate; curve 4, eggs pretreated with 2×10^{-6} M sodium periodate and inseminated in the presence of 0.018% inhibitor; curve 5, eggs with jelly layer removed. From Runnström (1957b).

Curve 2 in Fig. 16 shows the fertilization rate in the presence of 0.018% fertilization inhibitor from *Fucus* (Fe Inh(Fu)) contrasted with that of the control, curve 1. Even after 180 seconds, not more than 5% of the eggs have been fertilized. The spermatozoa survive excellently in the presence of the inhibitor, and, after 1–2 hours, the number of fertilized eggs approaches 100%.

A number of different substances added to the egg suspension have an enhancing effect on the fertilization rate. This holds, for example, for a number of amino acids tested and for serum albumin (Hagström and Hagström, 1954a). In this case, the effect is both on the eggs and on the spermatozoa, the motility of which is enhanced under the influence of the added substances. In some experiments, the eggs were pretreated

with albumin. The eggs were then washed and transferred into normal sea water where insemination took place. The rate of fertilization was enhanced, but to a lesser extent than when both eggs and sperm were subjected to the treatment.

Very obvious effects were obtained after pretreatment of the eggs with periodate (cf. Fig. 16). In this experiment, the 50% fertilization time (FT_{50}) for untreated eggs was about 38 seconds (curve 1), whereas in eggs previously treated with 2×10^{-6} sodium periodate it decreased to 18 seconds (curve 3), i.e., the rate was doubled. In this case, the action is wholly on the eggs, as the periodate was washed away before insemination (cf. Runnström, 1957b).

It was demonstrated that the physical properties of the surface layer of the unfertilized eggs changes upon treatment with periodate

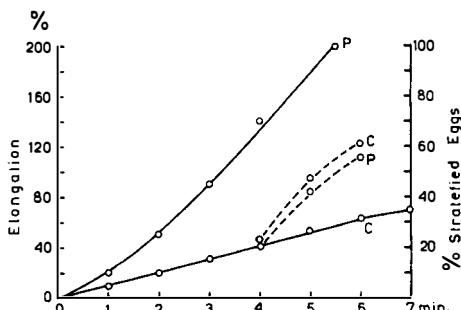


FIG. 17. *Psammechinus miliaris*. Centrifugation of unfertilized eggs. Continuous lines: elongation of the eggs; broken line: percentage of stratified eggs. Acceleration about 4.400g. C, control eggs; P, eggs pretreated with 5×10^{-5} sodium periodate. From Kriszat (1956).

The eggs were suspended in microcuvettes on the border between sea water and an isotonic cane sugar solution and centrifuged for 8 minutes at about 4400g. In the centrifuge microscope (E. N. Harvey, 1931; E. B. Harvey, 1931); the elongation of the eggs could be measured each minute. The measurements started when the centrifuge had reached constant speed. Curves like those drawn with continuous lines in Fig. 17 were obtained in this way. The elongation of the eggs in the centrifugal direction is given in per cent of the diameter of egg, e.g., 40% on the ordinate means that the longer axis of the centrifuged egg is 1.4 times the diameter of the nonelongated egg.

In the present experiment the control eggs (C) reached a final elongation of 64%, whereas the eggs subjected to periodate (P) exhibited a much more rapid elongation reaching a value 200% after 5.5 minutes.

There was practically no difference in the rate of stratification of the interior, as shown by the curves in Fig. 17, drawn in broken lines (cf. Runnström, 1957b). The results indicate a decrease in the rigidity of the surface layers of the egg.

This description applies to about 20% of the treated eggs. A smaller portion remained similar to the control whereas the rest were activated. As a consequence the rigidity of the egg surface was increased. The enhanced elongation reflects an intermediate state between the non-activated and the activated one. It was designated as the preactivated state (Kriszat, 1953).

Addition of thioglycolate, versene or serum albumin causes a decrease whereas the inhibitor from *Fucus* causes an increase in the rigidity of the surface layers (cf. Wicklund *et al.*, 1953).

The jelly coat swells considerably after treatment with periodate. Likewise the viscosity of a solution of jelly substance decreases very quickly upon addition of periodate (Runnström and Kriszat, 1950). The action of periodate is, however, not due only to its splitting action on the jelly coat, as follows from such data as those presented in Fig. 16. The mere removal of the jelly coat (curve 5) does not bring up the rate of fertilization to the same level as that of the periodate-treated eggs. Other experiments likewise prove that periodate acts not only on the jelly coat, but also on the cytoplasmic egg surface.

Eggs pretreated with periodate ($2 \times 10^{-6} M$) were less sensitive to the *Fucus* inhibitor than nonpretreated eggs (cf. curve 4 of Fig. 16). FT_{50} is here 120 seconds, a very considerable increase in fertilization rate compared with the nonpretreated eggs, curve 2.

Periodate is an oxidizing agent, but its effect on the egg proved to be rather specific. No other oxidizing agent gave the same improving action as periodate. The following agents were tested: hydrogen peroxide, ferricyanide, iodosobenzoic acid (Runnström and Hagström, 1954), and particularly the high-potential oxidation-reduction indicators, porphyrindine and porphyrexid (Runnström, 1957; Runnström and Kriszat, 1957). Most of these had a pronounced inhibitory effect. In the presence of, for example, $5 \times 10^{-6} M$ porphyrexid, the rate of fertilization was lowered, whereas in the presence of the same concentration of periodate it was considerably enhanced. The rigidity of the surface layers of unfertilized eggs was increased in the presence of porphyrexid. As already mentioned, a pretreatment with the high potential indicators may block the penetration of the spermatozoon.

Periodate and also serum albumin may remove underripeness of the egg (Section II, D, 2). Probably these agents act in different ways but, directly or indirectly, they seem to lower the activity of an inhibitor

present in the cytoplasmic surface, whereas the *Fucus* inhibitor and other substances with inhibitory action reinforce in an additive way the natural inhibitor. The decrease of the effect of the *Fucus* inhibitor on periodate-pretreated eggs is of interest from this point of view.

As is well known, periodate oxidizes in the first place α -glycol groups, a configuration present in carbohydrates. The strong effect on the jelly is mainly due to a depolymerization of the polysaccharide moiety. Also in the action on the cytoplasmic surface, polysaccharides may be involved.

d. The cortical changes. According to Allen and Griffin (1958), the first 17–20 seconds after the effective sperm attachment represent a period of apparent latency. The fertilization rate was determined with the lauryl sulfate method. In a parallel test, the progress of change was interrupted by immersing the eggs in 4% formaldehyde in sea water at different time intervals after insemination. Figure 18, curve 1, shows

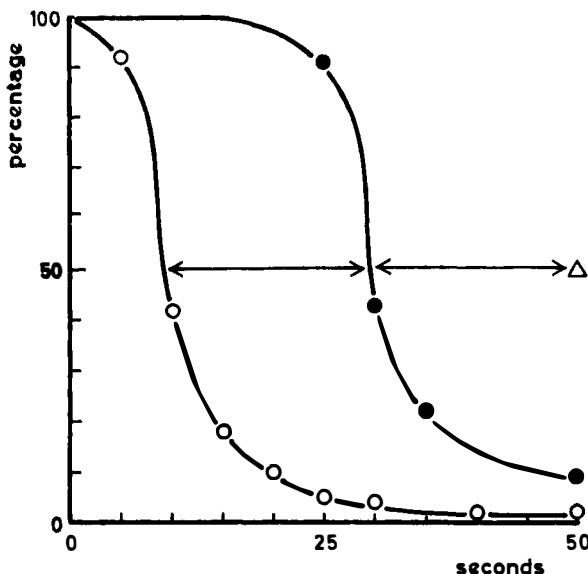


FIG. 18. *Paracentrotus lividus*. Curve 1 (open circles), decrease of number of unfertilized eggs after insemination; curve 2, (black circles), decrease of eggs showing no cortical change after immersion in 4% formaldehyde. Triangular point corresponds to the 50% value of an ascending curve which gives the number of eggs with a separated fertilization membrane. Cf. also Allen and Griffin (1958).

the decrease of unfertilized eggs according to the lauryl sulfate method; curve 2 gives the decrease of eggs without any visible cortical change in the formaldehyde test. The distance between the 50% values of curves 1 and 2 is a measure of the latency time. This was considered to end as

soon as the beginning of the membrane elevation was manifested by a small, thin blister. Such blisters appear earlier in formaldehyde than in the living egg and indicate that the expulsion of the cortical granules has started (cf. diagram, Fig. 19). Their attachment to the vitelline membrane could be followed by transferring the eggs after different time intervals into $2 \times 10^{-4} M$ solutions of porphyrexid. The membrane is thereby stiffened in the state which prevailed at the moment of immersion. The granules are at first rather loosely attached to the separating membrane, but they soon flatten and form eventually a continuous layer (Runnström, 1958). The vitelline membrane covering the cytoplasmic surface of the unfertilized egg is about 100 Å. thick (Afzelius, 1956). By the incorporation of the cortical granules, the thickness of the mem-

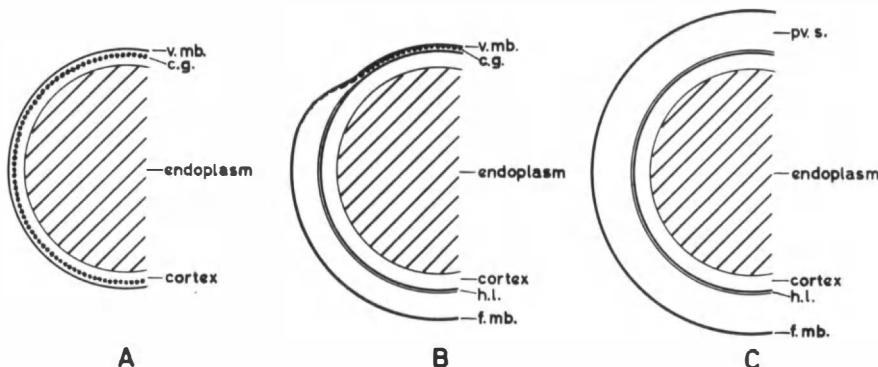


FIG. 19. Diagram showing the surface layers in sea urchin eggs and their changes upon fertilization. A. Unfertilized egg. B. The cortical changes. C. Fully elevated fertilization membrane (*f. mb.*). *c.g.* = cortical granules which are expelled and gradually merge with the vitelline membrane (*v. mb.*) *h.l.* = hyaline layer; *p.v. s.* = perivitelline space.

brane increases to 500 Å. (Mitchison, 1953; Afzelius, 1956). The incorporation of the granules is also accompanied by an increase in the birefringence of the membrane (Runnström, 1928a, 1958).

Figure 20 is an electron micrograph of the cortex of an unfertilized egg of *Echinus esculentus*. The cortical granules exhibit a rather complicated structure which varies in different forms. Figure 21 is an electron micrograph of an egg where cortical granules are seen to be coalescing with the vitelline membrane.

Moser (1939) observed the wavelike "breakdown" of the granules starting from the point of sperm attachment. Motomura (1941) Runnström *et al.* (1944, 1946), and Endo (1952) made clear that the cortical granules are incorporated with the vitelline membrane. According to

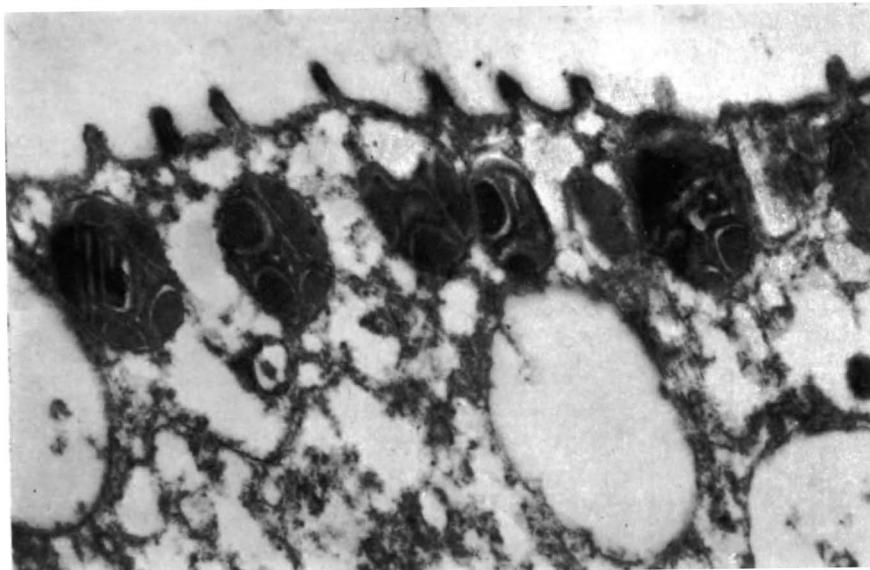


FIG. 20. Electron micrograph of the cortex of an unfertilized egg of *Echinus esculentus*. Below the cytoplasmic surface (furnished with a number of protrusions), a row of cortical granules. From Afzelius (1956). (Magnification: $\times 32,250$.)

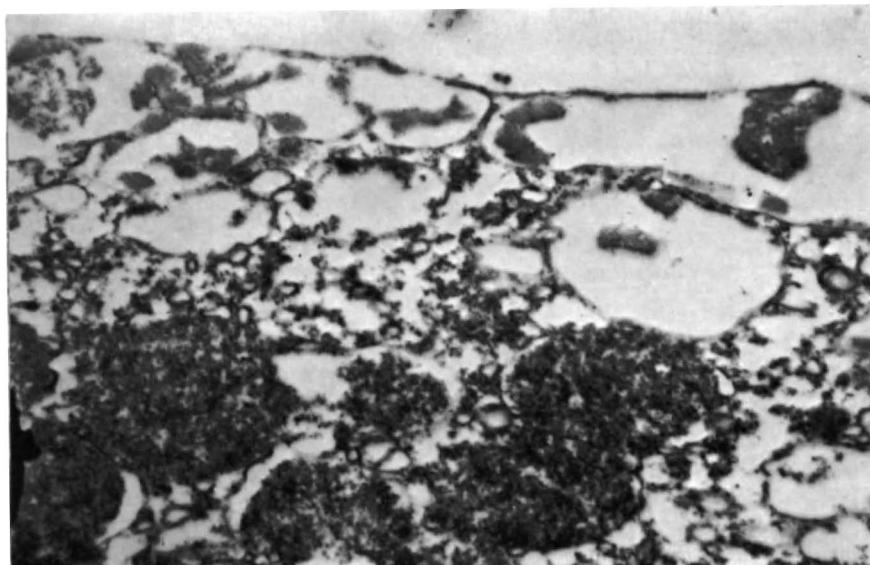


FIG. 21. Electron micrograph of an egg of *Echinus esculentus* shortly after fertilization: cortical granules coalescing with the vitelline membrane; cf. particularly the upper right side of the micrograph. Afzelius, from Runnström (1957b). (Magnification: $\times 18,705$.)

estimations made by Afzelius (1956), the total original volume of the cortical granules is greater than the volume they occupy within the membrane. There is a possibility that the cortical granules contribute to the osmotically active substance present in the perivitelline space. The presence of this substance was postulated by Loeb (1908), and it is considered by Hiramoto (1954) to have the character of a gel. Its colloid osmotic pressure was supposed to be the active factor in the membrane elevation. Chambers (1942) and Runnström (1928a, 1948) have considered that molecular rearrangements contribute to the membrane elevation, which results in about a doubling of the surface of the membrane. The flattening and toughening of the cortical granules may at least play a role in the elevation and smoothing of the vitelline membrane. Before fertilization, its area is enlarged by the papillar protrusions present in the unfertilized egg (cf. Figs. 11 and 20) and may equal that of the final fertilization membrane.

In Fig. 18 the triangle to the right indicates the time when 50% of the eggs had a membrane which was elevated over the whole surface of the egg, i.e., about 40 seconds after the attachment of the effective spermatozoon; 120 seconds after insemination, 95% of the eggs had elevated membranes. The progress of solidification of the membrane was followed by centrifugation (Motomura, 1950b) or by filtration of the eggs under light pressure through a fine net of bolting silk (Markman, 1958). After 5 mintues, no further increase in toughening could be noted in eggs of *Psammechinus miliaris* by the latter method. With toughening, the membrane becomes resistant against proteolytic enzymes (Kopac, 1941).

The spermatozoon certainly exerts more than a trigger action in fertilization. The quality and extent of the cortical changes were shown to be dependent on age of the sperm, or on individual or species characteristic properties of the male (Runnström, 1949; Tyler, 1950; Hagström, 1956e; Markman, 1958).

Before their expulsion, the cortical granules are lodged within membranes forming pockets which are opened upon fertilization. Papillar protrusions appear which are more numerous and more richly vacuolated than the protrusions in the unfertilized egg (cf. Fig. 20 and 22). These protrusions enter probably as structural components of a new surface layer appearing in connection with the cortical changes which occur in fertilization. This is the hyaline layer which sometimes appears to be striated when observed with the light microscope in the living state. The space between the protrusions is filled with a hyaline substance which probably is exuded from the egg cortex. An outer limiting membrane becomes more visible after fixation in formaldehyde, which also makes it possible to study the first appearance of the hyaline layer. This

becomes visible during the period of the membrane elevation and is at first very thin and adheres to the egg surface. Previous to the toughening of the membrane, the hyaline layer becomes wider, as shown by studies on formaldehyde-fixed material.

When the membrane begins to be elevated around the attached spermatozoon, a concavity is formed. This reaches its greatest depth approximately 50–60 seconds after insemination in eggs of *Psammechinus*

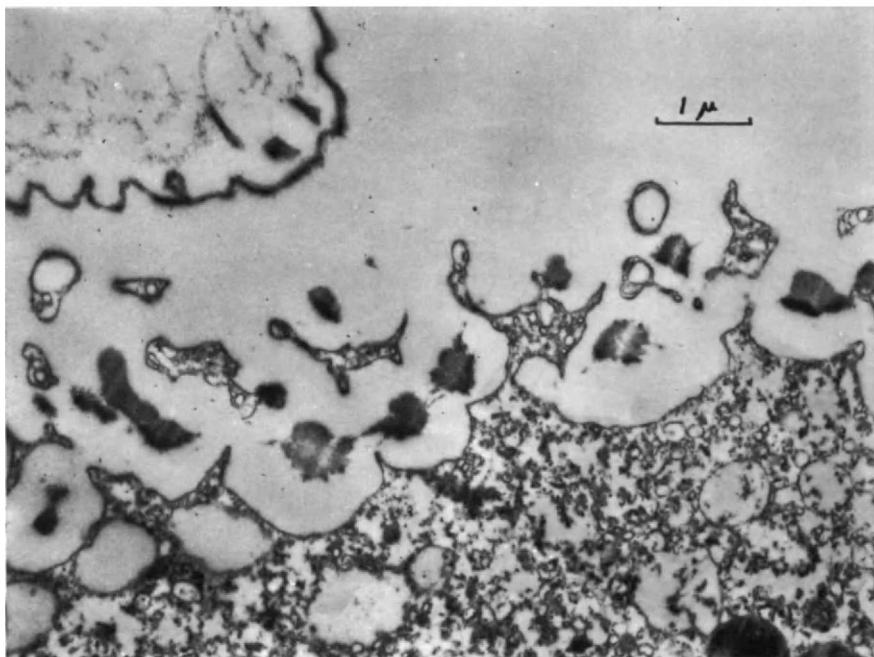


FIG. 22. *Echinus esculentus*. Electron micrograph showing surface of the egg about 5 minutes after fertilization; in the upper left of the figure, with numerous vacuolated protrusions, the fertilization membrane. From Afzelius (unpublished).

miliaris or *Paracentrotus lividus* (Kuhl and Kuhl, 1949; Runnström, 1958). It seems rather probable that the formation of the concavity involves a secretion from part of the egg (Runnström, 1949, p. 278). This secretion may initiate the formation of the matrix of the hyaline layer. The final fertilization membrane often seems denser above the region of effective sperm attachment owing to an accumulation of cortical granules in this region.

Under normal conditions, the concavity vanishes and the egg becomes spherical 60–100 seconds after insemination. In the presence of soybean trypsin inhibitor, the fertilized egg often was observed to remain in the

stage with concavity, which may become deeper than is normal. This favors the idea that the release of a proteolytic enzyme is closely connected with the normal course of the cortical changes (cf. Section III).

In the unfertilized egg fixed with Zenker's fluid, for instance, one can demonstrate a layer in the egg surface (Fig. 23) stainable by the acetic ferric method of Hale (1946). The ferric ions are bound to acid groups such as phosphate and sulfate. Even after extraction of the nucleic acids, the staining of the surface layer remains. It is therefore probable that the positive Hale reaction indicates a layer of acid mucopolysaccharides (cf. Runnström and Immers, 1956). After fertilization, the layer undergoes obvious changes. It is apparently split up into granular units which stain more intensely with the Hale method than before fertilization. It seems that the acid polysaccharides after fertilization become located

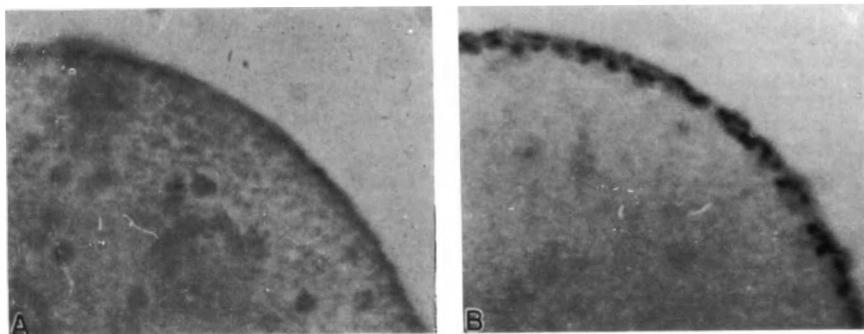


FIG. 23. A. Surface layer of mature unfertilized egg without jelly coat. B. Surface layer of egg 10 minutes after fertilization. Stained by Hale's method (1946). From Immers (1956).

in the hyaline layer. In the electron microscope, the hyaline layer appears rather empty if one disregards the protruding papillae and the outer membrane. Probably the empty space corresponds to the acid mucopolysaccharides demonstrable with the Hale reaction. If this is correct, at least one important component of the hyaline layer can be derived from a precursor present in the cortical layer of the unfertilized egg. It was demonstrated that larvae reared in sea water devoid of sulfate gradually developed a hyaline layer which was negative to the Hale reaction. This is evidence that the acid character of the polysaccharide of the hyaline layer is due to esterification with sulfate. According to Nakano (1956), the hyaline layer also contains lipids, and Lansing and Rosenthal (1949) have presented evidence that nucleic acids also are closely associated with polysaccharides in the egg surface. It thus appears probable that

the hyaline layer has a composition which is rather similar to that of the hyaline ground substance of the cytoplasm. The hyaline layer may only be richer in acid mucopolysaccharide.

It is of great interest that Austin (1956) has found characteristic cortical granules in hamster eggs and that these granules "break down" on fertilization. Their fate and role has not yet been elucidated. Another intriguing question is whether or not a hyaline layer appears in mammalian eggs. This layer plays a great role as the common cover of the ecto- and mesendodermal epithelia of sea urchin embryos.

e. *The activation impulse and its propagation.* Just (1922a) pointed out that an impulse of activation spreads from the point of sperm attachment, and Runnström (1923, 1928a) observed in dark-field illumination a color change from yellow to white which also spreads from the point of sperm attachment. It probably reflects a submicroscopic change. The time of propagation of the color change was found by Rothschild and Swann (1949) to be about 20 seconds. Runnström (1928a) reported that the color change may sometimes undergo a decrement so that the "distal" egg pole, i.e., that opposite to the site of sperm entry, remained yellow for a longer period. The color change immediately precedes the expulsion of the cortical granules.

It is conceivable that, during the preceding period of "latency," there is an interaction between the acrosome filament of the spermatozoon and the cytoplasmic egg surface. This interaction may lead to a local change in this surface and, when a certain threshold has been reached, the propagating impulse starts. This process has proved to be more accessible for study in experiments on artificial parthenogenesis. Yamamoto (1939, 1949a, b, 1956) carried out highly significant studies on the unfertilized eggs of the fish *Oryzias latipes*. Like frog eggs (Bataillon, 1910, 1911), *Oryzias* eggs may be activated by pricking with a needle of suitable thickness, e.g., 15–20 μ . A wave of breakdown of mucopolysaccharide-containing alveoli starts from the point of puncture. When eggs are treated with isotonic oxalate solution in order to remove calcium and pricked while in this medium no immediate effect is apparent but when they are returned to a Ringer solution, the cortical response occurs without further treatment. This experiment dissociates the primary stimulus caused by wounding the egg surface from the propagating impulse. The former occurs even in the absence of calcium, whereas the latter requires the presence of calcium.

Pricking of the *Oryzias* egg in normal medium with a thinner, approximately 5 μ , needle gave no visible response, but repeated pricking with this needle elicits a propagating impulse. The repeated treatments bring the local change above the threshold necessary to get the impulse

started. In earlier work on starfish eggs, Lillie (1941) had adduced important evidence of such additive effects.

The needle technique is not satisfactory for sea urchin eggs, but other means were developed to assure a local action of different activating agents on sea urchin eggs. It is of interest to know whether the effect of the agent will cause an impulse which spreads over the whole egg or whether the action of the agent may remain local. Sugiyama (1953) showed that butyric acid, distilled water, and urea solution act in the former manner on eggs of the sea urchin *Hemicentrotus pulcherrimus* and thus imitate the action of the spermatozoon. Conversely, wasp venom, sodium choleinate, and detergents bring about only a local effect. Allen (1954) found that application of periodate on *Psammechinus* eggs held in a capillary tube causes an impulse which spreads over the egg surface, whereas hypertonic medium only gives the local effect. Sugiyama (1956) made an interesting application of the capillary method, illustrated in Figure 24. He drew up an unfertilized egg into a

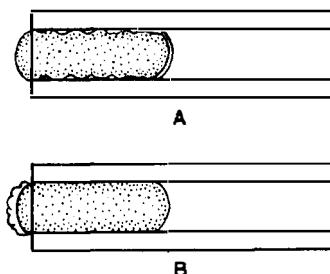


FIG. 24. Eggs held in capillary tubes. A. Treated with butyric acid. B. Treated with a detergent. After Sugiyama (1953).

capillary tube and moved it so that a small part of its surface was exposed to an outer medium. When the capillary tube and the protruding part of the egg are immersed in sea water containing butyric acid, no cortical change occurs in the immediately exposed region of the egg. Cortical changes including membrane separation do occur, however, in the parts of the egg not directly exposed, owing to the impulse which spreads from the exposed region (A). After the egg has been blown out of the capillary pipette into normal sea water, a low membrane forms around the whole egg, which retains its elongated form. Immersion of the capillary tube with the slightly protruding egg in detergent, on the other hand, brings about only a local reaction (B).

An electrical current sent through a sea urchin egg held in a capillary tube causes cortical changes first at the anode and later at the cathode, but no impulse is propagated from the activated regions. This means

that the electric current in this case only acts locally and does not seem to be an adequate stimulus for the artificial activation of the sea urchin egg. In these experiments, the cortical changes caused by the electric current seemed to be delayed in the neighborhood of the nucleus (Allen *et al.*, 1955). It may be added that the cortical changes following a regular fertilization were delayed in the region adjacent to the egg nucleus (Allen, unpublished). From the description of Yamamoto (1949a), it seems to follow that the electric current is able to produce a stimulus in the *Oryzias* egg which gives rise to a propagated impulse. Allen (1954) brought about a localized fertilization of *Psammechinus* eggs held in capillary tubes (cf. Fig. 25). If the surface of the elongated egg increased more than 30%, the cortical change did not cover the whole surface of the egg. The "distal" part of the egg remained unfertilized. There was an intermediate zone (*b*) between the fertilized and the nonfertilized regions. In this zone, a certain number of cortical granules

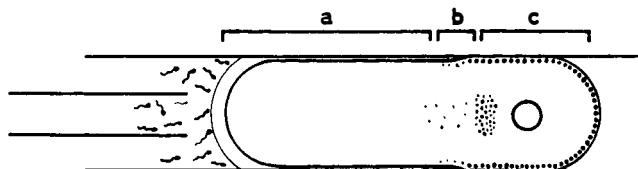


FIG. 25. Egg of *Psammechinus miliaris* drawn up into a capillary tube and inseminated from one side: *a*, fertilized zone; *b*, intermediate zone; and *c*, unfertilized zone. From Allen (1954).

were expelled, whereas a number of granules remained unchanged. The impulse decreased within this zone to a level not sufficient to cause the expulsion of all the cortical granules. An interaction between the male and female nucleus was observed in such cases as that represented in Figure 25. When the nuclear membrane disappears in the male pronucleus, the same occurs in the female one. This interaction may be mediated by diffusion. Nevertheless, no ordered cortical changes occurred on the unfertilized "distal" part of the egg. In order to get further material for the analysis of the activation impulse, Allen and Hagström (1955) subjected *Paracentrotus* eggs, at different intervals after insemination, to a temperature of 32° C. for 2½–3 minutes. Thereafter the eggs were brought back to room temperature, 18°–20° C. The exposure to the elevated temperature interrupts the impulse and the earlier the exposure, the greater is the part of the egg which remains unfertilized. In the fertilized part, the elevation of a membrane occurs. The membrane may become smooth and solidify, as is apparent from its refractility in phase contrast. Only its borders are less refractile and, moreover,

granular—a sign of deficient incorporation of the cortical granules. It was observed that solidification of the membrane occurs only when a well-developed hyaline layer is present in the fertilized region. This may indicate that the "toughening factor" (Motomura, 1950b) is released through the hyaline layer.

Why does heating stop the cortical changes? When one considers this question, it is useful to know that heating of unfertilized eggs for 2–3 minutes to 32° does not injure them; on the contrary, eggs so treated show an increased rate of fertilization. The unfertilized zone of the partially fertilized egg reacts normally to reinsemination. The egg is particularly sensitive to the elevated temperature in the zone where the wave of activation impulse is progressing. In this zone, processes may occur which are intimately coupled. Such coupled processes are very exacting from the stereochemical point of view and may easily come out of gear even by moderate elevation of the temperature. This is also in keeping with Just's (1922a) demonstration that the eggs of the sand dollar, *Echinorachnius parma*, are particularly sensitive to hypotony during the progress of membrane elevation. It seems most probable that the activation impulse spreads by a change of certain units which successively cause the change of other units, so that a sort of chain reaction is established. Secondarily, the changes spread to the interior. This was the view taken by Runnström and Kriszat (1952) and by Allen (1954). As an alternative to the successive cortical conduction of the impulse, Rothschild and Swann (1949) and Rothschild (1949) assume a diffusion through the endoplasm. On basis of this view, it would be difficult to understand why a complete block can persist in the partially fertilized eggs, although these are in possession of a complete activation system, sperm nucleus, and middle piece.

f. *Mechanism protecting against polyspermy.* The problem of protection against polyspermy in sea urchin eggs has been raised several times in the preceding part of this review. Even if, under natural conditions, the jelly coat eliminates a great fraction of the spermatozoa, a second mechanism of protection seems to be necessary at the level of the cytoplasmic egg surface. It follows from the evidence presented in Section II, D, 4, c and d that the cortical changes which could bring about a relative, and later a complete, protection start only 15–20 seconds after effective sperm attachment. It is tempting to assume that prior to this time, a block is established, i.e., an impulse which is considerably more rapid than the one which was described in the preceding section spreads over the egg surface. Gray (1931) assumed, in fact, that a block passed over the egg surface in 10^{-5} seconds.

The degree of polyspermy will depend among other things on the

concentration of the spermatozoa. In an experiment referred to in Figure 26, Rothschild and Swann (1952) used a rather dense suspension of spermatozoa, 2.8×10^8 per milliliter, in a fertilization experiment. After different intervals, samples were taken from the egg sperm mixture and these were diluted about forty-five times. This gives a sperm density of $2-3 \times 10^6$ per milliliter at which practically no polyspermy occurs. The percentage of polyspermy found after each interval is plotted in the curve as a function of the time. After a certain period, about 85 seconds after fertilization, no further increase of polyspermy occurs. This is regarded as the time when an absolute block against polyspermy is established. This time corresponds rather well with the time of com-

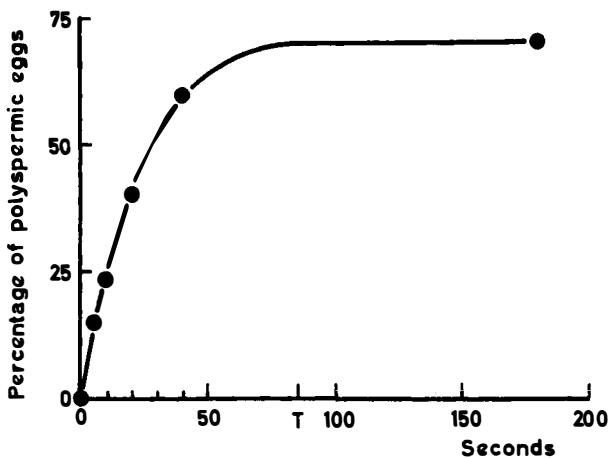


FIG. 26. Curve showing the percentage of polyspermic eggs in *Psammechinus miliaris* at different intervals after insemination with a dense sperm suspension, 2.8×10^8 per milliliter. At time T , approximately 80 seconds, no further increase of the polyspermy occurred, i.e., at this time the protection against polyspermy was complete. Data from Rothschild and Swann (1952).

pletion of the visible cortical changes. It seems therefore reasonable to assume that the cortical changes constitute the mechanism of protection against polyspermy. The curve in Figure 26 shows no doubt that the tendency to polyspermy is highest immediately after the primary fertilization, which was assumed to be 100% after 2 seconds. From zero to 20 seconds 40%, from 20 to 85 seconds 35% of the eggs became polyspermic. In view of our previous description, the incidence of polyspermy is higher during the period of "latency" than during the period of the cortical changes. Nevertheless there seems to be a certain protection also during the first 20 seconds.

From an analysis of their material, Rothschild and Swann infer that there is one slow block which reaches its full efficiency in around 80 seconds. This is, however, preceded by a more rapid block which is initiated in the egg surface and begins to be active as early as some few seconds after fertilization, perhaps earlier. The rapid block would provide the relative protection prevailing in the early period after fertilization.

Rothschild and Swann based their discussion on the assumption that the whole egg surface is equally receptive to spermatozoa. There is, however, evidence that a certain equatorial or subequatorial region of the egg responds more readily to the spermatozoon than do other regions (Runnström, unpublished). This could for example be demonstrated when the activation impulse was interrupted by heating eggs of *Paracentrotus lividus* soon after insemination. The pigment girdle described by Boveri (1901) served as landmark.

The protection against polyspermy has evidently not been confined to one single factor. There is a "multiple insurance" brought about by the consonance of the sperm-eliminating action of the jelly layer, the exacting threshold conditions in the interaction between spermatozoon and egg surface, the formation of the hyaline layer, and finally the fertilization membrane. The two first-mentioned factors may be sufficient to provide protection during the early period after effective attachment of one spermatozoon, whereas the cortical changes bring about the more efficient and definitive protection.

It has been possible to induce polyspermy by pretreatment of the eggs with various agents, e.g., nicotine (cf. Rothschild and Swann, 1950; Hagström and Allen, 1956). Immersion of the eggs at different intervals after fertilization in soybean inhibitor (0.025%) promoted polyspermy (Hagström, 1956f). The incidence of polyspermy decreased with time of transfer to the inhibitor. Hagström demonstrated that the polyspermy in these experiments was correlated to a defective hyaline layer. As shown by Sugiyama (1951), sea urchin eggs may be refertilized if transferred to calcium- and magnesium-free sea water, some minutes after fertilization. The still-soft fertilization membrane was removed mechanically immediately upon fertilization. B. E. Hagström and B. Hagström (1954b) pretreated the eggs for 5–10 minutes with 0.0005% crystalline trypsin. As this treatment inhibits the membrane formation, the egg could be refertilized at practically any interval after the first insemination subsequent to treatment with calcium- and magnesium-free sea water. Calcium-free or magnesium-free water were also active, the former somewhat more strongly than the latter. Under the conditions of these experiments the hyaline layer dissolved, so the experiments gave evidence of the protecting role of the hyaline layer.

Nakano (1956) brought the hyaline layer into solution and demonstrated that it inhibits fertilization in the dissolved state.

As was pointed out previously, the formation of the hyaline layer starts at the same time as the other visible cortical changes, i.e., 15–20 seconds after fertilization (Fig. 19). At this time, the resistance to polyspermy no doubt increases according to the curve of Figure 26. In some organisms, the shielding of the egg surface with a membrane must be of importance, the entry of the spermatozoon being limited to a micropyle, as seems to be the case in the eggs of teleost fishes.

According to T. Yamamoto (personal communication) a change in the membrane which brings about closure of the micropyle is an important factor in the protection against polyspermy in fishes. No rapid block contributes to the protection, as was shown in experiments on *Oryzias* eggs. A change may, however, take place which gradually renders the cytoplasmic surface nonreceptive to spermatozoa. In the herring (*Clupea pallasii*) eggs deprived of their membrane become polyspermic upon insemination (Yanagimachi, 1957).

In the lamprey and in the teleost *Oryzias*, responsiveness to stimuli and conduction rate of the impulse are highest at the animal pole and decrease along an animal-vegetal gradient (Yamamoto, 1949a). Possibly a certain differential receptivity towards spermatozoa will prove to occur in the eggs of numerous animals.

In Anura and in mammals, the mechanism of protection against polyspermy may be rather similar to that prevailing in sea urchins.

g. The engulfment of the spermatozoon and the action of the centrosome. In the sea urchin, the attached spermatozoon becomes invisible from the outside after 50–60 seconds, although the tail may still be visible for some minutes. Above the place of engulfment the reception cone becomes visible. It soon assumes a flamelike appearance and disappears after some minutes. It seems to represent a part of the hyaline layer which accumulates at the site of sperm entry. The changes following engulfment are well illustrated by Wilson's (1895) classical drawings, Figures 2 and 3. In the *Nereis* egg, the engulfment seems to occur only about 48 minutes after sperm attachment; the details of the process may therefore be studied with advantage in this material. Accordingly, some of F. R. Lillie's (1912) pictures pertaining to *Nereis* are reproduced in Figure 27.

As early as in the stage when the spermatozoon is merely attached with the acrosome filament, the cortical cytoplasm becomes denser at the site of attachment. The cortical cytoplasm seems to flow together toward the site of sperm entry; but later the condensed cytoplasm is carried down with the sperm head into the interior of the egg. The commencement of this inward movement is marked by the withdrawal of

the reception cone. The rotation of the sperm head seen in Figure 2 must be causally connected with the inward streaming. The confluence of cytoplasm toward the site of sperm entry is probably due to a general movement in the cortical layer, analogous to the confluence of cortical material toward the future division plane in mitosis (cf. Mitchison, 1952).

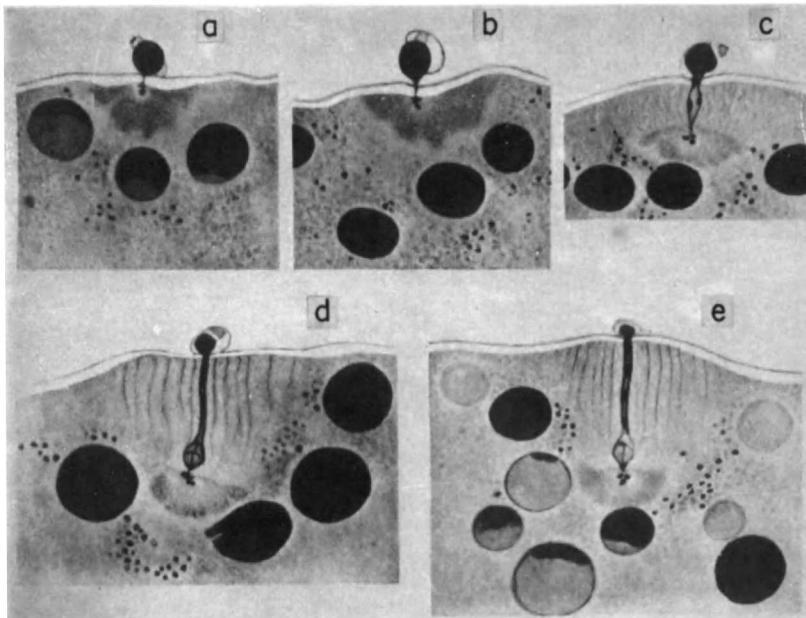


FIG. 27. *Nereis limbata*. Different stages in the engulfment of the spermatozoa. a and b. Thirty-seven minutes after insemination; spermatozoa fixed by the acrosoome filament; concentration of cytoplasm to the site of sperm attachment. c, d, e. About 48 minutes after insemination; different steps of engulfment with centripetal movement of cortical cytoplasm. From Lillie (1912).

Chambers (1921) removed micrurgically the cortical layer in unfertilized starfish eggs and showed that after this operation no effective fertilization is possible. The same can be shown by producing exovates on the surface of the egg. These exovates are covered with an extremely thin cortical layer or none at all. Numerous spermatozoa may penetrate into the exovate—in the absence of cortex the protection against polyspermy is lowered. Moreover, no asters develop around the middle piece of the spermatozoon (Runnström and Kriszat, 1952). According to Boveri (1887), the essential point in the activation of the egg at fertilization is the introduction of the new centrosome with the spermatozoon.

It seems probable that the centrosome or rather its more condensed part, the centriole (Fig. 11), is a replicating cell organelle which is not wholly autonomous. It has to interact with the cortical layer in order to be able to display its activity in a normal way. F. R. Lillie (1914) has stated: "The spermatozoon needs itself to be fertilized." This is true in particular for the centrosome function.

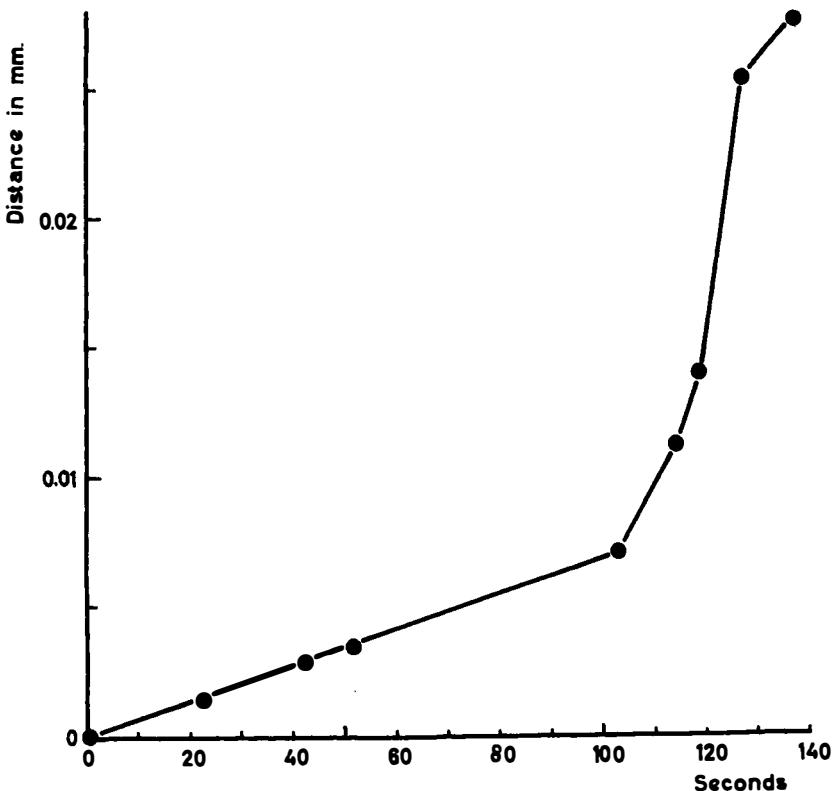


FIG. 28. *Psammechinus miliaris*. Migration of the female toward the male pronucleus; graphical record from time-lapse photographs. O, marks the beginning of the recording. From Kuhl and Kuhl (1949).

5. Karyogamy

The union of the pronuclei, "Karyogamy," in the sea urchin has been studied by Kuhl and Kuhl (1949) with the aid of time-lapse photography. They found, as Fig. 28 shows, that the egg nucleus migrates toward the male nucleus. The migration is rather slow at the beginning, but later accelerates. After the fusion of the pronuclei, the "synkaryon"

moves rather slowly to the center of the eggs. The Feulgen reaction of the female pronucleus is negative, whereas that of the male pronucleus is positive (Marshak and Marshak, 1953). After the union of the nuclei, there is in the *Paracentrotus* egg a period, 45–70 minutes after fertilization, when both male and female nuclear materials are Feulgen negative (Immers, 1957). Thereafter the Feulgen-positive chromosomes appear and cell division is completed approximately 85 minutes after fertilization. It is difficult to interpret these results, but probably there is a very fine dispersion of the Feulgen-positive material which may have some physiological implication. In mammals, a certain synthetic activity in the pronuclei seems to be indicated. The synthesized nucleolar material passes to the cytoplasm through the nuclear membrane, or it may be released upon the breakdown of the pronuclei (cf. Austin and Bishop, 1957).

The migration of the female nucleus can be studied very well in *Paracentrotus* eggs when the fertilization impulse is interrupted by warming the eggs to 32° C. 10–30 seconds after insemination. As described previously, the impulse covers then only a part of the egg surface. Nevertheless the head and middle piece of the spermatozoon penetrate, but not beyond the region in which cortical changes have occurred (Allen and Hagström, 1955). A radiation develops around the nucleus, but it remains limited to the activated region. The female pronucleus migrates with formation of a bulge or pseudopodium in the direction of the male nucleus. The union of the pronuclei is considerably delayed in these eggs, and the chromosomes of the female pronucleus may become basophilic even before the union with the male pronucleus. The stainable material of the nucleus is found to be concentrated in the direction of the male nucleus. Finally a union occurs within the region of the egg which has undergone cortical changes. After the union, however, the synkaryon is released from its dependence on this region. The synkaryon sinks down toward the center of the egg. It may even be dislocated somewhat in the direction of the pole opposite the sperm entry. But such eggs as those described usually do not divide or they divide into two cells or at most undergo a few irregular divisions. The partial character of the cortical changes precludes a normal course of division. This points to the primary importance of the cortical changes. Even when a synkaryon with a mitotic apparatus has developed, a completion of the cortical activation cannot occur by an action from the interior. On the other side, the course of cell divisions is upset. It seems rather probable that this is due to disturbances in the replication of the centrosome. As the centrosome has to react with the cortex during the penetration of the spermatozoon, its replication also seems later to

be dependent on an interaction with the cortical layer.

Gradually an expulsion of the cortical granules may occur in the nonactivated part of the cortex in partially fertilized eggs, but a hyaline layer is not formed. Even if a subdivision into cells occurs in the egg region with nonactivated cortex, the cells are loosely aggregated and rarely undergo any real differentiation (Runnström and Hagström, unpublished).

6. Species Specificity

The species specificity in fertilization has been emphasized in Section I, E. The principal way of testing the species specificity is cross-fertilization between different species. The incompatibilities may, however, appear at different levels—at fertilization, in embryonic development, or in the adult stage, in which sexual sterility may occur. We are here concerned only with the incompatibility at the level of fertilization. Many different degrees of incompatibility may be found. Refinement of methods often allows the discovery of low degrees of incompatibility earlier unnoticed.

Cross-fertilization of *Psammechinus microtuberculatus* ♀ × *Paracentrotus lividus* ♂, for example, gives nearly 100% fertilization. The rate of fertilization is, however, found to be lower in the cross-fertilization than in self-species fertilization, as may be seen in Fig. 14. If the jelly layers are removed from the egg previous to insemination, the rate increases in both cross- and self-species fertilization. The difference in rate is very much lowered after the removal of the jelly layer. The sieve-like action of the latter is here much stronger in cross- than in self-species fertilization. When *Echinocardium cordatum* ♀ is crossed with *Psammechinus* ♂, the rate of fertilization is extremely low. Upon removal of the jelly coat, the rate of self-species fertilization shows the usual increase, but the rate of cross-fertilization increases to a level equal to that of the self-species fertilization. The resistance apparently resides wholly in the jelly layer (Harding and Harding, 1952b). In many other crosses, the inhibitory role of the jelly coat is less pronounced or insignificant. In cross-fertilization between *Arbacia* egg and sperm from a number of different species such as *Genocidaris maculata*, *Paracentrotus lividus*, and *Psammechinus microtuberculatus*, the incompatibility is complete and no improvement occurs upon removal of the jelly coat (Hagström, 1956e). In these latter cases, the incompatibility resides in the cytoplasmic surface and possibly also in the jelly coat.

The sperm concentration used in these experiments was comparatively low, 2×10^6 per milliliter. It is known that a considerable increase in sperm concentration will allow a certain percentage of cross-fertiliza-

tion. This was shown for example by Lillie (1921) in crosses between the two *Strongylocentrotus* species, *franciscanus* (F) and *purpuratus* (P). At sperm concentrations which in self-species fertilization gave 100%, no cross-fertilization occurred. By increasing the sperm concentration 40–160-fold, a percentage of cleaving eggs was obtained, which in F ♀ × P ♂ combination amounted to as much as 65%, in P ♀ × F ♂ combination to 25%. The necessary augmentation of the sperm concentration in order to obtain cleavage was regarded by Lillie as an approximate measure of specificity.

Trypsin increases cross-fertilization in concentrations which decrease the self-species fertilization (Bohus-Jensen, 1952).

Some damage of the egg surface seems to be involved in the enhancing action on cross-fertilization. A strong increase in sperm concentration may act in the same way (Hultin and Hagström, 1955). Doubts may thus be raised as to whether the sperm reception in heterologous fertilization is mediated by exactly the same mechanism as in the self-species fertilization.

In many cases, enhancement of cross-fertilization is due to an unspecific stimulation of sperm motility. This holds for example for the action of egg water or jelly solution in combination with glycine or serum albumin (Harding and Harding, 1952a; Hagström, 1956d).

In genetics, inferences are made concerning a normal mechanism from observed cases of mutational deviations from the normal. Likewise, in experiments on cross-fertilization we infer from incompatibilities that an exacting genetically based complementariness is a prerequisite for normal fertilization. According to a case recorded by Vasseur (1952b), two alleles of complementarity genes united in a male hybrid (*Strongylocentrotus pallidus* × *S. droebachiensis*) exhibit no dominance phenomenon, in this respect resembling the alleles underlying human blood group AB. For a more detailed account of specificity on the fertilization level, the reader is referred to F. R. Lillie's (1919) still useful survey and to Rothschild (1956).

It may be assumed that, on the molecular level, the conditions determining compatibility in sperm attachment are similar to those responsible for specificity in connection with bacteriophage attachment or virus agglutination. For reviews of these fields, cf. Hershey (1957), Tolmach (1957), Burnet (1955).

7. The Immunological Approach to the Fertilization Problem

The specificity discussed in the preceding sections is bound to macromolecules in the surface layers of the gametes. Hence, for an understanding of the mechanisms of sperm attachment and egg activation, it

is necessary to know the molecular architecture of these layers. For the chemical characterization of the macromolecules in question, specific antibodies may be useful as analytical tools of a high power of resolution.

Antibodies have been used occasionally with the aim of studying mating substances (cf. Metz, 1954) or the surfaces of the gametes from higher animals (cf. Landsteiner, 1946). In the sea urchin, the use of antisera in studies of fertilization originates from Tyler (1946, 1948, 1949) and Tyler and O'Melveny (1941). Upon injection into rabbits of certain extracts from sperm and from eggs, Tyler obtained antisera which specifically agglutinated sperm and impaired fertilization. Since the extracts employed for the preparation of the antisera contained antifertilizin (cf. Section II, D, 3 and 4, c), the results were considered to support the view that antifertilizin is of importance for fertilization. However, the immunological homogeneity of the extracts employed for the production of antibodies was not established and the type of sperm agglutination in the various cases was not analyzed (cf. Henle *et al.*, 1938). Therefore, the bearing of these results on the validity of the fertilizin theory of fertilization appears doubtful. Similar objections may be raised against conclusions of this sort found in a later work of Tyler and Brookbank (1956).

A more complicated picture emerges from the recent studies carried out by Perlmann. Some of Perlmann's data will be referred to in order to exemplify this kind of work. The analytical tools in these studies were antisera obtained from rabbits injected with homogenates of unfertilized eggs, sperm, and isolated jelly substance.

When a small number of eggs are exposed to homologous anti-egg serum, a number of microscopic changes are induced in the eggs (Perlmann, 1954, 1956). The most important among those are precipitation of the jelly coat and parthenogenetic activation. When too large a number of eggs is used per volume of antiserum, these changes no longer appear. However, eggs treated in this way behave abnormally when fertilized after removal of the antiserum (Perlmann and Hagström, 1955, 1957). Thus, their fertilization rate is reduced, the formation of the fertilization membrane is more or less inhibited, fertilization may become polyspermic, and development is abnormal or completely suppressed. None of these response reactions is induced in normal serum. Hence, it can be concluded that the effects are due to the reaction of specific antibodies with eggs. However, comparison of the antiserum actions on eggs from different sea urchin species demonstrated a considerable species specificity of the antibodies.

For further analysis, relationships were established between the anti-serum doses and the various response reactions of the eggs. This per-

TABLE I
COMPARISON OF VARIOUS POTENCIES OF ANTI-*Paracentrotus lividus* SERA,
TESTED WITH EGGS OF THE SAME SPECIES^a

Antiserum ^b	Activity ^c			
	Jelly precipitation	Activation	Fertilization rate depression	Cortical damage
P-9	+	++ ±	+++	+++
P-10	±	++	++	++ ±
P-11	±	+++	++ ±	+
J-3	+++	±	0?	0
J-4	+++	++ ±	+	±
Sp-6	0	0	0	0

^a From Perlmann (1957).

^b Each antiserum was the serum pool of three or five rabbits. *P-9*, *P-10*, *P-11*: antiegg sera obtained from rabbits injected with homogenates of jelly-free eggs; the mode of preparation of *P-11* differed somewhat from that of *P-9* and *P-10*. *J-3*, *J-4*: antijelly sera; *J-3* was obtained from rabbits injected with a purified preparation, whereas *J-4* was obtained from rabbits injected with a crude preparation. *Sp-6*: antisperm serum, obtained with sperm homogenate.

^c 0 = inactive; ± to +++ = very weak to strongly active.

mitted a more detailed comparison of the various potencies of antisera prepared in different ways (Perlmann, 1957). The results of this study are summarized in Table I. As can be seen, the antijelly sera were much more potent jelly-precipitants than were the antiegg sera. Conversely, the latter were more potent as fertilization inhibitors. The parthenogenetic activity of the antisera was variable. Moreover, whereas the two antiegg sera *P-9* and *P-10* strongly affected the cortical reaction in the fertilized eggs (cortical damage), the activity of *P-11* was low. When tested in precipitin tests with egg extracts as antigen (Fig. 29, a), it was found that *P-11* also was deficient in a number of precipitating antibodies present in *P-9* or *P-10*. In addition, Fig. 29b shows that the strongly parthenogenetic antijelly serum *J-4*, obtained with a crude jelly preparation, contained some antibodies against egg-antigens, whereas *J-3*, obtained with a purified preparation, did not.

These results suggested that a number of different antigen-antibody reactions are responsible for the various response reactions. This could be fully confirmed with the aid of antiserum-inhibition techniques (Perlmann and Perlmann, 1957a, b). Thus, when various preparations, obtained from the eggs, are added to the antisera, antibodies are precipitated or bound by antigen. When tested on the eggs, various activities

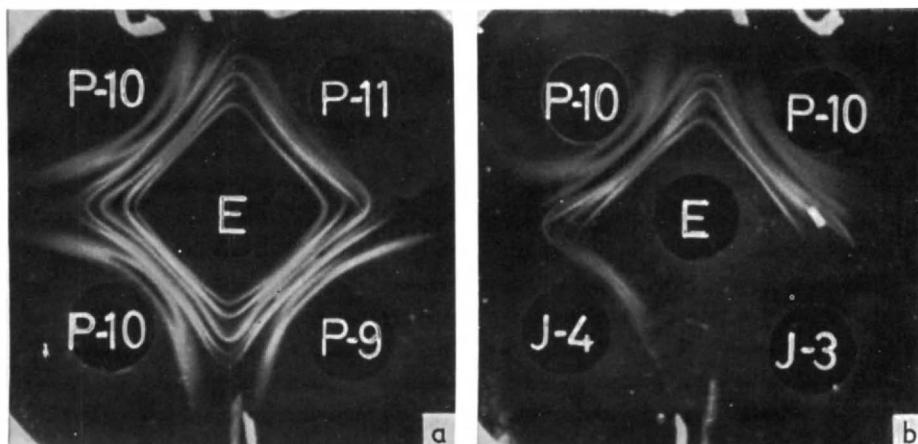


FIG. 29. Precipitin tests in agar. Dark-field photographs, taken after 2 days' incubation at 38° C. followed by 2 weeks in the refrigerator. *E* = soluble part of egg homogenate, diffusing from the central reservoir. Various antisera (cf. Table 1) diffused from the peripheral reservoirs. The lines represent minimum numbers of different antigen-antibody precipitates. From Perlmann (1957).

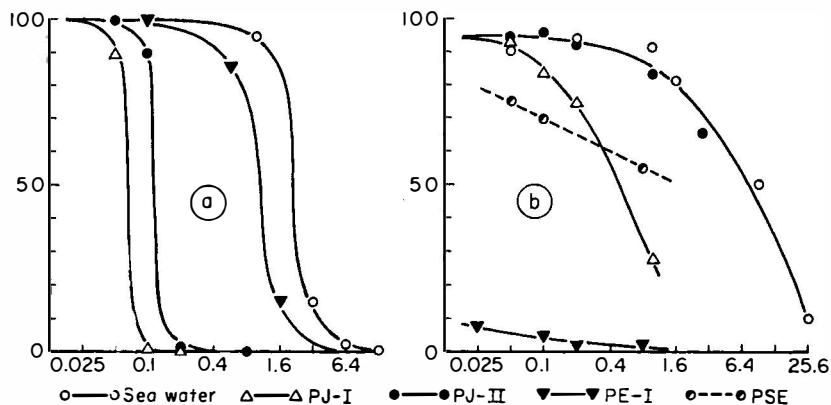


FIG. 30. Unfertilized egg of *Paracentrotus lividus*, treated with aliquots of anti-serum, previously absorbed with antigen or diluted with sea water.

Abscissae: milliliters of various antigen solutions used for absorption of 1-ml. aliquots of an anti-jelly serum (a) or an anti-egg serum (b). PJ-I = solution of *Paracentrotus* egg jelly, purified with alcohol, 20,000 eggs per milliliter; PJ-II = solution of *Paracentrotus* egg jelly, neutralized, 10,000 eggs per milliliter; PE-I = homogenate of unfertilized, jelly-free *Paracentrotus* eggs, 125,000 eggs per milliliter; PSE = homogenate from jelly-free unfertilized eggs of *Psammechinus microtuberculatus*, 125,000 eggs per milliliter.

Ordinate: per cent jelly-precipitated eggs (a) or per cent activated eggs (b). Each symbol represents 200 counted eggs. From Perlmann and Perlmann (1957a).

of such antisera are inhibited, depending on which of the antibodies were eliminated by absorption. The procedure is illustrated in Fig. 30. From the results of the inhibition experiments, conclusions could be drawn concerning the number, location, and chemical nature of several distinctly different surface antigens.

In the eggs of *Paracentrotus lividus*, four different antigens have been determined so far. Thus, jelly precipitation is caused by the reaction of antibodies with a special antigen, the *J*-antigen. The *J*-antigen is a heat-stable carbohydrate, constituting an integral part of the jelly coat. It does not seem to occur in the cytoplasmic egg surface and its immunological reactions do not inhibit fertilization. Parthenogenetic activation is due to the reaction of antibodies with a particular antigen, the *A*-antigen. This is soluble, heat stable, and susceptible to oxidation with periodate and to mild acid hydrolysis. It seems to consist of a glucose-sulfate-containing polysaccharide or mucoprotein. It is part of the egg cortex, but seems to be bound in a rather labile fashion, often occurring in the jelly coats as well. Cortical damage, both in eggs which have been fertilized after pretreatment with antiserum and in antiserum-activated eggs, is caused by another antigen-antibody reaction, involving a soluble but heat-labile antigen, the *C*-antigen. This may be a protein and is probably also located in the cortex. It must be an important link among the molecules which participate in the cortical changes (Section II, D, 4, e). The fertilization rate-depressing action of antiserum involves several components. At least one of these is heat labile and may be identical with the *C*-antigen. In addition, one or more heat-stable factors, distinct from both the *J*- and *A*-antigens, are also involved in this response. This *F*-antigen may be a specific, sperm recepting carbohydrate of the egg cortex. It seems to be less soluble than the antigens mentioned above.

Similar results were obtained with other Mediterranean sea urchins, indicating significant similarities in the surface structures of the eggs. However, considerable differences may occur in the chemical nature of the antigens and in their arrangement in the surface of various types of eggs.

These briefly reported results indicate a complicated antigenic structure of the egg cortex which has very little in common with that of the jelly coat. Moreover, several of the antigens have important functions in fertilization. This does not imply, however, that the chemical determinants responsible for the reactions with the antibodies are necessarily responsible also for the possible functional specificities of the various macromolecules in connection with fertilization (cf. Lillie, 1919). Nevertheless, it seems obvious that further immunological and immunochem-

ical research may contribute to an elucidation of the mechanisms of egg activation and sperm attachment.

8. Metabolic and Ultrastructural Changes

In the growing oöcyte a strong synthetic activity occurs. In the fully developed oöcyte and in the mature egg, the synthetic activity decreases. Even after the maturation divisions, certain synthetic processes may, however, occur in the sea urchin egg, as shown by Kavanau (1954). He found an increase of about 25% in proteins and a corresponding decrease in amino acids in unfertilized eggs of *Paracentrotus lividus* which were kept for 7 hours in sea water. The proteins formed belong to the yolk material.

Fertilization means a rechanneling of the metabolic processes in the egg. The production of yolk proteins stops and a pronounced breakdown of these proteins starts (Örström, 1941; Kavanau, 1954). Also ribonucleic acid seems to be broken down (Elson *et al.*, 1954). Örström (1941) demonstrated a formation of ammonia, recently confirmed by Ishihara (1956). The source of ammonia proved to be adenylic acid. The ammonia production may be an indicator of breakdown of adenosine triphosphate (ATP) occurring in connection with the cortical changes. A direct demonstration of an ATP-breakdown has not been presented.

The breakdown processes mean that stored material is made available for the synthesis of new compounds. Kavanau (1954) has made clear how splitting of yolk and synthesis of embryonic proteins alternate in sea urchin development.

In no other respect is the rechanneling as obvious as in the production of nuclear material. In an early period, this takes place at the expense of relatively complicated precursors stored in the cytoplasm. From a certain stage of development on, deoxyribonucleic acid (DNA) is built up from low-molecular precursors (Zeuthen, 1951; Agrell, 1956).

Synthetic processes require energy which is made available by the oxidations in the cells. It is therefore not surprising to find a certain general correlation between the intensity of the oxidations, measured by the O₂ consumption, and the rate of synthesis in the cell. In the oöcytes of the sea urchin, the oxygen consumption is comparatively high, whereas after maturation the oxygen uptake sinks gradually to a rather low value. After fertilization, the oxygen consumption increases rather suddenly. The periods of increase of oxygen consumption which are observed during the early development correspond to periods of synthesis (cf. Runnström, 1956). An acid is formed in the sea urchin egg upon fertilization (Runnström, 1933). This acid formation starts very early (Fig. 31), simultaneously with the visible cortical changes, and

outlasts these for some time; it certainly reflects enzymatic processes the exact nature of which is unknown. The changes in acid polysaccharides occurring upon fertilization may contribute to acid formation. The rise in acid formation occurs earlier than that of oxygen consumption, as Figure 31 shows.

Nevertheless, one must regard both acid formation and rise in oxygen consumption as rather rapid changes in contrast with the more gradual decrease or increase observed in unfertilized or in fertilized eggs (cf. Fig. 31).

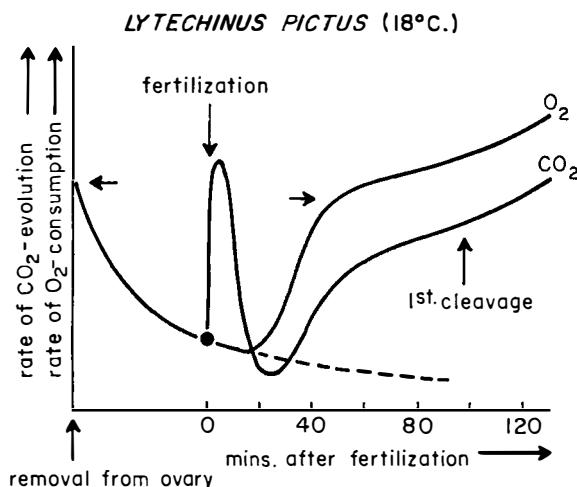


FIG. 31. *Lytechinus pictus*: acid formation and respiration in connection with fertilization. The diver method was used and fertilization brought about within the divers. The left horizontal arrow points to the oxygen consumption prevailing in oöcytes and mature eggs still in ovary. The right horizontal arrow points to the value reached after fertilization. From Borei (unpublished); see also Borei (1948).

The metabolic changes occurring upon fertilization are due to rapid structural rearrangements, acid formation probably directly representing the splitting of macromolecular complexes. The rise in oxygen consumption, on the other hand, reflects the removal of a block within the chain of oxidative enzymes. In this way, the flux of electrons from the more negative part of the oxidative chain to the cytochrome system is increased without synthesis of new enzyme molecules (cf. Runnström, 1956).

Oxygen consumption before and after fertilization has been compared in many different organisms (cf. in particular, Whitaker, 1933). The apparent deviations from the conditions described for the sea urchin are

due to differences with respect to the stage in which the attachment of the activating spermatozoon occurs. If fertilization in sea urchins occurred in the oöcyte stage (Section II, D, 1 and 2) oxygen consumption would not increase, but rather decrease slightly, as follows from Figure 31. (See the points marked with horizontal arrows.) Even in forms where the quantitative changes in oxygen consumption are small or nil, a rechanneling may occur, involving, for instance, a change in substrates oxidized as indicated by the decrease in respiratory quotient found by Brachet (1948, p. 116) in the frog egg. The oxidative process may, in a fully developed oöcyte, be of an essentially nonphosphorylating type, whereas after fertilization a greater portion of the oxidative processes may become engaged in the production of the energy-rich phosphate bonds which certainly play an important role in the developmental processes.

The cytoplasm of the mature unfertilized egg has a coarser structure than the fertilized egg. In dark field or phase contrast, this difference is very obvious. Even in sections, the endoplasm looks more homogenous in fertilized than in unfertilized eggs (Runnström, 1928b, 1949). If unfertilized eggs are put between coverglass and slide, and the tonicity of the medium is slowly increased by evaporation, birefringent fibrous structures appear in the cytoplasm. The birefringence is negative with respect to the longitudinal direction of the fibers. The fibrous structures appear below the cortex and their direction tends to be tangential. On continued exposure to the hypertonic medium, an artificial activation may occur. A membrane separates from the cell surface and at the same time the birefringent structures disappear. In fertilized eggs or in eggs where meiotic division occurs, the negatively birefringent fibers do not appear upon hypertonic treatment. The interpretation is that certain colloids of the ground cytoplasm of the unfertilized mature egg have a more hydrophobic character and therefore tend to aggregate, which gives the more opaque aspect of the egg. After fertilization, groups are exposed which give to the same components a more hydrophilic character, manifested also in the more rapid swelling of fertilized eggs in hypotonic medium, as compared to unfertilized eggs (Runnström, 1955).

The permeability of the eggs to anelectrolytes (ethylene glycol, glycerine) increases upon fertilization (Stewart, 1931). With the help of isotopes, ions, e.g., potassium or phosphate ions, were also found to penetrate more rapidly into fertilized than into unfertilized eggs (Abelson, 1947; Chambers, 1949).

If eggs are inseminated in normal sea water and then transferred after 10–20 seconds to sea water containing $10^{-3} N$ potassium cyanide, the cortical changes are completed in the fertilized eggs. The cortical

changes include the formation of a hyaline layer. The union of the pronuclei and the further progress of mitosis is stopped (Immers and Runnström, unpublished). It is evident from this and other experiments that the cortical changes are independent of oxidative processes and direct oxidative phosphorylation. The energy necessary for the rather complicated cortical changes may be stored in the structures of the cortical layer of the unfertilized eggs, and the splitting processes manifested in the acid formation may release the necessary energy. The problem of coupling between chemical processes during the cortical changes has been considered previously (Section II, D, 4, e).

Hultin (1950a, b) homogenized eggs in calcium-free sea water. By adding calcium to the homogenates, he was able to bring about many of the changes characteristic of fertilization. If the calcium is added under anaerobic conditions, an acid formation can be measured manometrically. Under aerobic conditions, he noted an increase in oxygen consumption. This increase seems to reflect an exposure of SH-groups which are oxidized without corresponding CO_2 output. Even considerable structural changes occur, e.g., the homogenate becomes more viscous but less light scattering. This is analogous to what happens in the cytoplasm of the living egg. Calcium plays an important role in activation, presumably by causing the release of certain enzymes. The temperature sensitivity of the calcium effect indicates that enzymes are involved. Addition of papain has, in fact, the same effect as that of calcium.

Numerous data pertaining to the metabolism of the sea urchin egg were summarized by Krah (1950).

9. Artificial Parthenogenesis

It has been known since the work of Hertwig and Hertwig (1887) and Morgan (1896) that the early steps of development can be brought about by submitting the egg to different treatments; but Loeb (1899, 1900) was the first to get a development to larvae from artificially activated sea urchin eggs. Soon other organisms also were successfully subjected to similar experiments. The method which proved to be successful in amphibia was pricking of the eggs with a needle of suitable size. Bataillon (1910, 1911), who developed this method, found that the inoculation of cellular material with the needle improved the result considerably.

Artificial parthenogenesis has demonstrated that the factors necessary for activation are contained within the egg. The methods applied remove a block. Normally, the spermatozoon exerts this action. So many different methods of artificial activation have been used that a single interpretation has been elusive. The key appears to reside within the egg system, which seems to have the general property of responding to

different stimuli. As mentioned previously, Sugiyama (1953), however, made a distinction between agents which have only a local effect and those which also induce the propagation of an impulse. It seems possible that the point of initial attack may be different in artificial than in normal activation of the egg. This would involve an analogy to the difference between conjugation and autogamy in *Paramecium* (cf. Section II, C, and Metz, 1954).

Let us consider Loeb's "improved method" of artificial parthenogenesis of 1906 (cf. Loeb, 1913). Sea urchin eggs (e.g., *Strongylocentrotus purpuratus*) are treated with butyric acid for some minutes and then returned to sea water. After an interval of 15–20 minutes, the eggs are put in hypertonic medium (50 ml. sea water plus 8 ml. 2.5 N sodium chloride). After 15–20 minutes, the eggs are placed at 5-minute intervals in normal sea water. The eggs exposed to the hypertonic medium for an appropriate time cleave normally and develop. The hypertonic treatment has a "correcting effect" (Loeb, 1913).

The treatment with the butyric acid alone brings about cortical changes and induces nuclear growth and cytoplasmic radiation centered on the nucleus, the "monaster." Cleavage may occur, but proceeds only a few steps. The hypertonic treatment is necessary to bring about further cleavage and development. This experiment gave rise to the conception that two steps are necessary in the activation of the egg.

Just (1922b) and later Bataillon (1929a) demonstrated that membrane formation and complete development may be obtained by one treatment with a sufficiently strong hypertonic solution. On this ground, Just has opposed a dual concept of the activation process. Bataillon, on the other hand, stressed the fact that the hypertonic solution must be alkaline. He maintained that two factors are at work, the hypertonicity and the alkalinity of the medium. The result of the experiments on the pricking of frog eggs seems to give further support to the concept of the dual nature of the activation process. The mere pricking with a clean needle brings about the cortical changes in the egg, but the introduction of cellular material favors the origin of a normally functioning division apparatus.

A kind of dual concept could be built up also from observations on the action of the spermatozoon. Its foremost part, the acrosome region, is able to cause the cortical changes (Section II, D, 4, a). These, on the other hand, induce synthesis of nuclear material and formation of a monaster—thus the action of the acrosome seems to be comparable to the butyric acid treatment in Loeb's improved method. After the engulfment of the spermatozoon, another of its parts plays the active role, namely, the centrosome which organizes the division apparatus.

After the maturation divisions of the sea urchin egg, its centrosome

retrogresses, but it is considered to be reactivated in artificial parthenogenesis. This is evidently a very exacting process which requires a fine balance between breakdown and synthetic processes.

Keltch and Clowes (1947) activated eggs of *Arbacia punctulata* by means of a hypertonic solution and found the same increase in respiration as upon fertilization. Despite this, the artificially activated eggs exhibited a cleavage much inferior to that of the fertilized control eggs. In the artificially activated eggs, the energy released in respiration could not be utilized to the same advantage as in normal eggs. This is probably often the problem in artificial activation. In certain eggs, like those of the starfish *Asterias*, the worm *Urechis*, and the mussels *Cumingia* and *Barnea*, which are activable before the meiotic divisions (Section II, D, 1), the artificial activation often brings about a submersion of the first meiotic spindle which then becomes the first cleavage spindle. In this way, the egg centrosome is kept from waning and development ensues after only one step of treatment. "Correction" is not needed.

Sometimes, however, artificially activated *Asterias* or *Urechis* eggs perform a complete meiosis. The conditions then become similar to those prevailing in sea urchin eggs. In frog and toad eggs, submerging occurs only in exceptional cases. The formation of a dicentric division apparatus is therefore a more exacting process also in these eggs. For further details the reader is referred to the accounts of Dalcq (1928) and Tyler (1941b), and papers by Dalcq *et al.* (1936) and Pasteels (1941).

The cell material used for inoculation in experimental activation of frog eggs has been replaced by fluid cell extracts (Einsele, 1930) or granular fractions (Shaver, 1953). Such studies promise to give some information about the nature of the processes which favor the formation of a dicentric mitotic figure.

King and Briggs (1957) activated frog eggs by pricking with a glass needle and thereafter removed the egg nucleus. A nucleus and some cytoplasm from a blastula or gastrula cell was then injected into the enucleated egg. The transplanted nuclei may be able to adjust themselves to the conditions in the egg cell and a development to larval stages occurs. In these experiments, a centrosome apparatus is probably brought into the egg cytoplasm along with the transplant (cf. King and Briggs, 1957).

The presence of calcium is compulsory in artificial activation, as was shown by Moser (1939). Hultin's work, which was referred to previously (Section II, D, 8), has given some insight in the mode of action of calcium. Increased calcium content induces parthenogenetic development of the eggs of the starfish *Asterias glacialis*, of the worm *Phascolion strombi*, and of the mussel *Cumingia* (Dalcq, 1928; Pasteels, 1941; Hollingworth, 1941; cf. also Heilbrunn, 1956).

Hollaender (1938) irradiated sea urchin eggs with ultraviolet light of different wavelengths. The optimum of activation was given by the short wavelengths below 240 m μ . This shows that the action is not exerted on nucleic acids but on other components of the egg.

III. CONCLUSIONS

In this account emphasis has been laid on the physiological rather than on the genetical aspects of fertilization. The physiology of fertilization is, however, to a large extent a study of mechanisms which ensure the union of one male and one female pronucleus, i.e., of two different haploid genomes which may undergo recombinations in an ensuing meiotic process.

The species specificity is a characteristic trait in fertilization (Section I, E) which prevails also in bacteria and unicellular algae (Section II, A and B). Even where 100% crossfertilization ensues differences in the rate of fertilization may be observed (Section II, D, 6).

In metazoa fertilization involves activation (Section I, D), i.e., a removal of a block which is established in the development of the oöcyte or egg cell. A similar block concerning cell growth and division is established in the development of the male cells; but, in the spermatids, a complicated differentiation process sets in, which results in the formation of the highly specialized male gametes.

In order to explain the block F. R. Lillie (1912) has formulated an hypothesis of great inherent value. He ascribes the block to a "lack of interchange between the egg nucleus and the egg cytoplasm" (Lillie, 1912, p. 447). The reception of the spermatozoon restores "the condition of active and intimate interchange between nucleus and cytoplasm. Aster formation and karyokinesis are evidences of such restoration" (Lillie, 1912, p. 449). More is known now about the structure of both the nucleus and, in particular, the cytoplasm and its inclusions. The endoplasmic reticulum of Porter (1956) seems to be of special importance in this context. It is a finely divided vacuolar system, which is present in all cells and therefore also in egg cells, although these may not be particularly favorable for the study of the system. In sea urchin eggs, a movement of certain structural elements in a peripheral direction takes place in connection with the meiotic divisions (Section II, D, 2). There are indications also that the reticulum is concentrated to a subcortical layer where it appears under the form of the birefringent fibers in eggs exposed to slight hypertonicity (Section II, D, 8). As electron microscopic evidence shows, these fibers represent in fact hollow membranous structures (Lansing *et al.*, 1952). The nucleus assumes also a peripheral position, but has probably in this position only a limited possibility of interaction with the cytoplasm.

Lillie's view regarding the physiological action of fertilization has probably the widest application. The "active and intimate interchange between nucleus and cytoplasm" can be limited or disturbed in different ways. As a consequence, synthetic processes become less vigorous and thus not sufficient to give rise to rapid divisions. In microorganisms, sexual behavior may be induced by such disturbances or limitations in the nuclear-cytoplasmic interchange.

In higher organisms, the conditions are more regulated by genetic mechanisms which, however, are also susceptible to exterior conditions. The block which is established in the development of the female gametes of metazoa, for example, is a well-regulated process of fundamental adaptive value. It maintains the female gamete in a relatively constant state until fertilization is brought about (Dalcq *et al.*, 1936). The sperm attachment involves a complementary reaction with surface structures, corresponding to some of the antigens which have been distinguished in the cytoplasmic egg surface. The A-antigen for example (Section II, D, 7) belongs probably to the acid mucopolysaccharides. These are known to function as enzyme inhibitors (cf. Esping, 1957b). The complementary reaction may lead to a release of enzymes, in analogy to what occurs in anaphylactic and allergic reactions (cf. Perlmann, 1956). Possibly the specific structures of the spermatozoon also contain enzymatically active sites which directly affect the egg surface. The acid formation in sea urchin eggs is certainly a sign of enzymatic breakdown processes (Section II, D, 8). A rapid activation of proteolytic enzymes upon activation has been demonstrated (Lundblad, 1954). The effect of soybean inhibitor on fertilization is dramatic (Section II, D, 4, f).

Acid mucopolysaccharides may act in an inhibitory manner in other ways than those considered. Heparin displaces the lipid constituent in lipoproteins (Chargaff, 1945). Let us assume the inhibited state in the egg before fertilization to be partly due to a displacement of this kind brought about by the presence of acid mucopolysaccharides! If, in one way or the other, these are eliminated upon fertilization of the egg, the lipoproteins are reconstituted. This would account for the impressive structural changes occurring after fertilization or artificial activation of the sea urchin egg (Section II, D, 8). The membranes of the endoplasmic reticulum consist of lipoproteins. There are strong indications of the role of these membranes in enzymatic processes, particularly such as involve protein synthesis (cf. Porter, 1956). Also this approach thus leads to the consideration of enzymatic processes which become activated in fertilization.

The structural changes first propagate themselves in the cortex, but from here they spread to the endoplasmic membrane system. An inward

movement occurs (Section II, D, 4, g) which culminates in the development of the sperm aster centered around the syncaryon. In this stage a maximum viscosity of the cytoplasm obtains (cf. Heilbrunn, 1956). In such a way, the "active and intimate exchange" between nucleus and cytoplasm may be restored. Signs of synthetic nuclear activity have been recorded in these stages (Section II, D, 5). Subsequent to pseudogamy in sea urchins (Section II, D, 4, a), the primary changes of the cortex spread to the interior, where monaster formation and growth of the nucleus occur. This is analogous to the first step in artificial activation produced by exposure to butyric acid in Loeb's method. A second step of treatment is necessary to activate the mitotic apparatus (Section II, D, 9). In normal plasmogamy also, two steps may be recognized; the action of the acrosomal region of the spermatozoon on the egg surface and the introduction of the centrosome. The latter, however, does not become effective unless it has interacted with a normal cortex (Section II, D, 4, g). An interaction between cortex and centrosome is necessary also in further steps of cleavage (Section II, D, 5). The union of the pronuclei is initiated by active movements, in particular of the female pronucleus. It seems to exert a liquefying effect of the cytoplasm (Section II, D, 5).

The changes ensuing upon fertilization do not restore only the capacity for cell divisions. Fertilization is also a step in differentiation, as shown by partially fertilized eggs. Even when provided with nuclei the nonfertilized region develops in an abnormal way (Section II, D, 5).

Nuclei with an incomplete assortment of genes are, on the other hand, not capable of maintaining a lasting interaction of normal character. Even the haploid chromosome complement in general does not suffice to maintain normal development in metazoa (cf. White, 1954, p. 209).

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CHAPTER 10

Sex Determination

By L. GALLIEN

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I. INTRODUCTION

Sexuality is a fundamental characteristic of living things. It occurs with wide distribution among the Protista, as well as among the Metazoa and Metaphyta. Sexuality is not absolutely necessary, however, to assure the perpetuation of the species, as evidenced by the examples of asexual reproduction.

Fundamentally, sexuality involves the existence of male and female haploid gametes which unite during fertilization to form the fertilized egg (see Chapter 9 and Vol. II, Chapter 13). The latter is a totipotent cell, normally diploid, which is the initiation of a new organism. In the Protista, sexual reproduction implies also the association of two elements having the value of gametes. From the outset, sexuality presents a problem of cellular biology.

In the Metazoa, with which we are concerned here, the phenomena of sex implicate the entire organism. In the historical sense of the words, a male may be defined as an individual that produces spermatozoa, a female as one that produces ova. The gametes are elaborated by specialized glands, the gonads (testis and ovary) which are the substratum of the germinal cells. In addition, there are somatic structures, including the genital tract and the secondary sexual characters, as well as psychic activities which are more or less directly involved in transport of the genital products and in fertilization. In the aggregate, they give the organism its sex.

The most refined state of sexuality is shown by the gonochoristic species in which the sex glands are carried by two different individuals. In hermaphrodites whose characters are complex, the somatic sexuality is impaired and sex characters may be limited to the existence of gametes.

Sex determination is the process by which the egg becomes genetically constituted for the role that the eventual organism will assume in gamete production. It may give rise to a male individual which produces spermatozoa, or a female which develops ova.

The process of sex determination is fundamentally genetic. Usually, the hereditary factors established at fertilization guide and control the embryonic differentiation in the male or female line so that the adult corresponds to the sexual type of the fertilized egg.

During development, the organism is exposed to environmental factors such as nutrition, temperature, or hormones which may oppose the genetic factors established at fertilization. These epigamous factors may alter or, in the extreme, completely dominate the primary genetic control. In other words, the *sex differentiation* and the resultant sexual phenotype of the individual may not correspond to its genetic constitution.

The phenomenon of sex determination is widespread, embracing both the animal and vegetal kingdoms. Within the scope of this chapter, we can take into consideration only the major points concerning the Metazoa.

II. CYTOGENETIC THEORY OF SEX DETERMINATION

It is from gonochoristic organisms that we may derive the clearest understanding of the sex-determination mechanism. Generally speaking, in species where sexes are separated, there are nearly as many males as females, i.e., the *sex ratio* is $1 \sigma : 1 \varphi$. Thus in the human species there are about 104 boys for 100 girls; the sex ratio is $1.04 : 1$. It is $1.07 : 1$ for the cat, $1.05 : 1$ for the mouse, and $0.98 : 1$ for the sheep. The slight inequalities observed are due to various causes, such as the higher prenatal death rate of one sex or its slower development.

The numerical equality of the sexes led Castle (1903) to suggest that sex determination could be controlled by a genetic mechanism. Indeed the ratio $1 \sigma : 1 \varphi$ is precisely the one observed in a cross between a dominant heterozygote and a recessive homozygote. If we refer to masculinity with the dominant symbol M, and to femininity with the recessive symbol f, the cross is written as in Diagram 1.

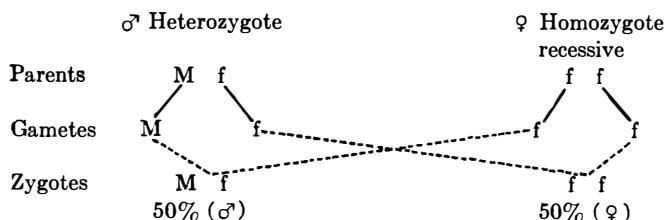


Diagram 1

According to this theory expressed by Correns (1907), one of the sexes has a hybrid constitution, with one of the factors of sexuality dominant; it is heterogametic (or digametic) and produces two types of gametes. The other sex is homozygous, recessive: it is homogametic and produces one type of gamete only. The reverse formula can also be imagined, i.e., $\varphi Fm : \sigma mm$. In both cases, the factorial explanation of sex determination is based on the digamety of one of the sexes, and the homogamety of the other.

Consequently, and especially after the discovery of sex chromosomes, general conventional formulas were adopted which presented this interpretation of the genetic constitution of the sexes:

$$\begin{aligned} \text{Male heterogamety: } & XY (\sigma) : XX (\varphi) \\ \text{Female heterogamety: } & ZW (\varphi) : ZZ (\sigma) \end{aligned}$$

The proposed theory means that there is a definite relationship between the quality of the germinal cells and the nature of the sex. Sex determination is established during the fertilization act, thus with amphimixis. Thus *sex determination is fundamentally syngamous*.

The mechanisms of sex determination are known not only in animals but also in plants, where sex chromosomes have been found.

III. KARYOLOGIC BASIS OF THE MECHANISMS OF SEX DETERMINATION

The cytogenetic theory of sex determination has been confirmed by two types of researches. These have been the karyologic studies, with the discovery of heterochromosomes, and the analysis of special crosses.

Henking (1891) in the hemipteran, *Pyrrhocoris*, Paulmier (1899) in *Anasa tristis*, de Sinety (1901) in the orthopterans, *Orphania* and *Dixippus*, and Montgomery (1902) in *Protenor* and *Alydus* had discovered among the chromosomes of these species an element that showed a peculiar behavior during meiosis. This element, which was pointed out as an aberration, was at first misinterpreted. MacClung (1902) was the first to understand the meaning of the odd chromosome he encountered during spermatogenesis of the orthopteran, *Xiphidium fasciatum*. He suggested that the two kinds of spermatozoa formed, with or without this element, were directly related to the sex of the offspring. Later, the works of Wilson (1905, 1906, 1928) and his students specified clearly the importance of the relationship suggested by MacClung. Various names were given to the chromosomes that determined sex: accessory chromosomes, X chromosomes, idiochromosomes, sex chromosomes, heterochromosomes.

Thus we have been led to distinguish two categories among the chromosomes of an organism. One category comprises paired chromosomes which are similar in both sexes. These are the autosomes (A). During meiosis, they separate into equal groups in all the gametes of both sexes. In the other category are chromosomes differing from one sex to the other. They are unequally distributed during meiosis in the gametes of one of the sexes. These are the heterochromosomes named according to the case X, Y, Z, W. Their arrangement achieves the visible digamety of one of the sexes.

A. General Mechanisms

1. Male Heterogamety

a. *Protenor type*: XO (♂) : XX (♀). This represents the simplest situation. The females have a pair of homologous heterochromosomes. The males have a single heterochromosome. Thus in the hemipteran

Protenor belfragei (Wilson, 1905) the female has 14 chromosomes including 12 autosomes (A) and two heterochromosomes or X chromosomes; its formula is AAXX. The male has 13 chromosomes including only a single X chromosome, i.e., AAXO. The heterochromosomes are easily recognized, for their size is at least twice that of the largest autosomes (Fig. 1).

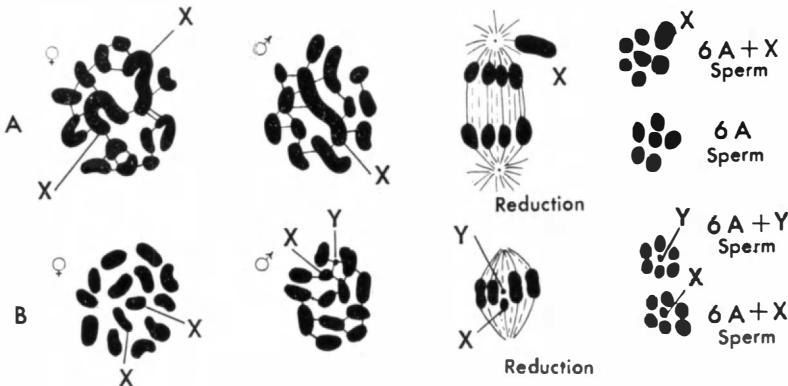
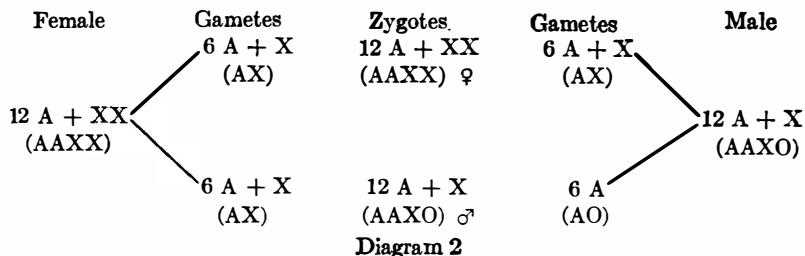


FIG. 1. Male heterogamety. A. *Protenor belfragei*. B. *Lygaeus turcicus*. From Wilson (1906).

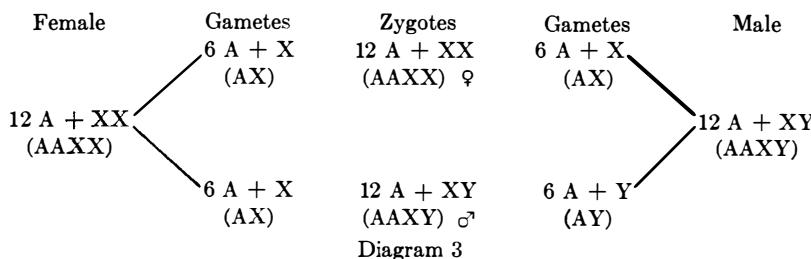
In the female, during chromosomal reduction, 6 autosomes and 1 X go to the polar body. All the ova receive 6 autosomes and 1 X and are therefore similar. In spermatogenesis, the single X chromosome passes to one of the poles during the reductional division so that two types of spermatozoa are formed. Half of them have the normal complement of autosomes and an X chromosome, totaling 7 chromosomes; the other half has only autosomes, for a total of 6 chromosomes. Fertilization occurs at random. Thus the male sex digamety is achieved (Diagram 2).



Zygotes with $12\text{ A} + \text{XX} = 14$ chromosomes possess the female formula and give rise to females; those with $12\text{ A} + \text{X} = 13$ chromosomes have the male constitution and become males.

b. Lygaeus and Drosophila type: XY (σ) : XX (φ). The second type of male digamety is characterized by the fact that, in the male, the X chromosome possesses a partner, called the Y chromosome. X and Y are nonhomologous or only partly so. The female always has a pair of X chromosomes which are homologous throughout their entire length. The chromosomal formulas are XY = σ and XX = φ . Since the autosomes are identical in both sexes, the diploid number of chromosomes is equal in the male and in the female. However, the shape and the structure of the Y chromosome are different from those of the X chromosome. Consequently, two categories of male gametes can again be identified (Fig. 1).

Thus in the hemipteran *Lygaeus turcicus*, the females possess 14 chromosomes: 12 A + XX, i.e., AAXX. All their ova are similar and receive 1 X. The males also possess 14 chromosomes, among which are 12 autosomes. But there are two different heterochromosomes. One of them is similar to the X of the female. The other is smaller; this is the Y chromosome. The male formula is 12 A + XY, i.e., AAXY. During the reductional division, the XY pair segregates. The X chromosome passes to one pole of the spindle and the Y chromosome passes to the other pole. The result is the formation of two types of spermatozoa: 6 A + X and 6 A + Y (Diagram 3).



This mechanism also achieves the digamety of the male sex. The Y chromosome characterizes the male lineage and comes from spermatozoa only. It is transmitted by the father. The X chromosome of the male comes from the mother. The female zygote receives one X from the mother, the other X from the father. These facts will assume their full implication when we consider sex linked inheritance.

The fruit fly, *Drosophila melanogaster*, belongs to the *Lygaeus* type. The female possesses 8 chromosomes (6 A + XX), the male has 6 autosomes + XY. The Y chromosome has the shape of the letter J (Fig. 2).

In some cases, the Y chromosome may not be different from X in shape or size, but the XY pair can still be recognized by its behavior.

Finally, in many species, the heterochromosomes cannot be identified cytologically within the entire chromosomal set. This is the case in many vertebrates. The urodeles are a good example of this situation. Only genetic studies based on appropriate crosses enable us to determine which is the heterogametic sex.

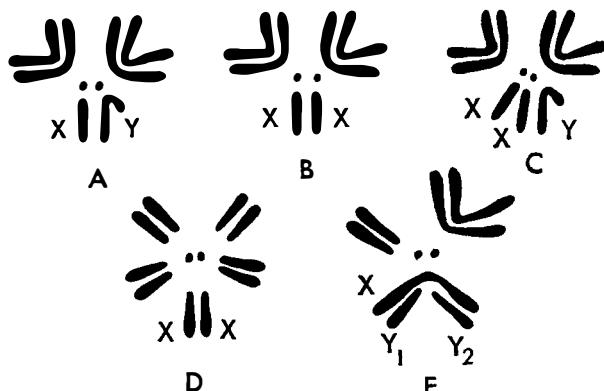
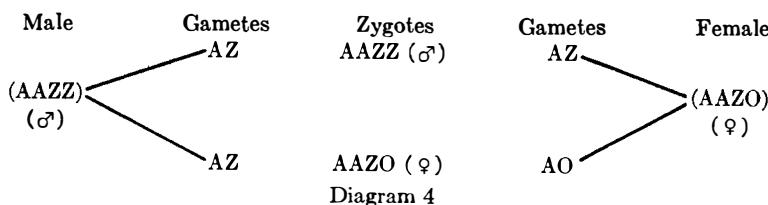


FIG. 2. Diagrams of chromosome sets in *Drosophila*. A, B, C *Drosophila melanogaster*: A = ♂; B = ♀; C = XXY type. D. *Drosophila virilis* ♀. E. *Drosophila americana* ♂.

2. Female Heterogamety

Abraxas type. In this type, which has been analyzed cytologically in Lepidoptera (Seiler, 1914a, b, 1920), the female is digametic, and the male is homogametic. The formulas are XX (♂) : XY (♀). However, conventionally and according to present usage the symbols ZZ (♂) : ZW (♀) have been adopted. In *Fumea casta*, the male possesses 62 chromosomes and produces spermatozoa with 31 chromosomes; the female has 61 chromosomes and produces two kinds of ova with 30 or 31 chromosomes. The male of *Talæporia tubulosa* has 60 chromosomes; the female has 59. Half of the ova have 29 chromosomes; the others have 30. The Diagram (4) of sex determination is the reverse of that of *Protenor*.



When, in the heterogametic sex, the Z heterochromosome possesses

a W partner, the formula AAZO is expressed AAZW. Generally in the case of female digamety, the heterogametic sex has been identified through genetic experiments.

B. Evolution of the Chromosomal Mechanisms of Sex Determination

1. Behavior and Properties of Heterochromosomes

The heterochromosomes that appear most clearly, i.e., in Hemiptera and Orthoptera, show particular characteristics which enable their identification cytologically. The absence of a chromosome (XO) or dissymmetry of the elements of a pair (XY) is readily noticeable in the XO or XY types of heterochromosome constitution. Size relationships between autosomes and heterochromosomes may vary. In *Pyrrhocoris*, *Protenor*, and *Orphania*, the heterochromosomes are larger than the autosomes. There is no great difference in size in *Oncopeltus*; the heterochromosomes can be identified, nevertheless by their behavior. In *Photinus*, they are the smallest elements of the karyotype. The rodent *Microtus agrestis* (Fig. 3) possesses giant-sized sex chromosomes (Matthey, 1950).



FIG. 3. Spermatogonial metaphase of *Microtus agrestis* ($2n = 50$) showing the giant-sized sex chromosomes. From Matthey (1950).

One of the two divisions of meiosis is equational: during this division, the heterochromosomes divide normally. The other division is reductional: each heterochromosome segregates and passes to one of the poles without dividing (Fig. 1). Generally in Homoptera and Orthoptera, the first meiotic division is reductional (prereduction). In most of the Heteroptera, the heterochromosomes disjoin during the second maturation division (postreduction).

The heterochromosomes often exhibit a peculiar behavior during segregation. At the anaphase stage (Fig. 4), they precede (precession) or follow (succession) the autosomes, during the ascension to the poles.

Precession is the rule among the Orthoptera, and succession is usual among the Heteroptera (except *Syromastes*). Both types of behavior can be observed among Coleoptera and Nematoda. In some instances where there is precession (*Gryllus*), the heterochromosomes manifest a relative independence if compared with the autosomes during interkinesis (Fig. 4).

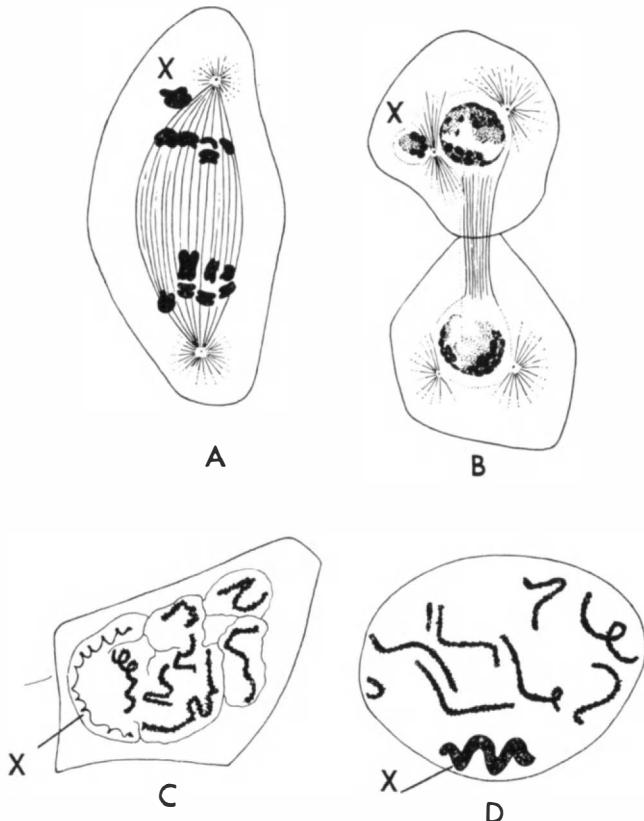


FIG. 4. Behavior of heterochromosomes. A. First spermatocyte anaphase in *Gryllus desertus*, precession of X. B. Same species, interkinesis; X a separate vesicle. C and D. Early spermatogonial prophase nuclei: in *Chortophaga viridifasciata* (C) and *Metrioptera brachyptera* (D); X chromosome negatively heteropycnotic in C, positively heteropycnotic in D. A and B redrawn from Brunelli (1909); C and D from White (1954).

During the growth of the spermatocytes, the heterochromosomes are often round and dense. They contrast by their stain affinity with the filamentous or diffuse autosomes. This property, initially called heteropycnosis, is particularly characteristic of the heterochromosomes, if not

specific to them. The heteropycnotic regions may be said to be composed of heterochromatin (Heitz, 1928). The chromophilic property of the same chromosome may vary according to the stage. The densely colored regions are positively heteropycnotic. The less densely colored parts are negatively heteropycnotic (Fig. 4).

Apart from species in which heterochromosomes can be identified by their properties, there are many cases in which it is difficult to identify the chromosomal pair that plays the decisive part in sex determination. They are genetically, but not cytologically, differentiated.

2. Relations between Heterochromosomes and Autosomes

As a result of translocation, autosomes or parts of autosomes are incorporated in heterochromosomes. Autosomal parts translocated to the X and Y elements are initially homologous, but their further evolution gradually leads to the constitution of new differential segments and to the development of heterochromatic properties. Such processes have been observed in grasshoppers, phasmids, and in *Drosophila*.

Among the grasshoppers, where most species are XO (σ) and the X chromosome acrocentric, there are species in which a centric fusion between X and an autosome may have occurred in the course of evolution, giving rise to the constitution of an XY system. In this case the unfused homologue of the chromosome having united with the original X plays the role of a Y chromosome. It is called a neo-Y. The X element which has become metacentric can be named a neo-X, to distinguish it from the original acrocentric chromosomes. Each neo-X is constituted with one limb representing the original X and with a limb which was originally autosomal. In meiosis, a chiasma is formed between the distal part of the latter and the neo-Y. These two segments were probably at first homologous on all their length; but eventually most of the neo-Y became heterochromatic, while the homologous part of X remained euchromatic. The gradual development of heterochromatin in the neo-Y may have happened, as Muller (1932) suggested, through the mutation of its genes into recessive or completely inactive alleles. This process, which achieves the passage from an XO (σ) type to an XY (σ) type consequent to centric fusions between X and an autosome, is known among grasshoppers in fourteen species according to Helwig (1941).

Patterson and Stone (1952) point out twelve species of the *Drosophila* genus in which there is a fusion between the X chromosome and one of the largest autosomes. In three species, X has become fused with the tiny dot chromosome. In both *D. prosaltans* (Spassky and co-workers, 1950) and *D. willistoni* (Spassky and Dobzhansky, 1950), the right segment of X contains genes suggesting a homology with the left arm of chromosome III in *D. melanogaster*.

The occurrence of the X-autosome fusions in *Drosophila* species having acrocentric chromosomes, e.g., *D. subobscura* or *D. virilis*, would lead to the formation of a multiple heterochromosome system (Fig. 2). In this XY_1Y_2 type the free homologous element of the chromosome linked to X would be associated to the male line. It becomes a Y_2 , whereas Y_1 represents the original Y. Such a case seems to be that of *D. americana americana* ($\sigma = XY_1Y_2$).

3. Multiple Heterochromosomes

The mechanism of sex determination is sometimes more complex than when there is a distinct XY pair. The X or Y chromosome, or even both elements may be represented by a set of multiple heterochromosomes (Fig. 5). Such systems are symbolized by the formulas X_1X_2O ,

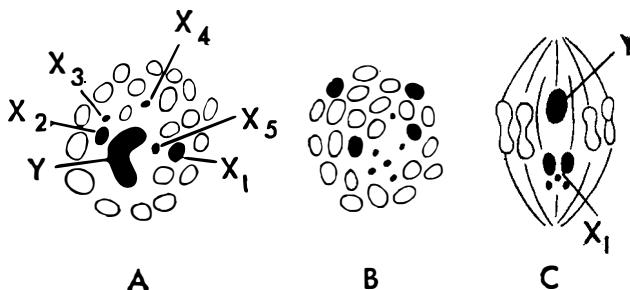


FIG. 5. Multiple heterochromosomes in the hemipteran *Acholla multispinosa*. Sex chromosomes in black, autosomes in outline. Y is the largest chromosome; X_1X_2 , X_3 , X_4 , X_5 -compound X chromosomes. A. Spermatogonial metaphase. B. Oögonial metaphase. C. Second anaphase in the male (side view), showing X_{1-5} segregating as a unit from the Y chromosome. From Payne (1910).

X_1X_2Y , $X_1X_2X_3Y$, XY_1Y_2 , etc. In this way we can express the situation when X or Y is double or triple. The general formulas are written X_nO , X_nY , XY_n , X_nY_n . The beetle *Blaps polychresta*, whose karyotype is $2n$ (σ) = $18A + 12X + 6Y = 36$ and $2n$ (φ) = $18A + 24X = 42$, probably shows the most complex system of multiple heterochromosomes (Guénin, 1953).

During meiosis, the group of multiple heterochromosomes acts as a unit segregating like a simple X or a Y. There are some exceptions to this rule (e.g., *Tegenaria domestica*).

The constitution of systems with multiple heterochromosomes seems to be achieved through two methods. The first is through incorporation subsequent to the translocations of one or several pairs of autosomes to the initial pair of heterochromosomes (XY); the other is through fragmentation into two or several elements of one initially single X or Y chromosome (White, 1954).

Drosophila americana americana is, as indicated earlier, an example of the first mechanism. Likewise, the conversion of the mechanism XO into X_1X_2Y probably occurred directly, without passing through the stage XY, in some species of mantids (Orthoptera), where the formulas are X_1X_2Y (σ) : $X_1X_1X_2X_2$ (φ). Indeed we do not know of any mantids of the XY type (Fig. 6).

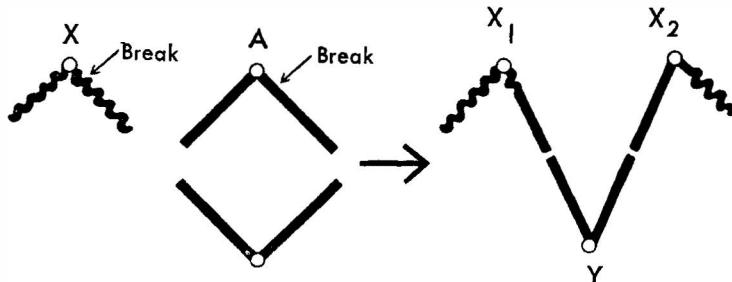


FIG. 6. Presumed conversion of the mechanism XO into X_1X_2Y in mantids by a mutual translocation between the X and a metacentric autosome (A). From White (1954).

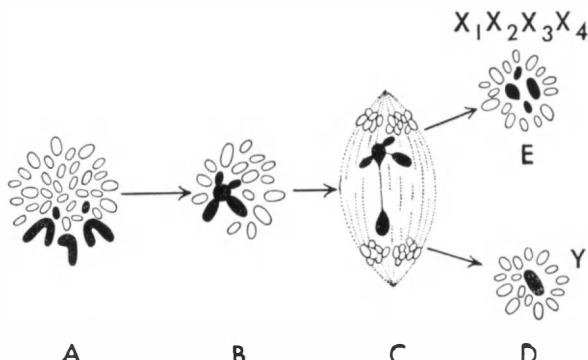


FIG. 7. Multiple heterochromosomes in the beetle *Blaps lusitanica*; $X_1X_2X_3X_4Y$ in black, autosomes in outline. A. Spermatogonial metaphase. B. Metaphase. C. Anaphase of first meiotic division. D and E. Second meiotic divisions. From Wilson (1928).

The formation of multiple heterochromosomes through fragmentation of a single chromosome probably occurred in Hemiptera (Heteroptera). In many species, belonging to about ten families of this group, multiple heterochromosomes have been reported (Schrader, 1947), as well as in species in which the heterochromosomes are single. The interpretation proposed is based on the fact that, in a single genus presenting species with simple heterochromosomes and other species with multiple hetero-

chromosomes, no decrease in the number of autosomes is noticed, in spite of the increase in the number of heterochromosomes.

The type X_nY is known in Coleoptera (Fig. 7) in the genera *Blaps* (Nonidez, 1915, 1920; Guénin, 1950, 1953) and *Cicindela* (Guénin, 1952), as well as in the insectivore *Sorex araneus* (Bovey, 1949) and the rodent *Gerbillus pyramidum* (Matthey, 1953).

XY_n arrangements have been described in the cricket *Eneoptera surinamensis* (Piza, 1946), in the marsupials *Potorous tridactylus* (Sharman and co-workers, 1950) and *Macropus ualabatus* (Agar, 1923). These species have the male formula XY_1Y_2 (Fig. 8).

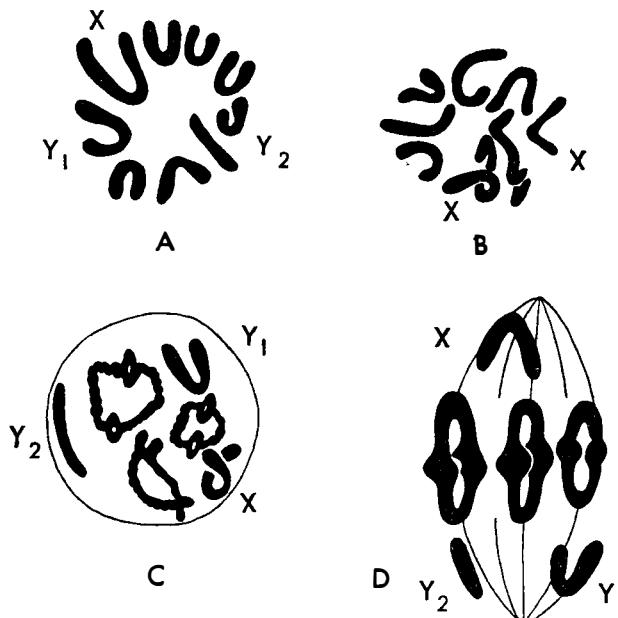


FIG. 8. Multiple heterochromosomes of the cricket *Eneoptera surinamensis* (XY_1Y_2 ♂). A. Spermatogonial metaphase. B. Female somatic metaphase. C. Diainesis in the male; three autosomal bivalents and three sex chromosomes. D. First metaphase in side view. From Piza (1946).

Systems of heterochromosomes of constitution X_nO have been identified (Table I) in insects, spiders, nematodes, and in the ostracod *Heterocypris incongruens* (Bauer, 1940). For example, in spiders, among 125 species studied by various authors (White, 1954), 103 belong to the type X_1X_2O (σ); 6 species are $X_1X_2X_3O$ (σ); and only 16 are of the type XO (σ).

Finally, in Nematoda, a series of species with multiple heterochromo-

somes (X_nO) is known. *Belascaris triquetra*, *Ganguleterakis spinosa* are X_1X_2O (δ); *Toxascaris canis* has the constitution $X_1X_2X_3X_4X_5X_6O$.

4. Heterogamety and Homogamety in Zoological Groups

The heterochromosomal control of sex determination has been observed in a number of zoological groups. The major results are grouped in Table I. The column titled "Genetic evidence" shows the cases for which the heterogamety of a sex was established by genetic data, although heterochromosomes could not be observed cytologically with

TABLE I
DISTRIBUTION OF SEXUAL TYPES IN ZOOLOGICAL GROUPS

Groups	Male heterogamety: Karyologic evidence (heterochromosomes)			Genetic evidence
	XO	XY	Compound types	
Psocoptera	+			
Embioptera	+			
Ephemeroptera	+	+		
Neuroptera		+		
Odonata	+	+		
Orthoptera	+	+		
Homoptera	+	+	+	
Heteroptera	+	+	+	
Coleoptera	+	+	+	
Diptera	+	+	+	
Myriapoda	+			
Arachnida (spiders)	+		+	
Crustacea	+	+	+	
Nematoda	+		+	
Fishes (3 Poeciliidae)				+
Amphibia (Ranidae)				+
Mammalia	+	+	+	+
Groups	Female heterogamety: ZO (φ) or ZW (φ): Karyologic evidence (heterochromosomes)			Genetic evidence
Lepidoptera	+			+
Trichoptera	+			
Fishes (1 Poeciliidae)				+
Amphibia (Urodela and <i>Xenopus</i>)				+
Reptilia		+(?)		
Birds		+(?)		+

certainty. In this regard, the vertebrates present an interesting situation. In his critical review on the matter, Matthey (1949) came to the conclusion that the actual observation of heterochromosomes in this group is limited to the mammals. However, the genetic analysis is extensive enough to permit the establishment of the sexual constitution of various types.

In cyclostomes, fishes, and amphibians the karyologic data are either contradictory or the heterochromosomes cannot be cytologically identified at all.

For reptiles, there are no substantiated observations concerning the existence of heterochromosomes. According to Oguma (1934), in the lizard *Lacerta vivipara* ($2n \delta = 36$, $2n \varphi = 35$), the female would be of ZO type. He reports the same situation (Oguma, 1937a) in the turtle *Amyda japonica* ($2n \delta = 64$, $2n \varphi = 63$). Makino and Asana (1948) conclude also in favor of female heterogamety in two other agamid lizards, *Calotes versicolor* and *Sitana ponticeriana*. However, Matthey (1949) contests the cytological proof of a female digamety in reptiles. In this class of vertebrates there is no genetic evidence establishing the heterogametic sex.

The birds present an exceptionally difficult subject for study because of their large number of chromosomes and the existence of many microchromosomes (in the hen, for example $2n = 77$ or 78). As a result of the very elaborate work of Yamashina (1944) in the chicken, we may conclude that the female would be ZO, with the Z element being fifth in size. Female heterogamety has been established in birds through genetic data.

In the mammals nothing is known concerning the sex chromosomes of monotremes. On the other hand, for marsupials, authors agree to the existence of an XY pair in the male. Counting of chromosomes is easier in this case because of the small number of elements (12 to 28). Valid evidence of XY maleness has been presented for eighteen species belonging to the genera *Didelphis*, *Lutreolina*, *Dasyurus*, *Sarcophilus*, *Isodon*, *Petauroides*, *Petaurus*, *Phascolomys*, *Potorous tridactylus* and *Macropus ualabatus*, as indicated earlier, possess multiple heterochromosomes.

The situation is complex and still controversial in the case of the Eutheria (see Matthey, 1949). In the best-known cases, it appears that the male sex is heterogametic and generally of XY type. However, Oguma (1937b), considers the following species to have the XO (δ) formula: *Apodemus speciosus ainu*, *Evotomys bedfordiae*, *Microtus montebelli*. Finally *Gerbillus pyramidum* (Matthey, 1953) and *Sorex araneus* (Bovey, 1949) show multiple heterochromosomes.

In man, all authors agree that there is male heterogamety and most

investigators count 46 autosomes. Some cytologists contend that there is a single X chromosome in the human male: XO (σ) : XX (φ), whereas others believe that there is also a small Y element, which leads to the formula XY (σ) : XX (φ). Genetic data (color blindness, hemophilia) confirm the heterogamety of the male sex.

Moore and co-workers (1953) demonstrated a sexual dimorphism of the resting nuclei in human skin cells. In the female there is a dense chromatic body, adjacent to the nuclear membrane, which is rarely observed in the male. The relation of this formation with the sexual chromosomes has not been clearly established.

C. Special Cases of Sex Determination

The phenomenon of sex determination generally manifests itself with the establishment of a 1 σ : 1 φ sex ratio, on the basis of the chromosomal mechanisms described above. There are, however, some exceptional cases which result in a variable sex ratio as a consequence of various alterations from the usual chromosomal cycles. This is the case in arrhenotokous parthenogenesis and in monogeny. The sciarid and cecidomyiid insects have entirely abnormal cycles. In the annelid *Dinophilus*, there is a progamous sex determination type, but its basis is not known. We shall consider only the characteristic facts about these unusual cases (for details see Bridges, 1939; White, 1954).

In some groups of animals the males are born from unfertilized eggs through a particular form of parthenogenesis called arrhenotoky, haploid parthenogenesis, or haplodiploidy. Although the males are haploid, the females are diploid and originate from fertilized eggs. The genetic system in effect differs from thelytokous parthenogenesis, in which females are born through a diploid parthenogenesis not involving a new mechanism of sex determination.

Arrhenotoky is the rule in Hymenoptera. It is known in some Acarina and rotifers and, among insects, in a few species of Homoptera (aberrant coccids), Coleoptera (*Micromalthus debilis*) and Thysanoptera.

The classical example of arrhenotoky is the case of the bee (*Apis mellifera*). The chromosomal cytology of this species has been thoroughly studied; however, the data do not agree exactly. According to most authors, the female has 32 chromosomes, the male 16. The gametes of each sex possess 16 chromosomes. Fertilized eggs produce females with 32 chromosomes; parthenogenetic eggs have but 16 chromosomes and become males. The sex ratio can vary widely. Alterations in the normal meiosis process must occur, since gametes produced by haploid males are themselves haploid. It seems that there is only a single mitotic maturation division which is equational (Walker, 1949; White, 1954). However, the modality differs among the various groups of Hymenoptera.

On first appraisal, the sex-determining mechanism in Hymenoptera might be interpreted simply on the basis of the number of chromosomes controlling the sex. This interpretation, however, is not easily reconciled with the concept of genic balance which states, as we shall see, that the sex depends on a balance between male and female determining genes. The studies of the Whitings, P. W. and A. R. (1925) and Whiting, P. W. (1939-1943) on a braconid, *Habrobracon juglandis*, helped to clarify the situation. Through inbreeding of this species, poorly viable diploid males have been obtained. They show genetic characters inherited from both parents, thus excluding the possibility of a diploid parthenogenetic origin. According to Whiting (1939-1943), the female *Habrobracon* is heterozygous for some sexual factors which can be written x_1, x_2, x_3, \dots . The haploid males may differ, each one having a different x factor, (x_1, x_2, x_3, \dots). The diploid males are homozygous for the x factors, ($x_1x_1, x_2x_2, x_3x_3, \dots$). The females are diploid and heterozygous, ($x_1x_2, x_1x_3, x_2x_3, \dots$). Whether or not these x factors are a series of multiple alleles at a single locus remains unestablished.

The ratio $1 \sigma : 1 \varphi$ implies that the X and Y chromosomes are arranged at random during the reductional division. If for any reason the distribution is selective, the result will be an alteration of the sex ratio. In the extreme, this could lead to monogamy, i.e., to the presence of a single sex in the offspring. This is seen in terrestrial isopods, although both sexes are diploid and the chromosomal cycle is normal. In *Armadillidium vulgare*, the populations possess a nearly equal number of gynogenic and androgenic females (Vandel, 1941).

Dinophilus (Archiannelida) shows a very peculiar type of sex determination (Korschelt, 1882; de Beauchamp, 1910; Shearer, 1912; Nachtsheim, 1914). The females lay large eggs which give rise to females and small ones which develop into dwarf males. Since the difference between the eggs is present long before meiosis, it is difficult to explain the phenomenon on a genetic basis. No sexual chromosomes have been identified in *Dinophilus*. Apparently sex determination depends on the amount of egg cytoplasm available for the development of the embryo. It is likely that an unequal oögonial division gives origin to the two types of eggs.

IV. EXPERIMENTAL VERIFICATIONS OF THE CYTOGENETIC THEORY OF SEX DETERMINATION

Sex determination must be interpreted on the basis of genic action. Consequently the study of the normal and atypical behavior of chromosomes is an essential method for the confirmation of the cytogenetic theory of sex determination. The sex chromosomes may be marked with genes which are not necessarily connected with sexuality. Thus the

genetic studies concerning sex determination started with the discovery of sex-linked inheritance. This process involves the transmission of inherited factors with a distribution following exactly that of the X chromosomes. Two types of sex-linked inheritance corresponding to the two kinds of dissymmetry of heterochromosomes have been discovered.

A. Sex-linked Inheritance

1. *Drosophila Type (Male Heterogamety)*

Morgan (1910) in *Drosophila melanogaster* studied crosses between *red*-eyed wild type (R dominant) and the *white*-eyed mutant (w recessive). The results which differ according to the cross, can be explained on the assumption that the white gene is carried by the X chromosome. Thus the white gene can occur only once, in the male, on the single X chromosome. The breeding tests are expressed in Diagram 5.

Cross I:

♀ Red: $X_R X_R$	\times	$X_w Y \sigma^a$ white
F_1 $X_R X_w$		$X_R Y$
		(♂ Red, homozygous)
		$X_w Y$
F_2 $X_R X_R$	$X_R Y$	$X_R X_w$
	(♀ Red)	(♂ Red)
		$X_w Y$
		(♀ Red, heterozygous)
		(♂ white)

Cross II:

♀ white: $X_w X_w$	\times	$X_R Y \sigma^a$ Red
F_1 $X_w X_R$		$X_w Y$
		(♂ white homozygous)
		$X_R Y$
F_2 $X_w X_w$	$X_w Y$	$X_R X_w$
	(♀ white)	(♂ white)
		$X_R Y$
		(♀ Red, heterozygous)
		(♂ Red)

Diagram 5

The results support Morgan's (1910) hypothesis. The analysis was extended to a number of other sex-linked mutants. The studies proved that the Y chromosome is genetically devoid of the genes controlling this group of mutants.

2. *Abraxas Type (Female Heterogamety)*

This type of heredity was demonstrated in the moth *Abraxas grossulariata* by crossing the *grossulariata* species with the *lacticolor* mutant. The latter shows a reduction of the spots of the wings (Doncaster and Raynor, 1906; Doncaster, 1908). The results of this cross (Diagram 6) are explicable with the assumption that the *lacticolor* allele (l recessive) of the wild-type *grossulariata* (G dominant) is localized on the Z chromosome.

The same type of heredity has been demonstrated in birds.

Cross I:

♀ Grossulariata	$Z_G W$	×	$Z_I Z_I \sigma^l$ lacticolor
F ₁	$Z_G Z_I$		$Z_I W$
	(♂ Grossulariata heterozygous)		(♀ lacticolor homozygous)
F ₂	$Z_G Z_I$	$Z_G W$	$Z_I Z_I$
	(♂ Gros.)	(♀ Gros.)	(♂ lact.)
			$Z_I W$
			(♀ lact.)

Cross II:

♀ lacticolor	$Z_I W$	×	$Z_G Z_G \sigma^l$ Grossulariata
F ₁	$Z_G Z_I$		$Z_G W$
	(♂ Grossulariata heterozygous)		(♀ Grossulariata homozygous)
F ₂	$Z_G Z_G$	$Z_G W$	$Z_G Z_I$
	(♂ Gros.)	(♀ Gros.)	(♂ Gros.)
			$Z_I W$
			(♀ lact.)

Diagram 6

3. One-sided Sex-linked Inheritance

This peculiar mode of sex-linked inheritance has been demonstrated by Winge (1922, 1927) in the fish *Lebistes reticulatus*. In this species some genes are absolutely linked to the chromosome Y. An example is the gene *Maculatus* (Ma), which produces a black spot on the dorsal fin. Since the Y chromosome is confined to the male line in standard races, the M gene is transmitted from father to son and grandson. It is never inherited by females.

Thus sex-linked inheritance is accomplished by two processes corresponding to the two types of dissymmetry of heterochromosomes and in accordance with the distribution of the X (Z) or Y (W) chromosomes.

B. Nondisjunction of X Chromosomes

The study of nondisjunction of X chromosomes in *Drosophila melanogaster* (Bridges, 1913, 1914, 1916) has also supported the theory of transmission of sex-linked factors.

When a white-eyed female (w) is crossed with a red-eyed male (R), a few white females and red males sometimes appear in addition to the red females and the white males normally expected. To explain these observations, Bridges proposed that the females producing exceptional offspring might have experienced a lack of disjunction of the pair of X chromosomes (see Diagram 7). Thus some gametes received 2 X, and others were devoid of X. This nondisjunction hypothesis was genetically and cytologically tested. It proved to be correct.

F ₁	♀ gametes	♂ gametes	
	$X_w X_w$	$X_R = X_w X_w X_R$	♀ Red
	$X_w X_w$	$Y = X_w X_w Y$	♀ white
	0	$X_R = X_R 0$	♂ Red
	0	$Y = Y 0$	lethal

Diagram 7

Y_0 is a lethal constitution. The $X_w X_w X_R$ females are poorly viable. The $X_R 0$ males are sterile, proving that Y is not completely inert, but contains at least one factor necessary for male fertility. Stern (1927) also pointed out the existence of a second fertility factor connected with the Y chromosome. The $X_w X_w Y$ females are fertile. It has been observed that they possess 9 chromosomes including 2 X and 1 Y (Fig. 2c).

In these $X_w X_w Y$ females, the disjunction of the system of heterochromosomes creates four types of ova: $X_w : X_w Y$ and $X_w X_w : Y$. When crossed with a normal red male, there are eight possible combinations (see Table II).

TABLE II
POSSIBLE COMBINATIONS FROM CROSSING $X_w X_w Y$ FEMALE AND
NORMAL RED MALE ($X_R Y$) IN *Drosophila melanogaster*

No.	Ova	Spermatozoa	Zygotes	Individuals
1	X_w	X_R	$X_w X_R$	♀ Red eyes
2	$X_w X_w$	X_R	$X_w X_w X_R$	♀ Generally dies
3	$X_w Y$	X_R	$X_w X_R Y$	♀ Red eyes
4	Y	X_R	$X_R Y$	♂ Red eyes
5	X_w	Y	$X_w Y$	♂ White eyes
6	$X_w X_w$	Y	$X_w X_w Y$	♀ Exceptional; white eyes
7	$X_w Y$	Y	$X_w YY$	♂ White eyes
8	Y	Y	YY	Dies

When additional crosses were made with these progenitors, the results were as predicted on the basis of the nondisjunction hypothesis, and the cytologic studies confirmed the genetic results. The observations in cases of nondisjunction thus gave direct proof of the relationship between sex-linked factors and distribution of the X chromosome.

It can be added that other experiments based on anomalies in the distribution of the X chromosomes, such as the attachment of X chromosomes to each other, and the analysis of aberrations of the X chromosomes (translocations) have led to the same general conclusions.

C. Gynandromorphism

Gynandromorphs are a mosaic of male and female sexual characters. The chromosomal content of the various regions of their bodies corresponds to the sexual type. Examples can be found among *Drosophila*, butterflies, bees, ants, spiders.

Gynandromorphism originates, as an aberration in the distribution of heterochromosomes. In *Drosophila* (Morgan and Bridges, 1919), an XX egg begins to develop in the female line. During embryonic mitoses, one of the X chromosomes is cast out of the spindle and lost (Fig. 9).

The resultant blastomere has the X0 constitution. Therefore, all progenitor cells of this blastomere will produce male structures. Since the X chromosomes are marked by different genes, it is possible to determine which of the two X chromosomes has been discarded. If this process occurs during the first cleavage, one half of the body of the fly is male and the other is female (Fig. 10). If the elimination occurs at a later

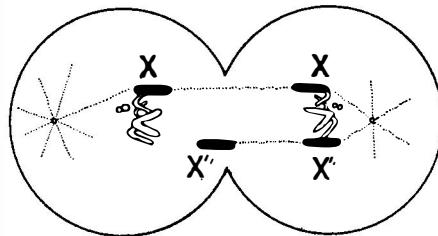


FIG. 9. Gynandromorphism in *Drosophila*. Elimination at an early cleavage division of one of the daughter X chromosomes of the paternal X(X'). The parts descended from the cell at the left will produce male structures, the parts descended from the cell on the right will be female. From Morgan and co-workers (1925).

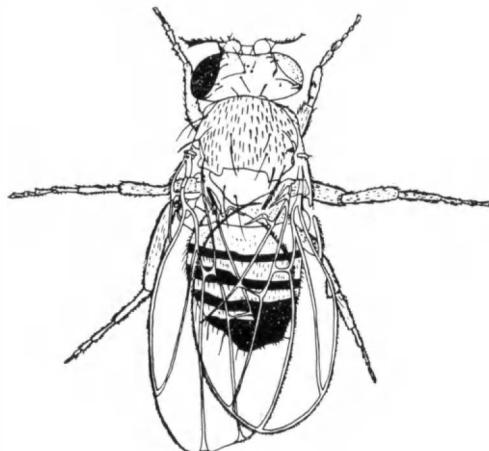


FIG. 10. A gynandromorph of *Drosophila* whose left side is female throughout and shows the dominant character Notch, and whose right side is male throughout and shows scute, broad, echinus, ruby, tan, and forked. From Morgan and co-workers (1925).

cleavage, female structures will be more prevalent. According to accumulated statistics, the anomaly occurs in *Drosophila* once in approximately 2000 individuals.

Gynandromorphism occurs as the consequence of double fertiliza-

tion in *Bombyx*. At fertilization the egg nucleus and the second polar body, which is retained within the egg, become each combined with one spermatozoon (Fig. 11). If one of the two egg pronuclei carries Z and the other W, the resulting moth will be a gynandromorph, since each sperm carries an X chromosome (Goldschmidt and Katsuki, 1927, 1928; Goldschmidt, 1931a).

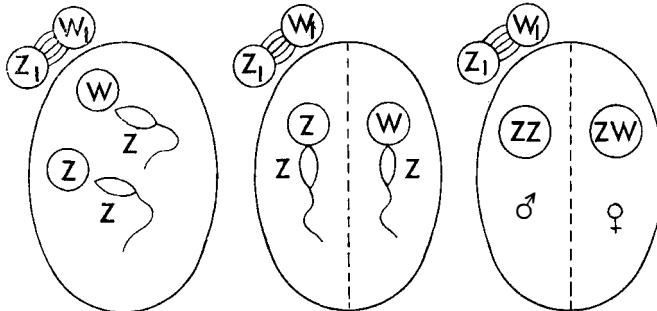


FIG. 11. Gynandromorphism in *Bombyx* (δ ZZ:ZW φ); double fertilization. From Goldschmidt (1931a).

D. Matings between Individuals of the Same Genetic Sex

The genetic theory that a given species has one heterogametic sex and one homogametic sex suggests a decisive experimental verification. What results from the successful crossing of two individuals of the same genetic sex? One would anticipate a disturbance in the sex ratio with the new numerical proportion of sexes predictable in advance. In an experiment of this type, the parents must have opposite *functional sexes* although their sexual genotypes are identical. This involves the experimental sex-reversal of one of the partners so that its sex phenotype becomes opposite to the genotype. These experiments have been actually performed as described in the following characteristic examples.

I. Progeny of Hermaphrodite Frogs

Witschi (1923) found *Rana temporaria* hermaphrodites (φ) with ovaries as well as a functional testicular nodule (Fig. 12). He obtained successful fertilization with male and female gametes originating from hermaphrodite individual. He also used sperm and eggs from normal frogs in crosses involving the hermaphrodites. The following sex distribution among the offspring was obtained.

φ Normal ova \times φ sperm	= 100%	φ (182)
φ Ova \times φ sperm	= 100%	φ (45) or φ (1)
φ Ova \times normal σ sperm	= 50%	σ (135):50% φ (132)
φ Normal ova \times normal σ sperm	= 50%	σ (127), 50% φ (128)

On the basis of the sex ratios, Witschi (1923) concluded that the frog is of the chromosomal type XY (σ) : XX (φ) and that the hermaphrodite was a female of XX constitution.

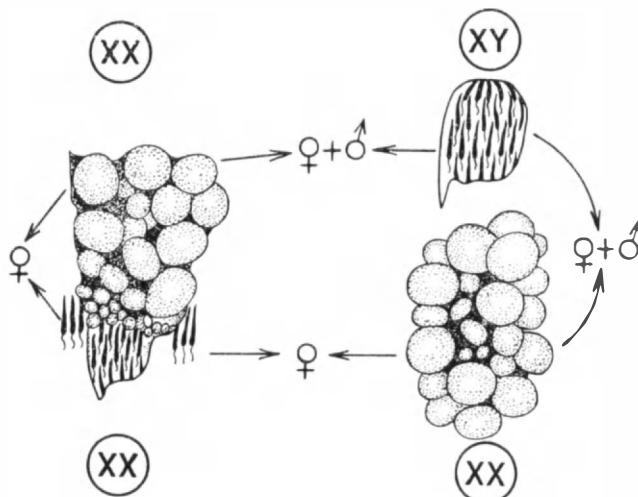


FIG. 12. Diagram illustrating the four possible combinations in *Rana temporaria* of eggs and sperms from a hermaphrodite, a normal male, and a normal female. Symbols between the arrowheads indicate the sex distribution in the offspring groups. The sex chromosome constitution of the parents is encircled. From Witschi (1956).

2. Progeny of Axolotl with Sex-reversed Phenotype

By heterologous embryonic graft (graft of testis on a genetic female), Humphrey (1945) succeeded in masculinizing genetic female axolotls (Urodele). The neomales obtained could then be crossed with normal females. The sex ratio of the offspring was approximately 75% (φ) : 25% (σ). Assuming the ZZ (σ) : ZW (φ) sex genotype for Urodele, a control cross would be written as follows:

$$ZZ (\sigma) \times ZW (\varphi) = 50\% ZZ (\sigma) : 50\% ZW (\varphi) \quad (1)$$

On the same basis, Humphrey's results could be expressed:

$$\begin{array}{ll} ZW (\sigma) \times ZW (\varphi) = ZZ (\sigma), ZW (\varphi), ZW (\varphi), WW (\varphi) \\ (\text{neomale}) \quad (\text{normal}) \\ (\varphi \rightarrow \sigma) \quad \text{female} \end{array} \quad (2)$$

In this analysis, 25% of the offspring would have the WW constitution, which is unknown under normal circumstances. To agree with Humphrey's (1945) observed results, these individuals should be phenotypic females, thus forming part of the 75% of females in the F₁.

generation. Humphrey (1945) could establish this fact in an important study of the F_2 generation, resulting from crosses of normal males with randomly selected females from the F_1 generation of the experimental lineage. Supposing that both ZW and WW individuals were represented among the F_1 females, some of the F_2 progeny should have a normal 1 ♂ : 1 ♀ sex ratio, while others should be 100% female. These two types of results were actually observed.

$$ZW(\text{♀}) \times ZZ(\sigma^*) = 50\% ZZ(\sigma^*), 50\% ZW(\text{♀}) \quad (3) \\ (370) \qquad \qquad \qquad (378)$$

$$\text{WW (♀)} \times \text{ZZ (♂)} = 100\% \text{ ZW (♀)} = 833 \text{ ♀} \quad (4)$$

Of 17 F₁ females, 6 gave the (4) type of crossing, and 11 showed the (3) type. This fits almost exactly with the expected 2 : 1 ratio of ZW and WW females in the F₁ generation.

With the same grafting method, Humphrey (1948) succeeded in masculinizing WW females. In crossing these neomales with ZW females, he obtained female offspring exclusively.

$$\text{WW}(\sigma) \times \text{ZW}(\varphi) = \text{ZW}(\varphi), \text{WW}(\varphi) \quad (5)$$

(neomale 100% φ
 $\varphi \rightarrow \sigma$)

3. Progeny of Pleurodeles and Xenopus with Sex-reversed Phenotype

Following Humphrey's experiments, similar studies were performed with a newt, *Pleurodeles waltlui* (Gallien, 1951, 1954) and a lower anuran, *Xenopus laevis* (Gallien, 1955, 1956; Chang and Witschi, 1955). In both species, the administration of female hormone (estradiol) to larvae, reversed genetic males into physiological females. Mating these neofemales with normal males produced a unisexual progeny.

$$ZZ(\text{♀}) \times ZZ(\text{♂}) = 100\% \text{ } ZZ(\text{♀}) \quad (6)$$

(neo-female
 $\text{♂} \rightarrow \text{♀}$)

In *Pleurodeles*, six neofemales gave birth to 940 males and no females. In *Xenopus*, 13 neofemales produced 1624 males and no females. The control experiments [type (1)] in both cases, proved that crosses involving normal females result in the normal sex ratio, 50% (σ) : 50% (φ). In connection with these experiments, it is interesting to note how comparatively easy it is for the epigamous actions of an exogenous hormone, in the course of sexual differentiation, to overcome the primary control of the sexual genetic constitution of the fertilized egg.

These experimental genetic analyses have established that, in amphibians, although heterochromosomes cannot be identified cytologically,

sex determination is based on the heterogamety of one sex and the homogamety of the other.

E. Sex Genetics in Fishes

The study of sex genetics in fishes is complicated by the labile character of sex determination and, in some cases, the difficulty in establishing the nature of the heterogametic sex. Nevertheless, some suggestive results have been obtained. We shall point out here three examples.

Oryzias (Aplocheilus) latipes: In this species Aida (1921, 1930) could establish the female homogamety (XX) and the male heterogamety (XY) by means of genetic studies. Yamamoto (1953, 1955) obtained the feminization of males (XY) into functional females with hormonal gynogenic treatment (estrone and stilbestrol). The inversion was demonstrated not only by means of statistics, but also because the coloration of the body serves as a marker of the genetic sex. In cross-breeding experiments, Yamamoto (1953, 1955) obtained a male of YY chromosomal constitution.

Lebistes reticulatus: The sex formula for standard races is XX (♀) : XY (♂). The X and Y chromosomes can be marked by body colorations. For example *Maculatus* factor causes a black spot on the dorsal fin and is consistently linked with the Y chromosome, as mentioned in Section IV, A, 3.

Winge (1934) discovered two races in which there is a strong tendency to produce masculinized females, i.e., formation of a gonopodium and patterns of male-type coloration. These two races possess *Coccineus-Vitellinus genes* (X_{Co-Vi} X_{Co-Vi}) and *Tigrinus-Luteus* (X_{Ti-Lu} X_{Ti-Lu}) genes which are localized on the X chromosomes.

In crossing these two races with *strong male* tendencies, the male Y chromosome being marked by *Maculatus*, Winge obtained an F₁ generation as predicted:

$$\begin{array}{l} P \text{ (♀)} X_{Co-Vi} X_{Co-Vi} \times X_{Ti-Lu} Y_{Ma} \text{ (♂)} \\ F_1 \text{ (♀)} X_{Co-Vi} X_{Ti-Lu} \quad X_{Co-Vi} Y_{Ma} \text{ (♂)} \end{array}$$

However, there appeared also three individuals which were morphologically and physiologically male, possessing the (Co-Vi) and (Ti-Lu) color spots, but not (Ma). This means that these males were in fact $X_{Co-Vi} X_{Ti-Lu}$, and that they did not possess the chromosome Y. These were 2X females, but very strongly masculinized. In this genotype, therefore, there are masculinity factors able to dominate the femininity effect of 2 X chromosomes. Since there is no Y chromosome in these individuals, it seems that these masculinity factors are carried by the autosomes.

Winge (1934) concluded that the autosomes carry both masculinity and femininity factors whose effects are in equilibrium in standard races,

so that sex is determined by the distribution of the X chromosomes. In the exceptional (Co-Vi) and (Ti-Lu) races, male determining autosomal genes occur in excess. Through selective crossings, Winge (1934) could accumulate sufficient doses of male-determining factors in some XX individuals, so that the autosomal male-determining tendencies were stronger than the female-determining ones, even when 2 X chromosomes were present. Finally, when such an XX male was crossed with his mother, the offspring were 50% ♂ : 50% ♀, all XX. This proved, in the opinion of Winge, that the mother had a genetic constitution very similar to that of a male, and that she had but one heterozygous autosomal pair (Aa). Hence, in this case, an autosomal pair controls sex determination. The Y chromosome has been eliminated from the system. The heterochromosome and all the other autosomal pairs are similar in both progenitors. Furthermore, the females are digametic (Aa), and the males are homogametic (aa). These experiments show that it is possible to go from male digamety to female digamety within the same species, and they emphasize the importance of autosomes in sex determination.

Winge (1934) could also select XY-type females by accumulating female-determining factors in the autosomes of individuals having the XY pair. By crossing with XY types, he obtained YY individuals which lack X and are males.

Xiphophorus (Platypoecilus) maculatus: The mechanism of sex determination is rather complex. According to the latest reports (Gordon, 1946, 1947, 1951, 1952, 1957) in some wild stocks from Mexican rivers (i.e., Rio Jamapa) the males are heterogamous (XY ♂, XX ♀), while in other members of the same species from British Honduras (Belize River) and for domesticated stock, the females are heterogamous (ZZ ♂, ZW ♀). These facts have been established by racial crosses, where the various sex chromosomes may be "tagged" by genes that produce specific color marking in female as well as in male. According to Gordon (1957) female heterogamety in the platyfish of British Honduras and male heterogamety in the platyfish of Mexico constitute stable sex-determining mechanisms in each natural population. Unfortunately we have no informations on the mechanisms by which two opposite mechanisms of sex determination in the same species may have arisen.

In his recent review, Gordon (1957) re-examines the original data of Winge (1934, 1937), Winge and Ditlevsen (1948). According to Gordon, it has not been demonstrated that a single pair of autosomes has taken over the role of the sex chromosomes in *Lebistes*. He considers that exceptional XX male and XY female guppies which are occasionally detected in the laboratory are unstable and could not be maintained in natural populations where members live in a state of panmixia. However

Gordon agrees with Winge's conclusions that female and male sex-determining gene are probably distributed over a great many of the autosomes with superior genes in the sex chromosomes and that these superior genes may, in exceptional individuals, be overridden by many opposing autosomal genes, each with small effects upon sex determination.

V. GENETIC BIPOTENTIALITY OF SEX

The study of genetics in fishes and the achievement of experimental sex reversal leads to the concept of *genetic bipotentiality of sex*. Since one individual can develop male or female somatic structures, it is apparent that two groups of sexuality factors, one male (M), the other female (F), are simultaneously present in the fertilized egg. In gonochoristic species, there is a prevalence of one of the groups of factors.

The existence of hermaphroditism leads to the same conclusion. In the most complete case, that of an organism carrying an ovotestis, capable of self-fertilization, and in which the gametes mature simultaneously, it is reasonable to assume that the ΣF and ΣM factors are in equilibrium.

There is no reason to expect any sex chromosomes in these cases. In fact, heterochromosomes have not been found in functional hermaphrodites, except those reported by Perrot (1930) in Gastropoda (Pulmonata) and discussed by White (1954). Either the sex chromosomes are cytologically not differentiated, or if their existence was proved, one would have to assume that fertilization creates an equilibrium between the female and male genetic conditions.

Hermaphroditism appears in various forms. When it is functional, the male and female gametes can mature simultaneously in a single gonad or in two glands, ovary and testis, carried by the same individual. It can be successive, with the male gametes maturing before (protandry) or after (protogyny) the female gametes. This situation leads to arrhenoidy. The aged females of some birds (hen, pheasant) sometimes reverse to maleness. These are fundamentally bisexual species; the *turning point* is marked by intersexuality.

Many bisexual species exhibit *rudimentary*, nonfunctional hermaphroditism of different degrees. The most interesting facts to be considered are those of *juvenile hermaphroditism*, encountered in the so-called "undifferentiated" races of frogs. Finally, among the vertebrates especially, but also in other groups, the condition of *ambisexuality* appears. The latter is characterized by the coexistence in one gonochoristic organism of structures of the functional sex, as well as of discrete primordia of the heterologous sex. The toad is a good example of this condition. In

the normal male, the testis is capped by a rudimentary ovarian structure (Bidder's organ). There can also be found vestigial oviducts in addition to a functional system of male gonaducts. Intersexuality is very often associated with these various forms of hermaphroditism.

From these facts we can conclude that, in Metazoa, the genetic constitution of sex must be fundamentally bipotential, even in gonochoristic species possessing a system of sex-determining heterochromosomes. This raises the problem of the expression, with suitable formulas, of the bipotentiality of the sex of individuals. This has been made possible by the study of intersexuality.

VI. INTERSEXUALITY AND GENETIC INTERPRETATION OF SEX: GENIC BALANCE

The concept of *genic balance* is basic in the genetic interpretation of sex. The development of maleness or femaleness results from the activity of a whole set of genes. The genes of masculinity (ΣM) and of femininity (ΣF) interact during development in precise accordance with the sexual genic balance (see Bridges, 1939).

A. Intersexuality in *Drosophila*

The study of intersexuality in *Drosophila melanogaster* led Bridges (1921, 1922, 1925, 1939) to consider sex determination as the expression of genic balance. Fruit flies with the chromosomal ratio of unity ($X/A = 1$) are characteristic females. This is true of the standard diploid type ($2 X : 2 A$), as well as those with the ratios $3 X : 3 A$ or $4 X : 4 A$. In the male, the value of the ratio is 0.5 ($X : 2 A$). This suggests that the X chromosomes are mainly carriers of femininity genes and that the autosomes carry a set of factors for masculinity. The masculinity-inducing capacity of a set of autosomes is weaker than the female tendency represented by 1 X ($X > A$). Thus in the formula $2 X : 2 A$, the genes of femininity outweigh the genes of masculinity ($2 X > 2 A$) and lead to female determination. Apparently, the masculinizing capacity of $2 A$ overcomes a single X factor ($X < 2 A$). On this basis, the constitutions $3 X : 3 A$, $4 X : 4 A$ must impose a female determination. Instances of the constitutions $3 X : 4 A$ and $2 X : 3 A$ (ratios of 0.75 and 0.66, respectively) have been obtained and these have proved to be intersexes. Finally, flies with the constitution $1 X : 3 A$ and $3 X : 2 A$ (ratios of 0.33 and 1.5) are known, and they have been given respectively the names "supermales" and "superfemales." These terms are not well chosen because these animals, though of male or female type, are actually sterile. This suggests that optimal male and female functional structures can be achieved, but not surpassed. The haploid type $1 X : 1 A$.

which should be female, has not been obtained, but in rare mosaic individuals, some parts of the body could be identified as being haploid by the size of the cells. These body areas were of the female type. The entire series of the various constitutions is shown in Table III.

TABLE III
SERIES OF GENIC SEX CONSTITUTION IN *Drosophila melanogaster*

Type	Formula	Ratio X/A
Superfemale	3X:2A	1.5
Female	4X:4A	1
	3X:3A	1
	2X:2A	1
	1X:1A	1
Intersex	3X:4A	0.75
	2X:3A	0.66
Male	1X:2A	0.5
	2X:4A	0.5
Supermale	1X:3A	0.33

Bridges has expressed these facts by giving the female and male tendencies a numerical value ($X = 100$, $A = 80$), thus establishing a sex index.

The femaleness factor, F, might be localized at one gene locus or might represent the interaction of various gene loci, F_1 , F_2 , F_3 , . . . Dobzhansky and Schultz (1931, 1934), in attacking this problem, introduced into an intersex (2X:3A) fragments of an X chromosome issuing from chromosomal breakage produced by X-rays. They demonstrated that, the longer the fragment introduced, the greater was the feminizing influence on the intersexes. Therefore, it is likely that several genes of femininity, and not only one, are active and are found distributed along the length of the X chromosome. The autosomal gene factors for maleness in *D. melanogaster* appear to be located on the II and III pairs of chromosomes, whereas the IV chromosomes have little influence. The general conclusion is that, in *Drosophila*, sex determination depends on a ratio between two systems of genes each having small effects, some being localized on X and the others on the autosomes. The genic system determining sex can be expressed by the following formula:

$$\begin{aligned} X_F X_F A_M A_M &= \text{female} = FFMM \\ X_F Y A_M A_M &= \text{male} = FMM \\ \text{supposing that } FF > MM > F \end{aligned}$$

The Y chromosome is considered as being inert; however, we have seen that it holds fertility factors. The genetic interpretation of sex determination established for *Drosophila* is, generally speaking, valid for the *Lygaeus* type and also for *Protenor* (where there is no Y). It can be applied also to standard races of *Lebistes*. It should be remembered that, in this last case, the autosomes bear at the same time M and F factors nearly balanced. In general, the *Drosophila* formulas apply to all types of sex determination in which the male is heterogametic, and the female homogametic, although some quantitative corrections have to be employed in the case of amphibians.

B. Intersexuality in *Lymantria*

In the moth *Lymantria (Porthetria) dispar*, studied by Goldschmidt (1931a, b, 1932, 1934), the female is heterogametic: ZZ (σ) : ZW (φ). From Europe to Japan, this species is represented by a series of races different in the power of their sexuality genes. A weak race will have masculinizing genes (Z) of weak potency while a strong race has genes (Z) of strong masculinizing capacity. Most of the European races and those of Hokkaido are weak. The races of southern Japan are medium or strong. Goldschmidt could distinguish eight Japanese races gathered in one collection on the basis of the potency of the Z factor.

When two individuals of the same race, weak or strong, are crossed, no intersexes appear among the offspring. Intersexes do occur, however, in cases of interracial crosses.

When a female (ZW) of a weak European race is crossed with a strong Japanese race male (ZZ), the offspring (F_1) are males and intersexual females. The latter are of ZW constitution, but the strong masculinizing factors (Z) carried by the paternal gametes dominate the female-determining maternal factors. The extreme example is a cross between a very weak female and a very strong male. The offspring are all apparent males although one half are ZZ and the others ZW.

To explain the facts observed, Goldschmidt proposed the following hypothesis:

In a given local race, males and females possess both male and female potentialities. The females have the formula FM and the males are FMM. It is recognized that F > M and MM > F. The M symbol is given to the genes of maleness carried on the Z chromosomes. The F factors would be transmitted by the maternal cytoplasm and thus carried in all eggs. Therefore all zygotes receive F and either M (Z) or MM (ZZ).

In strong races F and M are strong; in weak races, F and M are weak. The normal sexual balance is achieved with an intraracial cross

According to races, the values of F and M change. The different values $M_1M_2M_3 \dots$ are considered as a series of alleles. The cross of a weak F female (Europe) with a strong M male (Japan) can shift the balance so that the F factor no longer predominates. The resultant female offspring are no longer standard females, but intersexual ones. The extent of their maleness would be proportional to the F : M ratio.

In the reciprocal cross (strong Japanese female with weak European male) both sexes are normal in F_1 ; but in F_2 normal females, normal males, and intersexes are obtained in the proportion 2 ♀ : 1 ♂ : 1 ♂. The poorly viable intersexes are of the F : MM type. Although they possess the male constitution, the strong F factor can manifest itself over the two weak M factors.

The matter of localization of the F factors is still controversial. If they are exclusively transmitted by the mother, the possibility of linkage with autosomes must be excluded. One could suppose that they are carried by W, but this interpretation does not comply with the facts of male intersexuality. Goldschmidt was thus led to conclude that the F factor is transmitted by the cytoplasm. However, Winge (1937) proposed another interpretation of the results by choosing arbitrary valences for M and F factors according to races, so that the theory would not have to involve the cytoplasmic factors hypothesis. The question thus remains controversial.

C. Genetic Interpretation of Sex Determination in Amphibians

Witschi (1932, 1939, 1951, 1956), after studies of hermaphrodite frogs and their progeny and after researches on sexual races, concluded that the chromosomal constitution of Ranidae is XX (♀) : XY (♂). He adopted the concept of genic balance for higher anurans and accepted, with slight modification, the formulas proposed for *Drosophila* MMFF (♀) : MMFF (♂). In this system, F is epistatic to M, i.e., FF > MM > Ff. The X chromosomes are F carriers and Y bears the f factor. The maleness factors are linked with the autosomes. The general formulas are:

$$(♀) X_F X_F A_M A_M : X_F Y_f A_M A_M (\delta) \\ FFMM = ♀ \quad FfMM = \delta$$

Some amphibians, particularly *Rana temporaria*, have sexual races, i.e., sexually differentiated or undifferentiated (Witschi, 1930). An individual of a differentiated race (Alps, Pyrenees) develops testes and ovaries directly. At metamorphosis, there are 50% ♂ : 50% ♀. In undifferentiated races (Fribourg), the male gonads at first pass through an ovarian stage and, at metamorphosis, there are 100% females. In the course of the first

year, the ovaries of the genetic males change into testes, so that, after an intersexual stage, the sex ratio is 50% ♂ : 50% ♀. The semidifferentiated races have an intermediate behavior, with the ovarian stage of males more or less shortened. Cross-breeding experiments between differentiated and undifferentiated races have proved that the sexual type is transmitted by the male (Table IV).

TABLE IV
RESULTS OF RECIPROCAL CROSSES BETWEEN DIFFERENTIATED
AND UNDIFFERENTIATED RACES IN *Rana temporaria*

Reciprocal crosses			
P		F ₁	
Differentiated	Undifferentiated	Genetic ♀	Genetic ♂
Davos ♀	Fribourg ♂	120 ♀	117 ♀ + 1 ♀ + 1 ♂
Davos ♂	Fribourg ♀	134 ♀	138 ♂

Witschi concluded that, in differentiated races, the f factor transmitted from father to son has a very different value from F. In the extreme, f may equal 0. In the undifferentiated races, f is very similar to, or even equal to F, so that the determination is weak or nil. The semidifferentiated races have an f value which, by various degrees, approaches F. According to this interpretation, the original F factor underwent a series of mutations F, F', F'', F''' . . . f, f', f'', f''' . . . Each has a quantitative value which influences the state of differentiation of the races in question. This explanation accounts for the bipotentiality of sex and the transitional intersexuality. The theory is compatible with the well-known phenomena of the lability of sexual phenotype and of the often prevailing actions of epigamous developmental factors in the differentiation of gonads (overripeness of eggs, influence of temperature, action of steroids).

The mechanism of sex determination in urodeles (axolotls, *Pleurodeles*) and in *Xenopus*, has been subjected to genetic interpretation by different means. As already indicated, cross-breeding between individuals of the same genetic sex has led to the conclusion that the chromosomal constitution of these species is ZZ (♂) : ZW (♀). This could be interpreted in terms of genic balance on the basis of MMFF maleness and MmFF femaleness, with the assumption that MM > FF > Mm. This postulate would suppose that the M and m factors are localized on the Z and W chromosomes, respectively, and that the F factors are autosomal. This explanation, however, does not cover all the observed facts.

As we have indicated, ZW and WW axolotl females can be obtained, which are essentially identical, physiologically normal, females. Males of the WW constitution are also possible. In *Pleurodeles* and *Xenopus*, physiologically normal females can be obtained experimentally which are of the ZZ constitution.

According to Humphrey (1948), the existence of males (WW) implies that the W chromosomes possess the genes essential to the formation of testicular structures. The standard genotype (ZZ) must possess all the genes necessary to the development of ovaries. The W chromosome does not seem more essential to ovarian development than is the Z chromosome for the organization of a testis. In other words, W is probably identical to Z, except for the gene or the genes localized on a short differential segment and responsible for the initial stimulus of the development of the gonad into male. It is certain that, in this primitive type of sex determination, the genic balance is near its point of equilibrium, as evidenced by the sensitivity of these organisms to experimental epigamous actions.

It has been possible to obtain in amphibians, especially in urodeles, heteroploid individuals: n , $2n$, $3n$, $4n$, $5n$, and aneuploids (reviewed by Fankhauser, 1945). Generally speaking, the addition of one or several sets of chromosomes to the diploid number induces total or partial sterility ($\text{♀ } 3n$, $\text{♀ } 4n$). In general haploidy is lethal. In triploidy, an alteration of sex determination in favor of femininity is observed.

VII: EPIGAMOUS FACTORS, INTERSEXUALITY, AND SEX DETERMINATION

A. *Epigamous Factors and Sex Differentiation*

When, in a species, the genic balance is near equilibrium, so that the prevalence of one of the groups of F or M factors over the other is weak, the epigamous factors exerted during development can alter sex differentiation and even lead to the inversion of the sexual phenotype. This point has been indicated previously, particularly with regard to amphibians.

The best-known case of epigamic actions dominating sex determination is that of the gephyrean worm *Bonellia viridis* (Baltzer, 1914, 1928, 1931). In this species there is an extreme sexual dimorphism. The body of the female is about the size of a walnut, and the extended proboscis may achieve a length of over a meter. The male is about 2 mm. long. Males live in the uteri of females, sometimes in considerable numbers. Fertilized eggs develop into indifferent larvae and, if they remain free living, metamorphose into young females. But the larvae normally have a tendency to settle down on the proboscis of adult females, and there

they differentiate into dwarf males within 3 days. Baltzer demonstrated that the masculinizing effect is in proportion to the duration of the proboscis parasitism. A substance elaborated by the proboscis, as well as by intestinal tissues, causes the male development. Even a simple increase or decrease of the hydrogen ion concentration of the sea water may modify the sex differentiation (Herbst, 1935).

B. Sex Determination and Cytodifferentiation of Gonocytes

One of the fundamental problems of cytology is that of understanding the mechanisms controlling cellular differentiation. The facts known about sex reversal of the phenotype provide some interesting information in this field of investigation.

When, in amphibians for instance, a genetic female is changed into a functional male or vice versa, the chromosomal structures are not changed. The study of the offspring of such animals proves this fact. We can conclude, therefore, that cytodifferentiation of a primordial germ cell into a spermatogonium or an oögonium, and eventually into a male or female gamete, does not depend on its genetic constitution.

The somatic components of the gonad, cortex and medulla, in which the germ cells mature, control the direction of their cytodifferentiation. The cortex acts as a gynogene inductor. Under its influence, the undifferentiated gonia become oögonia. The medulla is an androgene inductor, whose activity directs the cytodifferentiation of gonia into spermatogonia. If, in the course of embryonic development, some gonia wander out of the inductor regions, they do not undergo any differentiation and they degenerate.

Normally, development of cortex and medulla is under the control of the genetic determination mechanism, established at amphimixis. The prevalence of cortex or medulla depends on the quantitative ratio between F and M genes. But various epigamous developmental influences can succeed in inhibiting the epistatic system, thus liberating the potentialities of the hypostatic system. Gonochorism is not simply the direct result of a genic quantitative ratio. It is ensured by the interplay of two antagonistic inductor systems, acting under the influence of inductive substances. The exact nature of these inductive substances has not yet been established.

VIII. CONCLUSIONS

Sex determination is a cytogenetic problem. It is dominated by three fundamental concepts:

- (1) Heterogametic constitution of one sex, homogametic constitution of the other.

- (2) Potential bisexuality of each sex.
- (3) Syngamous character of sex determination.

The asymmetric genetic structure of one of the sexes is manifest in the existence of heterochromosomes whose interplay controls sex determination.

Studies (1) of properties of heterochromosomes (sex-linkage), (2) of their abnormalities (nondisjunction, gynandromorphism), (3) of crosses involving animals with sex-reversed phenotype, have confirmed the theory of sex homo-heterogamety, even when heterochromosomes cannot be cytologically identified.

There are some special cases of sex determination (arrhenotoky, monogeny, progamous determination).

The existence of hermaphrodite animals, the facts of ambisexuality and intersexuality, and the achievement of sex reversal lead to the concept of bipotentiality of each sex and to the proposal of genetic formulas indicating this bipotentiality. Sex determination is the result of a genic balance between maleness and femaleness factors present at the same time in each sex.

Usually the genetic factors controlling sex determination direct embryonic differentiation toward male or female development. However epigamous factors in the course of development can alter or even dominate the action of genetic factors. In this case, the sexual phenotype is not necessarily the reflection of the genotype.

Cytodifferentiation of gametes into spermatozoa or ova does not depend on their genic constitution. It is under the control of an inductor mechanism originating with the somatic components of the gonad (cortex and medulla for vertebrates) where the gonocytes develop.

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CHAPTER 11

Differentiation of Vertebrate Cells

By CLIFFORD GROBSTEIN

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This is a strategic, if difficult, time to consider our knowledge of cytodifferentiation. The problem is being attacked with renewed vigor, arising from the availability of more highly resolving biochemical and

biophysical instrumentation and the recent striking advances of microbial genetics. In addition, the availability of indefinitely propagable vertebrate cell populations in culture and as ascites tumors, and the expansion of techniques for dissociation and reassociation of tissues, have opened new horizons. There is, too, a growing sense of urgency as allied fields find our rudimentary understanding of cytodifferentiation increasingly more limiting in developing comprehensive theories of cellular activity.

Early rapid empirical advance therefore may be expected, and the life of general conclusions drawn now is likely to be short. On the other hand, because we are on the threshold of more penetrating and precise information, it is important to restate rigorously the state of our knowledge of differentiation at the cell level. A good deal of the literature on differentiation was accumulated during a period when technical methods were inadequate for study of individual metazoan cells, and data and concepts based on tissue behavior were less than rigorously extrapolated to the cell. This is a time, therefore, for winnowing—in an effort to preserve the very real foundation laid in the past, without carrying over conceptions which earlier were useful but now may be misleading or restrictive.

Most recent speculations about the nature of cytodifferentiation center on a number of assumptions: (1) That the several instances of cytodifferentiation are sufficiently common in character so that statements may be made about a *general* mechanism. (2) That cytodifferentiation is invariably irreversible; cells which have assumed a differentiated state cannot revert to what they were before. (3) That apparent reversibility, i.e., loss of differentiative properties, is either illusory—because true differentiation persists in a cryptic state—or indicates that the cells were not differentiated in the first place. (4) That differentiated states are mutually exclusive. Coupled with the second assumption, this means that interconvertibility of types cannot occur. (5) That the differentiated state is propagable through cell generations and, hence, is genetic or quasi-genetic in nature. (6) That the nucleus is not significantly altered in development. This set of assumptions, particularly the last two, has placed a heavy burden on the geneticists—who have responded with a number of ingenious models to account for controlled hereditary variation within a common genotype.

Though widely held, all of these assumptions are not universally accepted as valid. It is the general purpose of this chapter to evaluate the evidence relating to them. This will require consideration of the following questions: (1) What is the general nature of the phenomena which we designate as cytodifferentiation and what relation do the phenomena bear to differentiation and development in general? (2)

What are the validity and significance of the several types of criteria by which we identify and—hopefully—measure the process of cytodifferentiation? (3) What is the relative importance of intrinsic and extrinsic determinants in the initiation, progression, and maintenance of cytodifferentiation? (4) Is the state of differentiation propagated through cell division and, if so, for how long and under what circumstances? (5) Do differentiated cells ever revert or convert?

For reasons of space, the discussion is limited to vertebrate cells—except where data on other cells are particularly relevant to the problems posed in vertebrates. All general statements, therefore, are to be qualified as relating to this context.

I. THE CONCEPT OF DIFFERENTIATION

All adult organisms, examined at almost any level, are profoundly heterogeneous. Grossly we distinguish head, limb, trunk, and tail; by dissection we separate nerve, muscle, and gut; microscopically we see such diverse cells as neurone, melanocyte, and leucocyte—each in its characteristic intercellular matrix. Below this level, heterogeneity continues into cell organelles and intercellular fibers, and on down to a patterned and frequently interlocking array of molecular species, the full variety of which is only now coming to be dimly appreciated. It is to this phenomenological heterogeneity—at all levels—that the term “differentiated” is applied. The transformations by which heterogeneity arises, or increases, make up the process of differentiation.

Changes occurring at the several levels of organization are profoundly interrelated. In concentrating on differentiation of cells, we must understand that we are abstracting—legitimately, since differentiated cells frequently are recognizable entities both within and outside supracellular organisms. But it must not be forgotten that in some degree we *are* abstracting. *In vivo*, cells of metazoa differentiate in a broader fabric of a developing organism, and important governing interactions between the cell and its surroundings must always be expected. And behavior and its changes at the cell level have their correlated changes at the levels below. Differentiation is a multileveled process; what we distinguish as cytodifferentiation is a horizontal slice.

We have special difficulties with the concept of differentiation as applied to cells. As noted, differentiation fundamentally means increase of heterogeneity, progression from relative simplicity to relative complexity. In this sense no cell is fully undifferentiated, all are heterogeneous in possessing nucleus, cytoplasm, organelles, etc. When cell and organism are coextensive, as in the noncolonial protozoa, cytodifferentiative processes fit the basic definition. The cell pattern is rendered more

heterogeneous by the formation of such structures as membranelles, trichocysts, etc. But in multicellular organisms, where cells are parts, differentiation of the organism frequently involves increasing *homogeneity* within a particular cell, e.g., in cornification. In developing metazoa it is the cellular *population* which becomes increasingly heterogeneous; for individual cells the process is one of changing properties concomitant with increasing *supercellular* heterogeneity.

Changes in cells, however, are not usually regarded as differentiative unless they are developmental in the broad sense, i.e., unless they are relatively stable and progressive in terms of the life cycle of the organism. Short-term functional changes which are repetitive without significant residue, like muscle contraction or dark-adaptation of the retina, are not differentiative; but the appearance of myofibrils or of the ability to respond to light are. Just how new, stable, and progressive the changes must be is not easy to generalize, but some fixity of ongoing innovation is essential.

Weiss (1939) has drawn a useful distinction between modulation and "true" differentiation. He designates as modulation changes of cellular properties which persist only as long as their initiating external influence, and reverse when it is withdrawn. Differentiation, in these terms, is change which is stable beyond the initiating circumstance, i.e., which involves the rise of some stabilizing mechanism. In extreme cases, e.g., reaction of a prostate epithelial cell to androgen on the one hand and differentiation of a neurone on the other, the distinction is easily made. In other cases, particularly during the course of a change whose initiating circumstance is unknown, it becomes more difficult to state to what degree the change is modulatory and to what degree differentiative. In fact, one may lead into the other (see Weiss, 1947, and below). The distinction has the great advantage, however, of focusing attention on the need for study of the relative roles of intrinsic and extrinsic factors in the stabilization of particular cell types.

Cytodifferentiative changes are in the direction of specialization, i.e., of a set of activities and materials initially present, certain ones are emphasized—or new ones may arise—while others are relatively de-emphasized or lost. In extreme form, e.g., the mammalian erythrocyte, actual cell components may be sacrificed—the differentiated cell being a part-cell with only one possible end point. Specialization is seen in appearance and modification of structural elements, in particular activity, in special metabolism, and in molecular specificity. Cells with more highly specific activities, materials, and products are thought of as highly differentiated. But there is no general quantitation available at the moment, there are no identified properties demonstrably common to all differen-

tiations by which they may be placed on a single scale. This may be due to lack of knowledge, or it may indicate that there are a number of *kinds* of differentiation, linked only by their common significance for the developing organism.

As a first approximation, then, we may consider cytodifferentiation as including relatively stable, maturational changes of cellular properties which progressively concentrate the activities and structure of the cell, or portions of it, in particular directions at the expense of others.

II. CRITERIA OF DIFFERENTIATION

Since differentiation affects the cell in many ways and at many levels, there are a number of sets of criteria for identifying and characterizing a differentiated state. Since they are not always concordant, it becomes important to recognize the significance if each, and to specify criteria when one is characterizing differentiation. Cells may be said to be differentiated by morphological, behavioral, chemical, or developmental criteria.

A. Morphological

Morphology, in the descriptions of classical histology and pathology, provided the first descriptions of differentiation. Emphasis was on size and shape of the whole cell, on the character, number, size, shape, and staining properties of various organelles, and on the amount and nature of the intercellular materials. With improved microscopy, and particularly with the heightened resolution of the electron microscope, morphological description has become increasingly detailed and precise.

For example, Burgos and Fawcett (1955) described the fine structure of differentiating spermatids of the cat and called attention to intercellular cytoplasmic bridges, with thickened boundaries, frequently connecting spermatids in pairs or even groups of four. The significance of these bridges has not been established, but clearly they conceivably have bearing on cytodifferentiative processes. Fitton-Jackson (1956, 1957) has contributed important new data on the site and mode of formation of collagen fibers—long a matter of controversy. Weiss and Ferris (1956) report provocative electron microscopic studies of the reconstituting basement lamella of amphibian larval skin, normally a 20-ply arrangement of collagen fibers. The elements of this structure first appear as randomly arranged fibers in a homogeneous matrix between the epidermis and underlying connective tissue cells. Ordering begins close to the epidermal surface where the fibers become arrayed parallel to the surface. From this beginning, orientation sweeps across to the connective tissue side with each successive ply oriented at right angles to

the one preceding. Here morphogenesis, resulting from cooperation of two unlike cell types, can be followed almost at the molecular level.

All components of the cell may be affected by morphological differentiation. In general, nuclear changes tend to be less striking than cytoplasmic, but, as has been emphasized most recently by Briggs and King (1955), the nucleus is by no means uniform in all cell types. Beyond variation in size, shape, and staining reaction, there are differences in nucleolar number and in levels of ploidy in various differentiated tissues of a number of species.

The cytoplasm, however, certainly is the primary area of differentiation, in terms of degree of morphological alteration. In addition to the appearance of sundry droplets and granules with specific characteristics, profound modification may occur of such universally present organelles as the Golgi complex and mitochondria (Dempsey, 1956). New cytoplasmic structures, e.g., striated muscle fibrils, may emerge in the cytoplasm in sufficient abundance to crowd out other components. Modification at still lower levels of organization is suggested by the characteristic appearance of the cytoplasmic membranes or endoplasmic reticulum in different cell types (Palade, 1955; Porter, 1956). The membranes are reported to become increasingly prominent during certain differentiations, though it is not yet clear whether this is related to heightened activity in general, or to the particular syntheses of the specific cell type.

It is important to emphasize that morphological differences extend beyond the cell in the usual sense, to the intercellular materials whose amount, structure, staining properties, and other characteristics sometimes are more diagnostic than the features of the cells themselves. This is true not only because of the abundance of intercellular materials in such tissues as cartilage and bone, but because in many instances these materials are products of functional activities whose specificity so far is undetected in cell structure. It is much easier to distinguish chondrocytes, osteocytes, and fibroblasts by their respective matrices than it is without them.

The grosser morphological criteria are sharpest, and most useful, when cells are in their usual environment. As the pathologist and tissue culturist are well aware, morphological criteria tend to blur and fade under unusual conditions—which is when they frequently are most needed. This has led to a strong tendency to deprecate morphology as a criterion of differentiation and to an intensive search for more revealing indices. Some re-evaluation of this tendency is now indicated on two grounds. First, with the new resolution of morphology, carrying it down close to the level of molecular ordering, it would be remarkable if fine structure and metabolic activity were without correlation. We are on the

verge of "seeing" the merging of form and function. Second, full and certain identification of many cell types is possible at the moment only through their ability to assume normal morphology (and function) *when placed in their normal associations*. Return of long-term cultured cells to the organism, for example, may prove the most sensitive test for cells of doubtful differentiative state. Although there are pitfalls, particularly in the use of grosser characteristics, morphology cannot be disregarded either as a tool or in formulating a complete conception of differentiation.

B. Behavioral

The term behavioral is used here to designate all aspects of *activity* at the cellular level under both normal and abnormal circumstances. *In situ*, in transplantation, and in explantation a particular differentiated state may be characterized in terms of mitotic activity, motility, phagocytosis, secretion, conduction, contractility, etc. Goss (1932), for example, identified cardiac muscle cells in tissue culture on the basis of the cells' ability to contract, even though no striation could be observed. And Crain (1956) was able to demonstrate action potentials in spinal ganglia of the chick after as long as 7 weeks in culture. So long as the behavior is characteristic of the cell type in question, cytodifferentiation is assumed to persist. Of particular current interest is "affinity" (Holtfreter, 1939; Weiss, 1947), the recognition of cell type in reaggregating cellular masses. Moscona (1957a) has recently given a particularly striking example of this in mixed combinations of mouse and chick nephrogenic and chondrogenic cell suspensions. Mouse and chick cells of the same tissue type (e.g., chondrogenic) formed a chimeric mass of both species types. But when mouse chondrogenic and chick nephrogenic cells were intermixed, the two tissue types formed separate masses, each of which differentiated according to type. These remarkable, specific reaggregations of like type—as well as the specific coupling of such unlike types as neurone and Schwann cell—not only raise interesting questions of the recognition mechanism, but give promise of very subtle criteria for distinguishing states of differentiation.

Functional behavior and morphological differentiation are usually sufficiently correlated in the organism to allow the histologist and cytologist to make estimates of the functional and differentiative state of a tissue from the morphology of sections. However, there are difficulties even here, and in unusual transplant sites or outside the organism the difficulties multiply. Morphological signs of the classic kind frequently are wanting in the presence of behavioral signs, due presumably to the greater sensitivity of the latter. The relationship of the two should be clarified in the next few years as the electron microscope is more exten-

sively applied to differentiating tissues. It is a matter of considerable interest to know how the appearance of new and stable cellular activities is related to the molecular polymerization and ordering which underlies fine structure.

C. Chemical

Differentiation may be detected by changes of metabolic rate, by changes in the input or output of specific substances, or by the appearance, or change of concentration, within the cell of such materials as enzymes, intermediates, and products of specific metabolic pathways. The techniques may involve histochemistry (sections of organized tissues), cytochemistry (fractions of homogenized tissues), immunochemistry, tracer chemistry, or the use of antimetabolites and other metabolic poisons. In all cases the effort is to characterize differentiation in terms of particular molecular species or their effects, and these in turn in terms of specialized pathways.

As recent examples, Argyris (1956) found histochemically that succinic dehydrogenase activity is low and esterase activity is high in "resting phase" skin of the mouse. During wound healing, following irradiation, or in fetal skin the reverse is true. The data are taken to indicate that oxidative activity is high in undifferentiated epithelium and that nonspecific esterase, while not necessary for growth and differentiation, is a biochemical index for differentiative level. Immunochemically, Ebert (1955) has shown that cardiac myosin is fairly generally present in the early blastoderm and is secondarily localized in the heart-forming areas—prior to morphological signs of cardiogenesis. Interestingly, De Haan (1956) finds that in regenerating limbs of *Ambystoma* immunochemical characteristics of fully developed muscle appear just when myofibrils are forming. Cytochemically, Herrmann and Barry (1955) have shown that the rates of accumulation of collagen and actomyosin in chick embryo muscle tissue are very similar between the 10th and 19th days of development, while Konigsberg and Herrmann (1955) showed that the accumulation rate of alkaline phosphatase is significantly lower. The data indicate, as might be anticipated in a differentiating system, that the controlling factors for protein accumulation during this period of rapid muscle formation "do not affect all proteins to an equal degree." Using tracer techniques, Bonnichsen and Akeson (1955) report that iron-59 uptake in myoglobin and hemoglobin during myogenesis and hematopoiesis follows a very similar pattern which differs sharply from that for cytochrome b. Markert (1956) has combined radiotracers with chromatography to demonstrate differences in synthetic activity of tissues taken from various sites and stages of the chick embryo.

These early applications of the newer tools of biochemistry to cellular differentiation illustrate that many additional "markers" for differentiative events are rapidly becoming available. Since these markers primarily are molecular, a new level of resolution is being attained, and important new opportunities are opened. With the rapid advances in macromolecular chemistry, entry is being made into what may prove the critical level for understanding cytodifferentiation—macromolecular structure and interaction (Weiss, 1947; Schmitt, 1956). But it must be kept in mind that high resolution is achieved by concentration on small areas; hence, on part-processes. The appearance of new immunochemical combining groups (or their loss), or the change of enzymatic pattern, are aspects of the differentiative process, but they may reflect rather than key it. No one criterion is likely to characterize completely the complexity of differentiation; it is for the immediate future to determine what set of criteria are diagnostic in each case. This will require constant correlation of the more highly resolving methods with the broader spectrum behavioral properties of cells in their normal environment.

D. Developmental

Morphological, behavioral, and chemical properties are *overt*; they are the manifest signs of an existing differentiated state. In distinction, developmental properties are inferred from subsequent overt properties; they are characterized by what things *become*. The conceptualization of becoming has long given rise to difficulties and was vigorously debated by the philosophically minded embryologists of the nineteenth and early twentieth centuries. Today it is less debated but no less troublesome.

Current literature shows a sturdy rejection of vitalism and preformationism in favor of epigenetic materialism. But some of the language of an earlier day hangs on, particularly in the concept of potency. In this language, what things become exists in some form in what gives rise to them—the zygote is totipotent because all of the cell types of the adult will appear among its descendant cells. Differentiation involves loss of potencies down to one, and there can never be gain of potency, since anything that happens existed potentially before. Now in fact, the zygote is itself formed by the union of two highly differentiated cells, neither of which would be expected, nor has ever been observed, to transform into any other cell type before fusion. And the zygote, itself, has never been known to transform directly into any specialized adult cell type, let alone each and every kind.

What a zygote can do is develop as a whole organism. In it, for the moment, cell and organism are coextensive. As a cell it is specialized and limited, but through cleavage, the production of a heterogeneous

cellular aggregate, and the whole subsequent developmental history, cytodifferentiative tendencies and properties *appear*. Unquestionably, some of the necessary antecedent materials and conditions exist in the zygote, certainly in the nucleus, and set limits for the character of cyto-differentiation—but, by the same token, necessary antecedents must have existed in the earliest and simplest living things. We do not think it necessary or satisfactory to explain, or even describe, the evolution of microbes, mice, and men in terms of loss or sorting of such potentialities. The limits set obviously are too wide, and our problem is to explain not the limits—as it is in genetics—but the diversity which occurs within them. Moreover, too much happens between times, particularly too much incorporation of the “potentialities” of the environment, to regard the properties of these remote antecedents as related in any simple way to processes at the moment.

Similarly, of more immediate developmental concern than potentiality is capacity. The concept of potentiality is not incorrect, but it is imprecise and it places the emphasis on loss while development is more strikingly characterized by gain. Also, in practical terms, potentiality is nonquantifiable. A particular differentiation is potential whether it has a high probability of occurrence or a vanishingly small one, and the absence of potentiality is almost impossible to demonstrate.

What does generally decline during development is plasticity—the range of developmental behavior open to a part of the embryo at a given time. Although generally declining, plasticity is renewed (as potentiality cannot be) in some developmental histories, and it can be experimentally increased in others. The particular developmental pathways immediately open to a region of the embryo may be referred to as its capacity. The ability to respond appropriately to a known differentiating environment, as in induction, has been given the useful name of “competence” by Waddington (1940). Capacity includes such competences, but it need not be limited to pathways for which the differentiating environment is known. If competence is not so limited either, then capacity is the sum of all competences.

The consequences of emphasizing capacity rather than potency are seen in the following considerations. While the zygote is thought of as totipotent, its capacity as a cell is very low, probably zero. Therefore, during cleavage and the early stages of gastrulation, capacity in the several regions of the embryo increases. If level of differentiation is measured by capacity, then the products of cleavage are less differentiated than the zygote, i.e., the early stages of development are dedifferentiative. This may seem no more than a play on words, but it emphasizes that something is occurring during these early stages which is not brought

out in the language of potentiality. It removes, too, the embarrassment of referring to the product of an obviously differentiative pathway, oogenesis, as a completely undifferentiated cell. In these terms there need never be, as there probably is not, a fully undifferentiated cell—in the sense of universal capacity.

Returning to the developmental criterion of differentiation, then, in practical terms it refers to the range and character of the demonstrable and immediate developmental alternatives. Portions of the embryo which, on exposure either to "neutral" environments or "inductive" ones, at earlier stages give rise to differentiations other than their prospective ones, are gradually limited in their behavior to their normal fate. Plasticity decreases as particular developmental pathways are established and stabilized. But it is important to realize that, though the trend of *plasticity* demonstrably is downward during development, *competence* appears to rise shortly before a particular differentiation is due to occur and—if for some reason it does not occur—declines thereafter. Clearly, competence involves the appearance of something operationally new, a phenomenon which tends to be obscured by the language of potentiality. It is this detection of the origin of new properties, before they are demonstrable by other procedures currently available, which makes developmental behavior a more sensitive indicator of the immediate antecedents of differentiation than any other criterion. It is, therefore, an important tool in detecting the earliest stages of the process.

It must be noted, however, that these early changes have been demonstrated in aggregates of cells—sometimes homogeneous and sometimes heterogeneous—and cannot safely be extrapolated to individual cells without further information (Grobstein, 1955a). It will be pointed out below that differentiative behavior is markedly affected by the size of the mass tested and that tissues stabilized for a particular differentiation by ordinary tests may fail to show that differentiation following fragmentation below a certain size. Developmental tests of individual cells, now becoming feasible, are badly needed to give information on the time of onset and stabilization of differentiative processes within the cell itself.

It should be repeated, too, that developmental tests are limited to demonstration of what cells *can* do, and rarely are conclusive in showing what they cannot do. They can detect changes in bias or stability for particular developmental pathways in particular environments, in tissues of different origin or stage, but they rarely can be sufficiently exhaustive to cover all conceivable environments (Harrison, 1933). Their results, therefore, are relative and not accurately expressed in the absolute language either of potentiality or determination.

Summarizing the criteria of differentiation briefly, we may say that the first steps toward differentiation are recognized in developmental bias without overt signs. The nature and site of these first changes are not known, but there is evidence to suggest that they reside in tissue rather than individual cell properties. The overt changes which subsequently appear in the cells or their matrices are characterizable morphologically, behaviorly, and chemically. Determination of the relation between these several kinds of criteria, using methods at current levels of precision, is only beginning, but it may be expected that ultimately they will be found to express several aspects of the same fundamental process. Any one or any combination of these criteria may be applied for particular purposes, but only the full complex characterizes the complete and normal differentiated state. It is important, therefore, to validate individual criteria against as many as possible of the others. The broadest validation, and often the most convenient and convincing one, is the ability of cells to show their characteristic behavior in their normal associations *in vivo*.

III. PROPERTIES OF CYTODIFFERENTIATION

A. *Diversity*

We assume that the many observed instances of cytodifferentiation have important properties in common, because they are linked in their significance for development of the whole organism. But viewed in terms of the cell, and what goes on within it during differentiation, diversity rather than similarity is what is impressive. Consider the number of kinds, and the very different directions of modification, of cells found in an adult vertebrate.

Current histological compendia do not usually answer—or raise—the difficult question of how many differentiated cell types there actually are. They give distinctive names, however, to well over one hundred kinds of cells in an adult human being. The distinctions are based largely on morphological and behavioral criteria, increasingly supplemented in recent years by chemical and quasi-chemical criteria, applied *in situ*. Only relatively few are validated developmentally. In many cases the distinctions may be superficial, reflecting differences that critical testing will show to be transitory rather than developmental. On the other hand, more sensitive methods may reveal stable differences not now recognized—between the fibroblasts of different organs, for example. In any event, these compendia provide the only data available which allow an estimate of the number of recognizable cell types in a living organism, and they indicate something of the order of 100.

Even if the number is off by half, this is still an impressive degree of diversification of a single basic cell organization. Moreover, the number of directions of diversification is equally striking: we seem to be dealing not only with many differentiations but with many kinds of differentiation. Compare a sperm cell, a chondrocyte, a liver cell, a neurone, and an erythrocyte. In the sperm cell the Golgi material has been converted to new uses, the excess cytoplasm has been cast off, and what is left is little more than a nucleus and a highly specialized apparatus for motility and penetration of the egg. The sperm cell will never divide again, and its life is limited to a relatively short period of intense activity. In contrast, the chondrocyte shows little evidence of internal modification or specialized activity, but is locked in a specialized matrix which it has itself produced. It seems likely that the chondrocyte can again become active and can divide if released from its matrix in amphibian regeneration.

The erythrocyte resembles the sperm cell in that differentiation has imposed an early end to its life as a cell, but it differs markedly in what it preserves and discards. The sperm cell is almost all nucleus, Golgi material, and mitochondria; the erythrocyte does away with exactly these parts, leaving only the hemoglobin-laden stroma. In the liver cell, unlike the preceding three, a high and diversified metabolic activity continues. But like the chondrocyte, despite the absence of an isolating matrix, it does not normally divide—though it may be induced to do so in regeneration. The neurone, on the other hand, though it resembles the chondrocyte and liver cell in apparently preserving all of its essential parts, differs from them in the presence of specialized cytoplasmic neurofibrils and in its apparent inability to divide in the adult organism.

These examples can be multiplied by anyone familiar with specialized cells. They emphasize that differentiation is diversification of many kinds in many different directions, about as many as appear to be open to the original cell organization. In some, the cell becomes incomplete, with death as the only possible end point. In others, the cell may be so altered internally that subsequent division does not occur, or occurs only with great difficulty and then atypically. There may be obvious internal change without permanent effect on the mechanism of division; or no drastic internal change, the chief effect appearing externally in the intercellular matrix. This diversity of visible manifestation has so far defied any “natural” classification. Although certain groupings can and have been made, there is no encompassing, generally accepted, taxonomy of cell types—perhaps because we are, in fact, still early in the natural history phase of cell biology. In the absence of any known common property for all instances of differentiation, the assumption of any single mechanism for all seems decidedly premature.

B. Discreteness of Cell Type

As Weiss (1939) has emphasized, our impression of major vertebrate cell types is one of discreteness. As cells differentiate they become clearly one thing or the other, either epidermis or lens, either cartilage or muscle, either erythrocyte or granulocyte. The properties which characterize normal differentiated states appear to occur in sets or constellations which do not intergrade. Moreover, these seem to be alternative, or mutually exclusive. The cell which becomes a muscle cell does not simultaneously, in whole or in part, become a cartilage cell. We do not, in higher vertebrates, find cartilage matrix depositing around striated muscle cells, nor do we find cells which contract at one end and produce cartilage at the other. We do not see either, it should be noted, cartilage and muscle cells differentiating interspersed in a common tissue mass. Major cytodifferentiation is discrete, exclusive, and involves not only the whole cell, but cell populations.

This is an important enough point, in its implications for mechanism, to deserve most careful consideration. First, it does not assert that the character of a cell is such that it must necessarily be equally differentiated throughout. In ciliates, cell organization allows the cortical region to be highly differentiated, with some structures sometimes disappearing whereas others persist, while the endoplasm seemingly remains relatively undifferentiated. In vertebrates variations in differentiative level certainly may occur within the same cell, e.g., the apical striated border of intestinal cells with relative lack of differentiation proximally. It is the *set* of properties labeled "type" which is exclusive, not difference of level of differentiation within the same cell.

Second, discreteness and exclusivity are the rule within the range of circumstances, and by the methods, so far tested. It is important to know whether they will persist as more searching procedures are applied to particular properties in a wider range of environments. It is especially important to know how early exclusivity appears and what properties it first affects.

Third, the fact that the appearance of one set of properties precludes the appearance of another need not imply that the individual properties of either set are invariably linked with one another. Absence of intergrades need not mean that differentiation is integral, i.e., unable to be fractionated into independently varying properties. In specific terms, because a liver cell is a discrete type under ordinary circumstances we cannot assume that it may not show only certain of its properties under others; nor that the demonstration of one liver property in an unknown cell type assures that it is a liver cell in all properties. In both tumors

(Foulds, 1954) and long-term tissue cultures (Evans *et al.*, 1952) there are suggestion that certain properties of an original differentiation may be lost whereas others are retained.

C. System-Dependency

Several lines of evidence converge in suggesting that metazoan cyto-differentiation is dependent upon unknown but relatively specific conditions existing in cell aggregates, and not yet realized in the environment of individual cells. This is particularly true of the initiation and progression of differentiation but, to some degree, of its maintenance as well. This is not to say that all cytodifferentiative properties are of this character or that the unknown conditions are of such nature that they cannot ultimately be identified and even reproduced apart from cell aggregates. But it is important to emphasize the evidence now available which indicates that cytodifferentiation is the product of mutual cell activities. In this sense cytodifferentiation is as much a function of the properties resident in cellular systems as of those of the individual cell.

1. Aggregate Size

A limiting effect of the size of a cellular aggregate on its subsequent morphogenesis and differentiation has been demonstrated repeatedly in studies of regeneration and reconstitution of sponges and coelenterates (Berrill, 1945). Similar facts for vertebrate tissues have been reported by a number of investigators [literature cited, Grobstein (1955a)]. The common finding in these cases is that the larger the size of a developing tissue, the greater the complexity and the larger the number of cell types subsequently produced. Moreover, there appears to be a lower size limit—Involving a number of cells considerably greater than one—below which particular cytodifferentiations fail to occur at all.

Two recently reported examples of the latter sort may be cited. A standard piece of the chick definitive primitive streak blastoderm, just lateral to the node, consistently produces nervous tissue when placed on the chick chorioallantoic membrane after 5 days in tissue culture (Grobstein and Zwilling, 1953). One-sixteenth of this piece, treated similarly, never produces nervous tissue although it remains viable. Sixteen such pieces placed in culture in a close cluster, so as to re-fuse quickly into a single mass, produce nervous tissues on reimplantation in about 30% of the cases. The same number of pieces placed in a dispersed cluster, so as to spread more and fuse less rapidly, never produce nervous tissue.

Similarly, if the presumptive somite region of *Ambystoma* larvae, after about 24 hours in a saline medium, is explanted into a ventral ectodermal

jacket, only about 1% of the explants differentiate muscle (Muchmore, 1957). If two such regions are fused in the saline medium and then similarly explanted, 89% produce muscle. When ten somite plates are fused all produce muscle. Several other tissue types also increase in amount and frequency as the mass is increased.

Experiments of this sort leave much to be desired. One would like to know the actual minimum number of cells, whether they must all be of one kind, and the effect of environmental factors in determining the minimum. Some information on the last point comes from the finding (Finnegan, 1953) that presumptive erythropoietic tissue of the salamander shows little differentiation in a simple saline solution, but that erythropoiesis is much enhanced under otherwise identical conditions if the tissue is confined in a restricted volume. Pending the more precise and quantitative experiments which this subject deserves, and which improved techniques of dissociation and culture now make possible, we may say simply that with decreasing tissue mass it becomes progressively more difficult to satisfy necessary conditions for cytodifferentiation. Whether environments can be provided in which single cells can initiate and progress through a characteristic differentiation is an empirical question urgently needing analysis. From such studies it may be possible to decide whether the observed effect of mass is the result of the kinetics of density as suggested by Foster *et al.* (1956), or is dependent upon processes whose magnitudes or complexities are inherently greater than can be provided by a single cell. This would be true, for example, if a larger mass of cytoplasm were required than could exist in relation to a single nucleus.

2. Interactions between Unlike Tissues

Not only is cytodifferentiation favored by conditions existing in aggregates, but it is particularly favored when two aggregates with appropriate properties are brought into intimate association. This is seen most strikingly in those instances referred to as embryonic induction, whether of the neural plate, of the lens, or of the epithelial tubules of the kidney.

The phenomenon has been extensively studied since its importance in normal development was demonstrated some thirty or more years ago, and the literature analyzing it has been repeatedly reviewed (Holtfreter, 1952; Holtfreter and Hamburger, 1955; Grobstein, 1956). We shall recapitulate here only the salient points as they relate to cytodifferentiation.

The superficial ectoderm of the early embryo is directed toward neural and associated differentiation if it becomes underlain by chordamesoderm, toward surface epithelium and its derivatives if it does not. Surface epithelium becomes lens if it is influenced by the optic vesicle,

other structures if it does not. Metanephrogenic mesenchyme, derived from the intermediate mesoderm, becomes glomeruli and secretory epithelium if it is associated with the ureteric bud; it becomes generalized mesenchymal tissue if the ureteric bud is absent. In each of these cases cells are turned in one or another direction of specialization, i.e., whatever is involved in the inductive process has important cytodifferentiative effects.

The mechanisms of these inductions are not known, and they are very likely not alike in each case. What the various inductions have in common is dependency, the fact that a particular differentiation occurs in the presence of a dissimilarly derived tissue and not in typical form in its absence. This implies that for certain differentiations it is not sufficient simply to satisfy the aggregate size requirement referred to above. Some additional requirement must be met, something beyond that provided by a homogeneous aggregate even when it is above the critical mass necessary for differentiation. Certain cytodifferentiations occur most readily in a *heterogeneous* system. Whatever is involved in the minimum aggregate requirement can be produced in the mutual behavior of cells of *like* type. Whatever is involved in the heterogeneity requirement is produced in the mutual or cooperative behavior of cells of *unlike* type.

Can the heterogeneity requirement be met in the absence of living tissue? Twenty years ago the answer seemed to be unequivocally affirmative; today we must introduce qualifications. The affirmative answer came from the well-established fact that urodele gastrular ectoderm, which in the embryo requires association with chordamesoderm to differentiate in a neural direction, can be induced to do so under experimental conditions by killed tissues and by a variety of chemically well-characterized but unrelated substances. When it was found that even relatively slight changes in the ambient medium which lead to "sub-lethal cytolysis" (Holtfreter, 1947) are also able to neuralize gastrular ectoderm, stocktaking became imperative. In the resulting more cautious atmosphere several points were re-emphasized.

First, the results of artificial inductions always are incomplete and abortive in comparison with those produced by chordamesoderm. In minimum cases they involve only "neuralization," as indicated by form changes or "palisading" in the gastrular ectoderm which is reminiscent of early steps in formation of the neural plate. In general, artificial inductions more closely resemble the normal when more complex materials are used—killed tissues rather than simple chemical compounds.

Second, the differentiative response of gastrular ectoderm is not simple and direct at the cell level, but involves important intermediate

processes at the tissue level which have been variously referred to as individuation, regionalization, self-organization, etc. The essential point for present purposes is that the reacting gastrular ectoderm does not remain a homogeneous population, with all of its cells differentiating equally as though each were responding directly to the inductor. Instead, the mass of cells rapidly becomes heterogeneous, and usually only certain portions become neural while others become something else. The inductive stimulus may, therefore, only initiate or evoke reorganization, leading to heterogeneity, and this in turn may set the actual conditions for cytodifferentiation. Included in these conditions may be interaction of the unlike tissues resulting from the differentials set up in reorganization. Induction experiments with gastrular ectoderm must therefore be carefully analyzed before it can be assumed that the inductive stimulus is *directly* involved in the resulting differentiation of individual cells.

Third, it now appears that the effectiveness of nonliving inductors is not so general and may not be as significant as was previously thought. It has recently been reported that anuran amphibian gastrular ectoderm does not respond to nonliving inductors which are effective for urodele gastrular ectoderm (Smith and Schechtman, 1954). Moreover, urodele chordamesoderm killed with minimum denaturation is reported not to be inductive, but it becomes inductive when subjected to procedures known to promote denaturation (Okazaki, 1955). The suggestion is made that the mechanism of action of killed tissues may be very different from the normal process. Finally, later-stage inductions, occurring in tissues whose reorganizational capacities appear to be considerably less than those of gastrular ectoderm, are far less readily—if at all—simulated by killed tissues or chemically simple materials (Grobstein, 1956).

Under the impact of these considerations, current investigations of induction are marked by enhanced respect for the complexities of the problem (Holtfreter, 1952). While they have not yet brought full understanding, or agreement among the investigators involved, some convergence in their implications seem to be appearing. Studies of the inductive effects of killed mammalian tissues on amphibian gastrular ectoderm have established that these are qualitatively different depending on the tissue source, the manner of killing, and the subsequent treatment of the inductors (Chuang, 1939). Alcohol-treated liver, for example, primarily induces brain, whereas bone marrow primarily induces mesodermal derivatives. When the two are administered jointly (Toivonen and Saxén, 1955), each gives its characteristic induction, but a new element, spinal cord, also appears—presumably through interaction in the responding system. The fact that these joint inductions more closely approach the normal than anything previously obtained with non-

living inducers favors the idea that the effect of the chordamesoderm is complex, but that the response is not simply "imprinted" on a passive ectoderm. The changes in the inductive activity of killed tissues resulting from treatment with trypsin, nucleases, and other reagents strongly suggest that the activity is linked to large-molecular materials (Yamada and Takata, 1955, 1956). Whether these are proteins or nucleic acids is not fully established, though the evidence favors proteins in the judgment of the investigators involved.

Fluids in which chordamesoderm has been cultured for a number of days in small volume are reported to induce the appearance of neural cells, pigment cells, or myoblasts in small fragments of gastrular ectoderm subsequently introduced into them (Niu and Twitty, 1953; Niu, 1956). Under conditions favoring concentration, therefore, active soluble materials can be demonstrated apart from the source tissue. Ribonucleic acid (RNA) is demonstrable in the active fluid. Although the data are not fully conclusive, the nature of the procedures which further concentrate the activity, together with the effects of exposure to proteolytic enzymes and nucleases, allow the interpretation that RNA is the active material. It is to be emphasized again that, both in these experiments and those with killed tissues, the multicellular reacting system is subject to reorganization and resulting heterogeneity which may provide the more immediate causes of cytodifferentiation. In this connection the recent reports that RNA-rich preparations, and serum proteins from different species, alter cellular behavior and aggregation are relevant (Murray, 1957).

In the later-stage induction of kidney tubules in mouse metanephrogenic mesenchyme, no activity has been detected in killed tissues (Grobstein, 1955b), nor has it been possible to concentrate soluble activity in the complex medium required for culture of mammalian tissues. However, it has been shown that the inductor tissue, embryonic dorsal spinal cord, does not need to be in actual cytoplasmic contact with the reacting metanephrogenic mesenchyme (Grobstein, 1955c; Grobstein and Dalton, 1957). If the two are separated by a porous membrane, which excludes penetration by cytoplasm, the activity passes across a 20- μ interzone. On the other hand, with a membrane of higher porosity which excludes whole cells but allows abundant cytoplasmic penetration, the activity still appears to be able to move only some 60–80 μ (Grobstein, 1957). Passage of the activity is blocked by cellophane. These and related facts are in conformity with the hypothesis that the complex large-molecular materials of the intercellular matrix are important mediators of inductive effects.

Thus, several lines of evidence point to a role of proteins, and their conjugates with nucleic acids or polysaccharides, in meeting the heter-

ogeneity requirement. Interest in the possibility that small-molecular materials are involved, however, has not completely waned. Wilde (1956) recently has reported experiments which he interprets on a small-molecular model. Amphibian neural crest, which in the embryo gives rise to pigment cells among other things, is found not to do so in protein-free nutrient media unless phenylalanine is supplied. Known precursor combinations do not satisfy the phenylalanine requirement when supplied to the neural crest alone, but the precursors are effective when given in the presence of the normal mesodermal inductor. It is suggested that the inductor acts by synthesizing phenylalanine and passing it on to its associated tissue. It must be noted, however, that these interesting facts do not include demonstration of the actual site of synthesis of phenylalanine. It is equally possible that what is transferred is not phenylalanine, but something which so alters the neural crest cells that they can themselves synthesize phenylalanine. It is to be recalled that the inductive activity accumulated from chordamesoderm in Niu's experiments is reported to be nondialyzable. Until more is known of the transmission characteristics of Wilde's effect, the assumption that it provides a small-molecular model for induction must be regarded with reserve.

It thus appears that the current trend in studies of induction suggests that a number of cytodifferentiations, beyond the favorable conditions supplied in aggregates of like type, are further favored by conditions set up in the close association of aggregates of unlike type. The nature of the influence of one aggregate on the other is not entirely clear, but accumulating evidence fits the hypothesis that it resides in macromolecular materials separable, under experimental conditions, from living cells. These materials, however, appear to have such low mobility in native and active form that they normally occur only in close association with the source tissues.

3. General Systemic Factors

The facts so far cited have emphasized that cytodifferentiation occurs most readily in cellular aggregates, particularly of some degree of heterogeneity. It seems reasonable to assume that in such aggregates a suitable, local microenvironment is supplied to the individual cell. Cytodifferentiation, however, is also markedly affected by what are usually thought of as systemic factors—nutritional, nervous, and humoral. Whether these act through alteration of the microenvironment, or independently and directly on the cell, is an important question for experimental study.

a. Nutritional. Several recent reports are of interest in showing

specific control of differentiation by particular nutrients. Wilde's finding, referred to above, that ventral ectoderm and neural crest cells only form pigment in the presence of phenylalanine is relevant. Equally striking is the fact that a normally keratinizing epithelium becomes mucous-secreting, and even ciliated, in the presence of excess vitamin A. An antikeratinizing effect of vitamin A *in vivo* was implied by deficiency studies, but Fell and Mellanby (1953) showed that embryonic chick skin, which normally keratinizes *in vitro*, can be inhibited from doing so by excess vitamin A in the culture medium. Instead of keratinizing, the epithelium becomes secretory and ciliated. On return to normal medium the distal differentiated layers slough following keratinization from the basal layer. The cells of the basal layer do not appear themselves to differentiate, but the cells to which they give rise are shifted in one direction or the other by the environmental level of vitamin A.

The studies of Fell and Mellanby were on organized skin continuously exposed to vitamin A. Weiss and James (1955) have repeated the experiment, exposing the skin only briefly (15–60 minutes) and in the dissociated state. When the reaggregated cells were cultured, the control suspensions produced typically organized skin with keratinizing cysts and feather follicles. The vitamin-treated suspensions also reaggregated and became organized, but the epithelium did not keratinize. Instead it resembled in appearance the early stages of formation of a mucous-secreting epithelium. When the single vitamin treatment in the dissociated state was supplemented during subsequent culture by several short exposures to high vitamin A, typical mucous-secreting epithelium formed. It appears probable therefore that the vitamin effect can be exerted on individual cells, and can have differentiative consequences beyond the duration of application of the stimulus, although the possibility of persistence of cell-bound vitamin A is not excluded. It is to be noted, however, that the overt differentiation by which the effect is recognized does take place in an organized mass including epithelium and connective tissue in characteristic relations. So far as the data go, therefore, the vitamin A requirement to form secretory cells seems to be *additional* to what has been referred to above as the aggregate size and heterogeneity requirements. It is to be noted, too, that topical application of vitamin A to the skin of adult rats has an acanthotic effect, but is not clearly antikeratinizing and does not induce mucous metaplasia (Bern *et al.*, 1955a). It would be interesting to know what the effect would be on younger animals or during wound healing in adults.

b. Hormonal. The widespread influence of hormones on differentiative processes has been extensively discussed elsewhere (e.g., Willier, 1955) and will be mentioned only briefly here. Certain cellular responses

to hormones clearly are temporary or modulatory, the induced change disappearing without apparent residue on withdrawal of the hormone. Others are more permanent, persisting after withdrawal of the hormone. In the former category are hypertrophy of prostate cells in response to androgen, or of thyroid cells in response to thyrotropic hormone. In the latter category are certain skeletal differentiations induced by sex hormones, or the extensive differentiative changes involved in the anuran metamorphic response to thyroid stimulation. Effects of this kind indicate that in later developmental stages, in a number of instances, tissues require for differentiation substances produced in distant tissues and transported to them via the blood stream. Local conditions necessary for the particular differentiation having been satisfied by earlier developmental processes, the presence or absence of the hormone becomes controlling and endocrine integration of the further development occurs. There also is evidence that hormonal influence may "plasticize" a tissue, making reorganization and new differentiations more probable (Schotte and Chamberlain, 1955).

c. Nervous. Dependency of developmental processes on the nervous system was once regarded as far greater than it is thought to be today (Singer, 1952). Observations following removal of the nervous system in early embryonic stages, and on the development of nonnervous structures isolated in culture, both indicate that morphogenesis and cytodifferentiation of many kinds can progress in the absence of neural connections. However, there are several lines of evidence suggesting that neural influences cannot be disregarded in discussing the system-dependency of cytodifferentiation (see Singer, 1952, for literature).

First, denervation leads to regression of the differentiated structure of striated muscle, taste buds, and perhaps other organs. Second, developing nervous tissue has striking inductive effects on associated tissues; e.g., optic vessel induce lens, ventral spinal cord induces axial cartilage, dorsal spinal cord induces kidney tubules (see Grobstein, 1956, for literature). Third, denervated forelimbs in amphibia not only fail to regenerate but undergo extensive regression from the site of amputation to the shoulder (Butler and Schotte, 1941). Moreover, anuran limbs which do not normally regenerate after morphogenesis, can be induced to do so by increasing their degree of innervation (Singer, 1954).

The mechanisms of these various effects are not known, though they do not appear to depend upon impulse conduction. Nor does the evidence demonstrate a direct action on the cell while in differentiation. In the case of regenerating limb, reinnervation allows accumulation of a blastema and does not appear necessary once it is well established. The current impression is that the neural contribution is toward establishing

and maintaining preconditions, rather than toward the specific needs of particular differentiations. This is seen in the nonspecific manner in which nerves deviated to different sites in amphibian larvae may stimulate formation of a supernumerary limb, crest, or tail—each appropriate to the site stimulated. The possibility seems worth considering that the neural effect is related to the aggregate size requirement for differentiation—also a general precondition. Whatever the mechanism, it is clear that in certain cases and in certain stages cytodifferentiation is at least indirectly neurodependent.

4. *In Tissue Culture*

From what already has been said about the importance of aggregate size, heterogeneity, and systemic factors in cytodifferentiation, it is not surprising to find that the overt signs of differentiation tend strongly to disappear when tissues and cells are cultured *in vivo* (Willmer, 1954). However, it is important to note that dedifferentiation of overt characteristics in culture cannot be regarded as an inevitable consequence of isolation *in vitro* (Fell, 1953). Rather it is a function of the particular conditions under which the cells and tissues are maintained in culture, conditions which, incidentally, have their parallels in the intact organism. Indeed, variation of culture conditions provides one of the most powerful available tools for further studies of the nature of the extrinsic factors involved in cytodifferentiation.

It is useful to distinguish four classes of situations which collectively are loosely referred to as tissue culture. All allow maintenance and proliferation *in vitro* of cellular populations, but they vary in the degree of complexity and integration within the population. At one extreme is *organ culture*, in which the original explant is an entire embryonic rudiment or a relatively large fraction of one. The conditions of culture are such that the integrity of the explant is preserved, with all essential components present and in reasonably normal relations. In organ cultures, which are least radical in their exposure of individual cells to altered conditions, existing differentiation not only may be maintained, but it may progress and new differentiation may be initiated. It is clear from such cultures that isolation *in vitro* is not, in and of itself, anti-differentiative.

At the other extreme are *cell cultures*, in which a population of metazoan cells is maintained and proliferates as a collection of individual cells, very much as with microorganisms. Any aggregation which occurs is haphazard and incidental, and assumed to be without effect on the properties of the population. Significantly, overt signs of differentiation are exceedingly difficult to demonstrate in such cultures, in which altera-

tion of the cellular microenvironment may be expected to be most radical. The determination of the actual differentiative status of these cells is one of the foremost current problems of experimental cytology.

Intermediate between these two situations are *tissue cultures* in the strict sense, and these may either be homogeneous or heterogeneous. The former refers to cell populations known to contain only one of the recognized tissue types of the intact organism, or their descendants. The latter refers to cultures containing more than one such type or their descendants. Homogeneous cultures, particularly of early embryonic cells, may become heterogeneous if differentiation occurs within them. Heterogeneous cultures may become homogeneous through differential survival or selection of one type. In both, the cell population is maintained not as individuals, but as aggregates of varying degrees of cohesiveness.

It is important to recognize that until recent years the bulk of available information on culture behavior came from the study of initially heterogeneous cultures, which gradually were rendered increasingly homogeneous by procedures for developing "pure strains" (Fisher, 1946). In the initial stages highly differentiated cells, such as neurones, usually die in the primary explant. Less highly differentiated cells move outward from the explant into the medium, either by individual migration or through spreading of cohesive sheets. With peripheral progression the original organization of the explant is disrupted, and the departing cells show few overt differentiative characteristics. Differentiation is recognizable longest in the central mass and declines centrifugally. Many early studies involved subculture by transfer of the central region of the culture, sacrificing the "outgrowth zone." By these procedures the differentiated state may be preserved through a number of "culture generations," but without information on the number of cell generations, if any, involved. Once the central zone loses its differentiated character, or if it is removed and the outgrowth zone carried on, specific identification of the cells in terms of original differentiative type becomes exceedingly difficult.

Cells in culture, outside of the primary explant, have been classified in three general types (Willmer, 1954): (1) cohesive sheets of epithelioid character (epitheliocytes); (2) less cohesive networks of fusiform or fibroblastlike cells (mechanocytes); (3) individual cells with considerable motility (amebocytes). Cultures may exist in one of these forms for extended periods, but discontinuous or gradual transformation have been recorded from one to the other (Bloom, 1937). The important dominating generalization, for present purposes, is that by the usual criteria of identification *in vivo* cells in culture become decreasingly

distinguishable. This takes place as the cells, through spreading and migration, lose their earlier relationships with other cells of like and unlike type and increasingly are subjected to a common, generalized environment. It is natural to assume, as a first approximation, that the decline in overt differentiation is related to the migration, spreading, and resulting simplification of the cellular system.

There are several lines of evidence which support this assumption. The simple sheetlike organization of homogeneous epithelial cultures is quickly modified when they are combined with fibroblast cultures. In one study (Drew, 1923), for example, epithelium originally derived from embryonic kidney was isolated as simple undifferentiated sheets. A small fragment from a connective tissue culture was added. In 2-3 days there were "kidney cells forming typical tubules." Epithelium from a mammary carcinoma behaved similarly; in neither case did the connective tissue source seem to influence the result. As the cultures were carried further, if the epithelium outgrew the connective tissue, the former reverted to simple sheets. From these, and other results, it is clear that the undifferentiated appearance assumed by epithelia in culture is related to their separation from connective tissue.

Further evidence comes from the separation of the components of mouse salivary rudiments prior to cultivation (Grobstein, 1953a, b). When the epithelial and mesenchymal components are separated by exposure to trypsin, neither one alone undergoes anything resembling salivary development. Instead, both spread as simple cellular masses. If immediately recombined after separation, however, the epithelium undergoes characteristic morphogenesis in culture. Only salivary mesenchyme has this effect on salivary epithelium; other mesenchyme prevents the spreading of the epithelium as a simple sheet but causes it only to round up as a quiescent mass—an effect reminiscent of Drew's result described above. The specific effect of salivary mesenchyme appears to be exerted only in intimate association, although the effect can cross a porous membrane 20 μ thick (Grobstein, 1953c). Since interactions of this kind appear to be general, and to continue into later developmental stages if not into the adult, it is not surprising that differential spreading and migration which separates two normally associated tissue types leads to regression of overt differentiation.

Cellular dispersion in culture tends to inhibit differentiative processes even when the cells are presumably of one type. Inhibition of neuralization by fragmentation and spreading in culture of presumptive neural tissue of the mouse and chick already has been mentioned (page 451). Possibly an even clearer case has been described by Weiss and Amprino (1940). Fragments of the coat of 4-day chick embryo

eyecup can form scleral cartilage *in vitro*. Fragments below a certain size, however, fail to do so. They proliferate like ordinary fibroblast cultures, "but soon become attenuated and fail to differentiate further." The failure is attributed to loss of "the tight texture which apparently is a prerequisite of cartilage formation." This interpretation is supported by the finding that somewhat larger pieces are prevented from forming cartilage if they are subjected to stress. There obviously is a possible relationship between these findings and the minimum aggregate-size phenomenon referred to earlier (page 451).

It should be noted that differentiation in culture is reported to be favored by conditions which limit proliferation. A number of investigators have shown that morphological signs of differentiation appear when embryo juice is withdrawn from the nutrient medium, and Gaillard (1935) has reported that differentiation can be furthered by substitution of juices of older embryos for those of younger ones. In this connection, Fell (1957) has noted recently that conditions favoring mitosis usually favor cell migration and that measures to promote differentiation which limit both mitosis and migration may be more effective through the latter effect than the former.

Overall, then, the behavior of tissues subjected to spreading and gradual dissociation in culture is generally dedifferentiative—suggesting that overt cytodifferentiation is dependent upon conditions existing within a tissue system and not present in culture media so far used. Initiation and progression of differentiation is inhibited in culture when rudiment components are separated, or when aggregate size or density is reduced below a critical level. The conditions under which differentiation occurs *in vitro*—in organ cultures—are precisely those which favor maintenance of mass and integrity. The facts from studies *in vitro*, therefore, are in accord with those previously cited in indicating the strong system-dependency of important aspects of cytodifferentiation.

5. Regeneration

Returning to the organism, the facts with respect to regeneration of the amphibian limb, for example, point in the same direction. Following amputation the reparative process occurs essentially in three intergrading phases (Singer, 1952); (1) wound healing, in which the neighboring epithelium migrates and re-covers the exposed surface; (2) blastema formation, in which structural disorganization occurs in the immediately adjacent stump and morphologically indifferent cells accumulate below the new epithelial covering; (3) growth, differentiation, and morphogenesis of the blastema giving rise to new structures directly continuous with the old ones. Certain aspects of the process have important similar-

ties with the differentiative and dedifferentiative behavior of tissues in culture.

It is generally agreed that the stump in the immediate vicinity of the cut surface normally is the source of the indifferent cells of the blastema (Weiss, 1939), as the primary explant is the source of the outgrowth zone in culture. Bone, for example, can be observed to undergo dissolution of its matrix, and its cells become indistinguishable from those of the accumulating blastema. Blastema cells, unlike culture outgrowth cells, are confined by the already covering epithelium, and they enter into an association with it which has been shown to be essential to their future growth and differentiation (Thornton, 1957). As has been noted above, some influence of nerves is essential to blastema accumulation. In denervated limbs dissolution of stump tissues progresses proximad, even from wrist to shoulder, until reinnervation is permitted (Schotte and Butler, 1941). When this occurs, indifferent cells accumulate near the ends of the ingrowing nerves, regression stops, and regeneration proceeds, replacing all structures distal to this point. The interesting question is raised to what degree the extensive disorganization of explanted tissues reflects the absence of innervation, and whether it might be controlled by the presence of suitable neural tissues.

The subsequent history of individual cells released in the structural dedifferentiation of the stump of a regenerate is unsettled. Most investigators concur in the belief that they make up the blastema, and participate in its subsequent growth and differentiation. Whether they differentiate in directions other than that of their source (Schotte, 1940), e.g., original bone cells becoming muscle, is more controversial and not relevant at this point. What is important here is that in the regenerating limb the level of overt cytodifferentiation, as in other situations described, appears to be a function of system properties. The local factors of aggregate size and interaction of unlike tissues, as well as such systemic factor as hormonal and neural influences, all appear to be involved.

D. System-Independency

Certain aspects of cytodifferentiation are system-independent, i.e., clearly resident in properties of the individual cell. This is true in all cases where differentiation renders the cell structurally deficient and hence terminal. More significantly, it is true of those characteristics of a differentiation which represent differences between individuals or species, rather than between tissues of a single individual. These are demonstrably controlled genotypically, and indicate that the mechanisms underlying genotype must at some point impinge on those of differentiation. Further, specialization of cell organization is intrinsically stabilized in protozoa,

by mechanisms which go beyond classical genotype and have been suggested as possible models for metazoan differentiation (Lwoff, 1950; Sonneborn, 1950; Weisz, 1951; Ephrussi, 1952). And in what has been called mosaic cleavage, in certain invertebrate eggs, there are facts which provided the original model for differentiation by segregation of intrinsic hereditary determinants (Lillie, 1929).

1. Genotypic Control

The preponderant evidence indicates that the differences between homologous cells of different individuals or species are largely determined by the intrinsic hereditary properties of the individual cell, though these may interact with the properties of the larger system in establishing the final differences. Perhaps the most striking illustration is the often-cited results of transplantation of ectoderm from urodele to anuran embryos (see Schotte and Edds, 1940, for literature). Anuran head ectoderm normally forms suckers; urodele head ectoderm forms elongated balancers. When urodele replaces anuran ectoderm, differentiation is induced by the underlying anuran mesoderm but the structure produced is a urodele balancer. The reciprocal experiment leads to an anuran adhesive disk on a urodele head. The induction can take place xenoplastically, but the character of the response is dictated by the hereditary constitution of the graft.

The test here is really of tissue, rather than cell, properties. But the results are very similar in principle in the extensive experiments dealing with pigment cell properties in amphibia and birds (Rawles, 1955). Presumptive pigment cells migrate individually from their source in neural crest before settling down and differentiating. Grafts between species show that local tissue factors may influence orientation and distribution of pigment cells, but in shape and size, and in the detailed characteristics of their granules, the cells always are distinguishable from their host. Thus, the properties of cytodifferentiation which are characteristic of species are largely determined within the cell, and are relatively system-independent.

2. Ciliate Morphogenesis

In recent years there has been extensive investigation of protozoan morphogenesis, particularly of the ciliates (see Lwoff, 1950; Faure-Fremiet, 1954; Weisz, 1954; Tartar, 1956). The protozoa can be regarded as cell-organisms, i.e., forms in which the general organization characteristic of a cell has been adapted to unitary existence. As such, they are in certain respects comparable with entire metazoans, in other respects with metazoan component cells. To the extent that their morphogenesis

represents specialization of the basic organization of a cell, there is justification for the working hypothesis that the underlying intracellular mechanisms may be similar to those involved in metazoan cytodifferentiation. But interpretation must be tempered by the fact that protozoan differentiation is circumscribed by the requirements of unitary existence, and this may impose important differences. The protozoan cell, for example, in its differentiation may not regularly sacrifice the ability to divide, as do many metazoan cell types. Cellular specialization in protozoa is simultaneously adaptive species morphology, subject to the demands of evolutionary dynamics. The protozoan cell is of higher integrity than the metazoan cell, in the sense of being more sharply bounded from its less highly organized immediate environment—which may mean greater importance of intrinsic factors in the stabilization of its differentiated state. For these and other reasons, caution is indicated in carrying over conclusions from protozoa to metazoa and vice versa.

Morphogenetic changes, the appearance or disappearance of specialized form and structure, occur in protozoa during reproduction, in reorganization accompanying encystment and excystment or following injury, in regeneration of lost parts, and in developmental transition between stages of complex life cycles. Differentiation occurs largely in the outer, more gelated, ectoplasmic or cortical layer of the cell, forming such structure as pellicle, trichocysts, simple and compound cilia, skeletal plates, and contractile fibrils. Specialization of parts may become exceedingly complex in the more evolved forms.

The more generalized and more fluid endoplasmic region also may contain, in addition to mitochondria, granules, droplets, and other organelles, such specialized structures as chromatophores. The nucleus may vary in form and number in different stages of the life cycle. Its significance in relation to morphogenesis is treated in Chapter 13.

Of immediate interest is the role of the cortical infraciliature, and particularly of the kinetosomes, in protozoan morphogenesis. These granules, one at the base of each cilium, are the source—either by conversion or incitement—of “most, if not all, differentiations of the ectoplasmic cortex” (Weisz, 1954). They are linked together in rows by kinetodesmal fibers, each row designated a kinety and all together designated the infraciliature. Certain regions of the infraciliature are, in many species, specialized and dominant over the rest, so that in the absence of these regions no major morphogenesis can occur. The infraciliature thus is an integrator of cortical differentiative behavior, with the specialized kinetics and their kinetosomes as the dominant center. The intact system has considerable intrinsic stability. When sufficiently disturbed, however, either by external damage or little-understood influences from elsewhere

in the cell, there is set in motion a morphogenetic sequence of dedifferentiation and redifferentiation of cortical structures common to physiological reorganization, regeneration, fission, or encystment and excystment.

In all of these processes of neoformation the kinetosomes are identifiable physical elements of continuity, their ability to divide making the infraciliature not only an integrative system but a duplicative one. The details vary in different species, but the general rule is that when neoformation occurs new specialized structures are never formed directly from old ones, i.e., a cilium or cytostome does not split or bud off a new one. Rather it is the kinetosomes which divide and then subsequently transform into, or incite the formation of, new structures. They may do this simply, *in situ*, by kinetosomal multiplication at the terminus of a kinety or, particularly in forms having more complex structuration, by formation of "anarchic fields." These are unpatterned, local collections of kinetosomes derived from the specialized kineties by multiplication and aggregation. They subsequently become ordered and give rise to major new structures. In some instances such anarchic fields are included in cytoplasmic buds which detach to become free-swimming "embryos" before transforming into new "adults."

Kinetosomal division thus is a process of duplication of determinants. Whether the divisions are always equal is not clear: a cilium-bearing kinetosome, for instance, may divide and one of its daughter granules may transform into a trichocyst—thereby losing its power to reproduce. It is not known whether this results from an originally unequal division, or from the daughter granules becoming unequal due to exposure to different microenvironments after division. This emphasizes that the kinetosomes are "self-duplicative" only in the sense that they do not arise *de novo* but from partition of existing kinetosomes. They are certainly dependent upon their environment, both cytoplasmic and nuclear, for a supply of building materials, and their character and activity is subject to influence by these sources as well.

The visible differentiation of the ciliate cortex, therefore, is largely subject to destructure. Its stability, what Weisz (1954) has called its morphostasis, is conferred by the infraciliature. Further, when the cortical structure is taken down, as it is in many species prior to each division, its reappearance is ensured by multiplication and transmission of its progenitors, the kinetosomes. These visible bodies in the cytoplasm appear to behave as required to provide a physical basis for differentiation, in the same way that chromosomal behavior was found to conform to the requirements of genetic data. It is not surprising that they have been looked upon as a model for all differentiation, with the suggestion

that in those protozoa and in metazoan cells which do not possess visible kinetosomes, the cortex performs a similar role without visible particularization. In Tartar's (1956) words, ". . . it is not impossible to imagine the ciliate pattern being stripped of its specialized structural units and organelles, leaving a highly flexible but determinative network, capable of developing polarities and anisotropies and of guiding the course of differentiation, which might be present in all cells."

3. "Mosaic Eggs"

Embryological thinking about cytodifferentiative mechanisms has been much influenced by studies of the development of certain marine invertebrates which began shortly before the turn of the century. These studies were conducted in the context of a theoretical controversy between those who saw development as the product of a guiding vital principle or entelechy (Dreisch) and those who rejected this view and sought to find determinants for development in the materials of the embryos (Roux, Weismann). The rediscovery of Mendelism, and the subsequent demonstration of the powerful role of the chromosomes in hereditary processes, gave decisive support to the materialists. The objective of the embryologist could now be defined it seemed, apart from the uncertainties of philosophical speculation. In the words of Lillie (1906), "It is no less an undertaking than to derive the entire body of the individual from the chromosome-complex of its germ."

In this mood the precise, regular cleavage of the eggs of annelids, mollusks, and ascidians was highly attractive. In these eggs the very machinery of segregation of developmental determinants seemed to be visible—much as chromosomal behavior provided a conforming physical basis for the regularities of genetics. Moreover, these blastomeres, on isolation, each proved in general to give rise to the very cells which it would produce when part of the whole embryo. It was as though a limited set of developmental determiners or "potencies" present in the zygote was being parceled out in the cleavage process. Indeed, in some species visible pigments and granules were differently segregated in cleavage in ways which strongly suggested that they were themselves the determinative substances, or closely linked to them. It was an easy step to assume that differentiation is the sorting of cytoplasmic determinants into the proper daughter cells. Corollary to this, differentiation became determinative and irreversible—a cell lineage gradually narrows down to only one kind of determinant and hence is precluded from undergoing any alternative differentiation.

Because these essentially static conceptions continue strongly to influence thinking about cytodifferentiation in this period of distinctly more

dynamic analysis, it seems important to analyze critically the status of knowledge of the phenomena which gave rise to them. There is no question of the validity of the beautiful, detailed descriptions of cell lineage provided for a series of marine eggs at the turn of the century (see Costello, 1955). Nor is there any doubt that isolated blastomeres of the so-called mosaic eggs develop as limited parts under the conditions provided; this has repeatedly been confirmed (Costello, 1948; Clement, 1956). The question which must be raised is whether these facts, and the newer information available, *require* the hypothesis that originally present determinants are being sorted out to individual cells, and that this process is irrevocable. Or can the facts be explained otherwise?

The answer depends essentially on two questions of fact: (1) How rigidly fixed as to developmental course is a blastomere and its derivatives? (2) What is the site and mechanism of the stability? Relative to the first question the following points are noteworthy. The conditions of test have usually been such as to minimize the effects of separation in order to obtain the maximum degree of differentiation. Descriptions of the cleavage process in blastomeres (Costello, 1945) emphasize that it is very similar to that in the intact embryo—with respect to cytoplasmic streaming, behavior of inclusions, position of cleavage planes, etc. Some initial rounding of the blastomeres takes place, and the resulting cellular mass tends to be spherical rather than preserve the shape of a part-embryo. Even under these conditions, blastomeres, while they never produce more than their presumptive fate, sometimes produce less. Further, when the procedure is such as to allow blastomeres to come into contact with a glass substrate, ameboid activity results and differentiation may be completely inhibited (Costello, 1945). The impression is strong that the blastomere is not much altered by the “optimum” process of isolation, which means that its properties are not dependent upon the presence of other blastomeres at this stage. It does not mean, however, that the blastomere’s differentiative behavior necessarily is irrevocably fixed under all conditions. It may mean, instead, only that the process of isolation does not represent any significant change of conditions.

The range of conditions so far tested has, in fact, been narrow. Efforts to “induce” change of differentiative behavior in isolated blastomeres by their artificial combination with morphogenetically active polar lobe cytoplasm in *Sabellaria* failed (Novikoff, 1938). But further study is needed of the effects of spreading at surfaces, increased cellular mass, longer-term culture, and the effect of “plasticizing” agents before it can be accepted that “mosaic blastomeres” are rigidly fixed as to fate (Trinkaus, 1956). Meanwhile, it is clear that twinning can be induced in

several mosaic eggs (see Novikoff, 1940, for references) and must involve some change of fate, and that *Tubifex* embryos initially deprived of ectoderm by suitable blastomere destruction in cleavage stages will produce ectoderm from their mesoderm if kept alive long enough (Penners, 1937). Raven (1952) found that *Lymnaea* embryos can be altered in their adult head development up to the 32- to 64-cell stage by exposure to lithium salts, and that in the process some cells participated in altered developmental courses. Further, he described the formation of shell glands wherever the tip of invaginating endoderm contacted ectoderm, both in normal embryos and abnormal ones derived from lithium exposure. These various findings raise considerable question whether the behavior of isolated blastomeres indicates irrevocable fixity of cell type, rather than a high enough level of stability so that their developmental course is not altered under the conditions so far tested.

Nor does the stability appear to be connected with the visible cytoplasmic inclusions whose segregation originally gave rise to the notion of assortment of determiners. While the effects of centrifugation have been variously reported, it now seems certain that cytoplasm centrifuged completely free of visible inclusions may continue development and show differential properties. The segregation may be invisible (Costello, 1945; Lehmann, 1956), but efforts to establish cytochemical differences clearly and generally correlated with morphogenetic behavior have so far not been successful (Berg, 1956). It is of interest that Raven *et al.* (1950) conclude, on the basis of cytochemical and centrifugal studies of the polar lobe in *Sabellaria*, that its peculiar character may reside in structural configurations of large molecules not demonstrable by cytochemical methods. Raven and associates suggest that these may lie in the cortical layer of the egg rather than in its endoplasm (cf. Lehmann, 1956). In these terms the relative stability of mosaic blastomeres reflects the stability of molecular organization of their peripheral regions during the process of cleavage. The mosaic egg may thus resemble the ciliate. In these terms, mosaic cleavage parcels out the stable periphery, and the properties of the blastomeres are stable as long as the peripheral organization is maintained. Whether replication of peripheral determinants must be assumed, as in ciliates, is not clear from the evidence.

In summary, while the hypothesis of segregating hereditary determinants has not been decisively eliminated, its force has been weakened by accumulating evidence. Modified or alternative explanations appear possible even for the facts which were its original domain. The difference between "regulatory" and "mosaic" eggs need reflect no more than a difference in their cortical stability in the face of altered environmental conditions. Mosaic eggs indicate that considerable intrinsic stability, or

differentiative bias, can exist in blastomeres. The relation of this stability of part-embryos to the site and mechanisms of increasing stability of type in cytodifferentiation deserves much further study.

E. Stability of the Differentiated State

We come now to what, in many ways, appears to be the current central problem of cytodifferentiation—the degree and nature of its stability. This refers to the extent to which it is reversible, renewable, transformable, and propagable. Does a particular differentiated cell ever revert, in whole or in part, to its status prior to differentiation? Does it redifferentiate in an alternative direction? Do cells in a differentiated state confer that state on their progeny and, if so, over how many generations and under what conditions? To the extent that differentiation is carried through cell generations, is this due to propagable determinants within the cells or to stability of conditions surrounding the cells? The validity of our conceptions of the nature of differentiative mechanisms can be no greater than the precision of our answers to these questions. We may take up the evidence relating to them under three headings: (1) Dedifferentiation and Redifferentiation; (2) Transformation and Metaplasia; (3) Propagability.

1. Dedifferentiation and Redifferentiation

Certain differentiations unquestionably pass the point of no return, they are *definitely terminal*. In this category, for example, are cornified cells and erythrocytes. Differentiations which sacrifice, or too radically transform, an essential cell component clearly are irreversible within the present meaning of the term. Other cell types, usually highly modified in structure but not obviously incomplete, have never been known to undergo a reversal of differentiation, e.g., nerve cells or granulocytes. Since the possibility cannot be excluded that they may prove reversible under experimental conditions yet to be devised, they may be referred to as *provisionally terminal*. From the existence of such terminalizing differentiations it may be assumed that the processes involved can lead to permanent and irrevocable results.

But need they always? There is a still larger group of differentiated cell types which have been reported to undergo some degree of dedifferentiation. We must examine these in detail, since recently accumulating evidence has raised considerable question about the correctness of their earlier interpretation. Information comes from a number of sources—regeneration, pathology, tissue culture—and there is not space to examine them all. We shall confine ourselves to the last named, since it is most

frequently cited in this connection and appears to offer the greatest hope for an unequivocal answer.

As has been noted, there is no question but that some degree of overt dedifferentiation of many cell types takes place in culture. Champy (1913) was among the first to emphasize this, and he referred to the phenomenon without reservation as dedifferentiation. The term was criticized (Ebeling and Fischer, 1922; Fischer, 1924) on the ground that it implied a complete reversion to an embryonic state. A number of investigators reported that cultured tissues which had undergone dedifferentiation could, under proper conditions, return to their original state. Since these experiments have become the primary basis for the conception of the irreversibility of cytodifferentiation it is important to examine them carefully and see exactly what they establish.

First, it should be noted that Champy, himself, regarded the dedifferentiation as a function of tissue isolation. The dedifferentiative changes in epidermis in culture were inhibited in the presence of dermis (Champy, 1914a). He suggested that two normally associated tissues exerted a differentiative influence on each other, and related this to the mutual influence of blastomeres of regulatory eggs, then recently described by Driesch. On the basis of studies (Champy, 1914b, c, 1915, 1920) of smooth muscle, thyroid, kidney, prostate, and other tissues in culture, he regarded dedifferentiation as functional as well as morphological, and to occur more readily in less highly differentiated cells. He believed that dedifferentiation represented the ascendancy of general protoplasm over "paraplasma"—the specialized ingredients of the cell.

Champy's critics did not question the occurrence of overt dedifferentiation, nor its correlation with the separation of tissues from one another. They denied, however, that the dedifferentiation was so far-reaching as to render all cultured cells alike in properties, or to alter their developmental behavior. Thus, Ebeling and Fischer (1922) placed side by side a fragment of a 10-year-old fibroblast culture and a 2-month-old epithelial strain, and obtained epithelial tubules with distinct lumina, sometimes filled with homogeneous colloidal material. The fibroblasts resembled connective tissue and the whole section was reminiscent of salivary gland. The authors concluded that reversion to an embryonic state certainly had not occurred in culture, since both epithelium and fibroblasts still showed different stable properties. The often-cited experiment of Drew (1923) is of similar character, involving combination of cultured embryonic kidney epithelium with a small fragment of connective tissue. "Kidney cells forming typical tubules" were observed, and comparable results were obtained with a cultured alveolar mammary carcinoma. These experiments, recently confirmed and extended to clone

cultures by Puck *et al.* (1957) certainly indicate persistence of epithelial character and argue against reversion to a single generalized embryonic type. On the other hand, they fail to indicate that the tissues had undergone no alteration of original type or that they had completely regained it (Policard, 1925). Conforming to this reservation, is the finding (Grobstein, 1953b) that salivary mesenchyme, which makes some specific contribution to the morphogenesis of salivary epithelium, gradually loses this specificity over several weeks in culture, preserving only the ability to cause the epithelium to round up as a cyst rather than spread as a sheet.

Strangeways (1924) also obtained results which led him to conclude that "dedifferentiated cells in normal cultures retain most, if not all, of their original potencies." A culture of adult fowl articular cartilage was carried through 23 passages and no trace of the original implant remained. The culture was grafted subcutaneously, and hyaline cartilage formed. Similarly, a culture of embryonic mouse skin, transplanted under the skin of an adult, assumed characteristic squamous epidermal structure with keratinization. Other details of these experiments were not given. With respect to the cartilage experiment, Burrows (1933) tested a strain of fibroblastlike cells from chick embryo mandibular bone after elimination of the original explant. On reimplantation under the skin he found no bone and could not confirm Strangeways. On the other hand, Bloom (1957) reports an unpublished experiment of J. H. Heinen in which outgrowth cells of embryonic rat tibia, taken on the fourth day of culture, formed good cartilage and bone in the anterior chamber of the eye.

There are several reports on differentiation of cartilage or bone in culture, following cultivation in an undifferentiated state. Fischer and Parker (1929a, b) recorded experiments with a 7 to 9-month-old strain of fibroblastlike cells from the frontal bone of a 10-day chick embryo. The strain was subcultured from the outgrowth zone so as to avoid all carry-over of original tissue. When growth was suppressed, by deletion of embryo juice from the medium, an abundant ground substance appeared—at first fibrous, but gradually becoming homogeneous and then hyaline. This was interpreted as redifferentiation, and hence evidence for preservation of the original differentiative type. Burrows (1933) repeated these experiments and obtained similar results. However, Burrows doubted the redifferentiation hypothesis, pointing out that the fibers gave collagenoid rather than osteoid staining reactions and were not associated with calcification. Rumjantzew and Berezkina (1944) were also doubtful that Fischer and Parker had demonstrated bone formation. They cultured osteogenic mesenchyme by conserving the central region

of the culture and discarding the outgrowth zone. After 30 days they suppressed growth, and the thick, dense center gave rise to collagenous fibers, chondroid and osteoid tissue.

The most that can be said from these results appears to be that the ability to differentiate as bone can be conserved for some period of time under some culture conditions. Careful studies of the nature of the favorable conditions, and the duration of conservation, in time and in cell generations, are very much needed.

A number of investigators have examined the pigment-forming capacity of cultured iris epithelium. Ebeling (1924) reported that after 18 months in culture the depigmented epithelium was unchanged in appearance. In the presence of serum, growth was diminished and large amounts of pigment, similar to that of the iris, were formed. The report does not indicate at what point in the culture history the repigmentation experiments were done. Doljanski (1930) confirmed and extended these findings. He took a pigment-free culture in its 8th-10th passage and halved it. One half was continued in the presence of embryo extract, the other in its absence. Proliferation continued in both flasks but was more intense in the presence of embryo juice where little or no pigment formed. In the absence of embryo juice, pigment masses appeared, abundantly at the culture center but grading away in the migrating peripheral membrane. Doljanski concluded that the cultured cell "simply and temporarily ceases its specific functions without loss of potentiality." I. Fischer (1938) in general concurred, but added some significant details. Using vital staining or osmication she demonstrated the presence of "propigment granules" in depigmented cells. She regarded pigmented cells, *in vivo* or following withdrawal of embryo juice *in vitro*, as in a functionless storage stage. Cells spreading *in vitro* were mitotically active and formed new propigment and pigment granules. Without transfer these cells entered the storage phase by the 6th day, but, if transferred, spreading and mitosis set in anew. The cultures, therefore, showed an alternation between production and storage of pigment; no real dedifferentiation occurred. However, even with continued transfer, propigment granule formation ceased at about the 25th-30th passage (4-5 months in culture). Alteration of mitochondria and disappearance of Golgi material were observed at the same time, so the change was regarded as degenerative.

Ebeling (1925) reported that chick embryo thyroid tissue could be cultivated for seven months without loss of differentiated type, as indicated by the continued ability to organize as a glandular organ with production of colloid. Demuth (1932) repeated the experiment, finding that original folliclelike formations can persist up to the 55th day or

15th passage. In cultures of outgrowth epithelium neither follicles nor colloid were ever observed. Demuth interpreted his results and those of Ebeling as indicating persistence of original thyroid structure and denied that thyroid redifferentiation occurs from cultured cells. While not believing that cultured cells return to an embryonic state, he wondered whether "it is possible that the state of the cell growing *in vitro* is something entirely new." More recent results seem to support Demuth. Gonzales (1956) found that functional differentiation of embryonic chick thyroid lobes occurred in roller tubes, but soon declined. Colloid was demonstrable at the base of the outgrowth sheet but not beyond. Seaman and Stahl (1956) could detect I¹³¹ uptake in adult thyroid explants only up to 6 days, and Oppenheimer *et al.* (1956) reported no uptake at any time in outgrowth sheets. But Roche *et al.* (1957), using organ culture methods which preserved tissue architecture for 25 days, found synthesis of thyroid hormone continuing to this time. The evidence suggests that specialized functional thyroid activity continues in culture only as long as organized structure persists, and redifferentiation following dedifferentiation cannot be regarded as demonstrated.

The work of Goss (1932, 1933) has been cited (Bloom, 1937) as indicating that redifferentiation of cardiac muscle can take place after dedifferentiation in culture. Actually, Goss worked with primary explants of embryonic rat heart ventricle, under conditions designed to impose minimum disturbance on the explant. Myoblasts, always distinguishable from accompanying fibroblasts, migrated from the explant to form a characteristic interlacing network, and striations appeared in these myoblasts after some 25–60 days of culture. Contractions occurred before, during, and after the appearance of striations. Although mitosis was occasionally observed, never in cells with striation, it was not as abundant as in the fibroblasts, and growth of the muscle cells seems to have been quite limited. The experiments demonstrate, along with others on a number of tissue types, that under proper conditions of heterogeneous tissue culture cytodifferentiation can occur and be maintained. But there is no indication that cardiac muscle can redifferentiate from homogeneous undifferentiated cultures derived from originally differentiated cardiac muscle.

Toro (1933) combined culture and reimplantation into a lentectomized eye in a study of the differentiation of gut epithelium and mesenchyme from 3–7-day chick embryos. In the first three transfers the original explant was eliminated. After one week epithelium and mesenchyme were separately cultured. On recombination these gave organoid formations. Epithelium implanted alone into the eye failed to survive, but when combined with gut mesenchyme, the two differentiated into recog-

nizable gut with columnar epithelium, goblet cells, and hematopoietic islands but no enterochromaffin cells. Generally similar results were subsequently reported by Toro with implanted cultures of heart fibroblasts, mesonephric epithelium and mesenchyme, and lung epithelium and mesenchyme. The general conclusion was that cultured cells redifferentiate in their original direction, but that local tissue interaction and microenvironmental factors play an essential role. It is to be noted that the period of homogeneous culture of the epithelium and mesenchyme is not precisely stated, but appears to have been relatively short—a matter of days rather than weeks.

Kasahara (1935a, b) found that cultures of anterior pituitary of young rabbits gave rise to sheets of cells which were morphologically recognizable neither as chromophiles nor chromophobes. These would not redifferentiate on addition of fibroblasts or deletion of embryo juice from the medium. Addition of placental extract after strong growth-promotion led to the appearance of fairly typical looking eosinophilic cells in the central, and occasionally the intermediate, zone of the sheets, but never in the peripheral zone. The report does not indicate the length of culture prior to the test.

Jones *et al.* (1943) maintained human placenta in roller tube culture and periodically tested the supernatant medium for gonadotropic activity. The cultures were tested after they "no longer represented primary explants but were made up of several generations of new cells"—usually of one general type. Positive assays for gonadotropins were obtained during the earlier stages of culture (mostly less than three to four months) but "the cells tend to lose their ability to produce the hormone after they have been in culture over long periods." Waltz *et al.* (1954) recorded continued production of gonadotropins by hydatiform mole in heterogeneous culture for as long as 413 days. The tissue was maintained with minimal disturbance and proliferation generally was low.

Frequently cited in connection with the question of dedifferentiation of cells in tissue culture are a series of studies by Fischer and Parker which culminated in Parker's (1932, 1933a, b) extensive summary under the general title, "The races that constitute the group of common fibroblasts." Here it was demonstrated beyond doubt that strains of cells isolated from a single embryo and carried in homogeneous tissue culture, though they were morphologically indistinguishable, possessed stable differences in nutritional and functional characteristics. This was a highly significant finding. Beyond it, however, it was asserted that "these properties of the various strains were in some way related to conditions existing in the tissues of the organism prior to their isolation." If this assertion were true, it would indicate that difference in connective tissue at various

times and places within the organism (differentiation) are persistent over long periods in culture, and hence that dedifferentiation, affecting these characteristics at least, does not occur.

Particularly with the advantage of hindsight, there is considerable question whether the assertion is correct. Parker, himself, noted (1933b) that although a particular set of strains isolated from different regions of the same embryo, or from the same region at different stages, showed stable differences in culture," . . . it could not be predicted from one particular series how the strains of a second series would behave. . . ." Differences always occurred within a set, but the corresponding members of a set were not always the same—there was no assurance that "two cell strains would react identically to the same conditions, even when they were isolated simultaneously from closely adjacent parts of the same tissue or organ and cultivated together on the same medium." Finally, "*the two halves of a single culture could not be relied upon to display the same properties*" (italics supplied) until it had been rendered homogeneous by several passages. With what is known today of the changes occurring in homogeneous cultures (see below), particularly in their early stages, it must be regarded as quite uncertain that the undoubted stability of the culture lines is related to differentiation within the intact organism.

What may we conclude about dedifferentiation and redifferentiation from these data based on methods of heterogeneous and homogeneous tissue culture? First, that overt dedifferentiation of some tissues occurs and that it is favored by increased duration of culture and by conditions which promote homogeneity and lead to more direct exposure of the cells to the medium. Second, that certain dedifferentiative changes can be reversed, most readily when the culture interval has been comparatively short and the degree of disturbance minimal. The best-documented cases appear to involve storage products, e.g., pigment granules of the iris or secretion granules of the anterior pituitary. Redifferentiation may also have been demonstrated with osteogenic tissues, though here the literature is conflicting and, the criterion of differentiation being extracellular, it is not clear that the cells themselves were ever dedifferentiated. Further study of osteogenic tissue would be welcome. Third, with increasing time in homogeneous tissue culture the evidence for redifferentiation becomes progressively weaker. However, certain properties are recorded to persist for relatively long periods, e.g., the difference between epithelium and fibroblastlike cells. Overall, the evidence does not favor the idea that cultured tissues revert to a fully indifferent state in the developmental sense.

What information is obtained from the newer techniques of cell

culture—strains carried in fluid medium as proliferating populations with relatively little aggregation? These cells, like those in homogeneous tissue culture, have properties of considerable stability, but they are in general so different from those of their tissue of origin that identification becomes exceedingly difficult. The nature of long-term cultured strains of metazoan cells, with respect to differentiation and other properties, has become a paramount question with their increasing use in studies of nutrition, virus propagation, chemotherapy, etc.

Evans *et al.* (1952) called attention to this problem in connection with a strain of cells derived from mouse liver. After a six-month period of adaptation and relatively slow growth, the strain underwent a sudden increase of proliferation and, thereafter, could be carried by inoculation of flasks with cell suspensions. The cells existed in culture on the glass substrate in the form of individual flattened cells or sheets reminiscent of liver parenchyma, or as smaller elongate cells or rounded, granular and vacuolated cells. These morphological characteristics were essentially unaltered for three years after the initial period of adjustment. Cells of this strain (Westfall *et al.*, 1953) failed to inactivate estradiol, an activity displayed by liver slices from normal animals but not by slices from vitamin-E deficient animals. On the other hand, the strain proved to have a glycogen content twenty-five times higher than a strain of fibroblasts which had been in culture for ten years. Although there are uncertainties in the interpretation of these data, some of which the authors noted, they regard them as "compatible with the view that [the strain is] still liver epithelium."

On the other hand, with respect to the Chang strain of human liver, Perske *et al.* (1957) have recently reported enzymatic studies which lead to the conclusion that "these cells do not behave, metabolically, like hepatic parenchymal cells." Of four enzymatic activities characteristically present in normal liver (glucose-6-phosphate, fructose diphosphatase, hexosemonophosphate "shunt," and fructokinase), only one could be detected in the cultured cells.

These are preliminary efforts to apply chemical criteria to the identification of differentiated type in cell culture, and further data will be awaited with interest. Meanwhile, it may be noted that where the older literature of *tissue* culture emphasized, at least preponderantly, stability of type, the growing literature of *cell* culture emphasizes departure from properties *in vivo*, variation, and the difficulty of establishing differentiated cell type. Included in the variation are morphological and behavioral characteristics, alteration in virus susceptibility, transplantability, and malignancy, as well as a tendency toward convergence of a number of properties studied. Berman and Stulberg (1956) and Berman

et al. (1957) report that eight epithelial-like strains derived from cancerous and noncancerous sources took on similar appearance in culture and all possessed features suggestive of malignancy. Leighton *et al.* (1957) report similar experience when a number of strains were grown in sponge-matrix culture, a technique regarded as favorable to differentiation. The authors suggest that properties of established strains "may bear little relationship to their tissue origin." Eagle and Foley (1956) found little difference in nutritional requirements, and response to chemotherapeutic agents, among several strains of "normal" and "malignant" cells from different sources.

Further, Sanford *et al.* (1954) isolated stable strains with differing transplantation characteristics from a clone culture derived from a single cell. And Puck and Fisher (1956) similarly derived two strains differing in nutritional characteristics in ways which recall the results of Parker (1933b) referred to above. The problems raised by increasingly frequent observations of this kind were the subject of extensive discussion at the Decennial Review Conference on Tissue Culture in 1956. Willmer (1957) emphasized that cells isolated as clones "almost certainly change their character and become very different from what they were in the body." Parker (1957) referred to the fact that "cell alterations (in long-term culture strains) have been observed over the past several years with almost frightening frequency." He briefly summarized his and other observations of discontinuous alteration, or transformation, of cultured cells extending back more than twenty-five years, and described a recent case occurring in progeny of a single cell isolated from cultures of unaltered monkey kidney epithelium. The altered cells are characterized by their considerably heightened mitotic activity and their ability to produce uniform populations which can be propagated indefinitely.

Harris (1957) emphasized the possible role of proteins in stabilizing cell properties, describing experiments with freshly isolated chick embryo cells grown on glass. These cultures developed giant cells and other variants most conspicuously in media containing heterologous serum, less so with homologous serum. When the medium was supplemented with a protein fraction from adult chicken spleen the populations were rendered exceedingly uniform and could be propagated this way apparently indefinitely. Harris suggested relationships among these observations, tracer results *in vivo* suggesting a dynamic equilibrium between intracellular and plasma proteins, and results *in vitro* implying possible direct passage of proteins from medium into cell.

Swim (1957) and Haff and Swim (1957) noted that "cultures frequently acquired the ability to proliferate in media which initially appeared to be inadequate," recalling the situation in microorganisms.

Dunnebacke Dixon (1957) reported that cultures of amnion changed from normal epithelial type to much enlarged fibroblastlike cells between the 5th and 9th passage. Accompanying the change was an altered cytopathic reaction to polio virus, the altered amnion cells behaving like stabilized strains of HeLa and monkey kidney (see Scherer, 1955, for related observations on HeLa cells). Chevremont (1957) pointed out that the promoting action of choline in transforming fibroblasts and myoblasts into macrophages is exerted on freshly explanted cells, but not after these have been transplanted four or five times. Hull (1957) added that two simian viruses were cultivable in primary explants of monkey kidney cells, but not in a stable long-term strain (Sheffield and Churcher, 1957, report growth of polio virus in long-term cell strains from rabbit embryos but not in original explants). Related recent indications of fairly rapid changes in freshly explanted tissues are the reports of Drew (1957) of transformation of fibroblast to epithelial-like cells in the presence of embryo juice, of Billen (1957) that bone marrow cells maintained *in vitro* and injected into mice will protect against radiation damage only at culture intervals of less than 21 days, and of Guillemin and Rosenberg (1955) that anterior pituitary production of ACTH continues in explants for only 4 days, except where hypothalamus is included in the explant. Longer-term changes were emphasized by Gey (1957) and Earle (1957), who remarked on the frequency with which cultural strains have become malignant and the continuing variations they show even beyond this point.

On the basis of all information on differentiative behavior in culture, both the older and the newer, what is the best answer to be given to the question earlier raised, i.e., does a particular differentiated cell revert, in whole or in part, to its status prior to differentiation? Clearly some cell types, the terminal ones, do not and presumably cannot. Equally clearly, certain tissues lose overt signs of differentiation and, after periods of varying duration, can recover them under conditions which promote tissue and higher levels of organization. But reliable information is too restricted, and relates to too few properties of too few tissue types, to allow any more detailed a generalization—particularly with respect to duration of reversibility and the precise role of culture conditions.

With respect to cells cultured by methods approaching or attaining true cell culture, it is evident that the cells are exceedingly difficult to identify as to type by criteria used *in vivo*. There is little evidence to suggest, however, that these cells have reverted to embryonic cells along an uncomplicated dedifferentiative pathway. Rather, decline of specialized characteristics is accompanied—whether coincidentally or causally

is not clear—by variation which is reminiscent of microorganisms and may introduce “something entirely new” (Demuth, 1932). There is urgent need for careful studies, beginning at explantation and with frequent return to the intact organism, of the differentiative status of various cell types under various conditions of culture. Until results of such studies are available, the extent and nature of dedifferentiative changes possible to various cell types, and the relation of these to other changes observed, cannot be generalized and must be regarded as an open question. Meanwhile, the at least superficial similarities between the course of events in long-term cultured cells and in tumorigenesis (Foulds, 1954) is provocative.

2. *Metaplasia*

Unexpected types of differentiation occurring in tissues of otherwise expected type have been referred to as metaplasia, a term clearly implying that transformation has occurred from the expected type to the unexpected. It seems useful to generalize the term to include all cases of transformation from one defined differentiated type to another. Examples abound in the literature of pathology (Willis, 1953), e.g., the appearance of bone in old inflammatory foci or in adenocarcinomas of the stomach or prostate, the occurrence of striated muscle in mixed tumors of the endometrium, the finding of sebaceous glands and hairs in the wall of the vagina and cervix. Willis (1953, p. 642) comments, on the basis of these and other examples that “students of normal histology have been too apt to assume that the different kinds of tissues and cells in the adult are permanently determined invariable structures, each a distinct immutable species capable of producing by proliferation cells of its own kind only. A study of pathological histology, i.e., of what the various cells can be and do in all manner of abnormal environments, soon corrects this error and shows that great transformations of cellular structure—metaplasias—are possible in most tissues.”

Intensive study of metaplasia unquestionably has been delayed by the deterministic assumption Willis refers to, and by the corollary inference that pathological metaplasia has some relatively superficial explanation. The easiest is that it does not truly represent transformation, but only an indication that undifferentiated cells persist and differentiate atypically in pathological states. The “why” of the atypical differentiation, however, would nonetheless be important. Or, it may be explained on the assumption that metaplasia does not represent changes of stable differentiated states, but evanescent reactions to altered environment—modulations. This, however, cannot be assumed without experimental demonstration. The fact is that, in an increasing number of carefully

studied cases, transformations resembling pathological metaplasia have been described, both within the organism and in tissue culture.

In considering such cases we must distinguish between *tissue* metaplasia (heteroplasia, Motyloff, 1950) and *cellular* metaplasia. The skin and its appendages represent an organized cellular population with a germinal component capable of giving rise to any of a number of specialized cell types (Montagna, 1956). If transformation of type takes place by sloughing of all cells of the old type, and their replacement from the germinal epithelium by cells of a new type, a tissue transformation has occurred. But no particular cell of the old type has transformed into a cell of the new type; no cellular metaplasia has occurred. The former seems to be the case in the vitamin-A induced transformation of embryo chick skin reported by Fell and Mellanby (1953). No transformation of cornifying cells to mucous-secreting, or vice versa, is recorded—when the vitamin level is changed, the old type is replaced by the new from the germinal epithelium. The plasticity of the population lies in its possession of ambivalent cells, not in plasticity of the fully differentiated cells themselves. This, then, is a possible model for pathological metaplasia which does no violence to the assumption of completely stable cell types.

There is, however, recorded evidence for cellular metaplasia, cases in which cells hitherto generally accepted as one differentiated type are said to lose their overt characteristics and assume those of another type. These involve cells both of epithelial and mesenchymal derivation. In the former category is the regeneration of sensory retina from the pigmented epithelium or tapetum of adult salamander eyes, described in detail by Stone (1950a, b). This can be demonstrated following degeneration of the original retina incident to transplantation of the whole eye, after surgical excision of all or part of the retina, or upon transplantation of retina-free wall of one eye into another. In all cases, soon after the pigmented cells have lost association with the neural retina they change in morphology and begin to divide. The more pigmented cells remain in the peripheral layer, the less pigmented ones form an inner membrane of clear cells which thickens through continued proliferation. Later, differentiation sets in and the characteristic sensory and ganglionic layers of the retina are reconstituted. Thus, the progeny of pigment-forming cells lose this characteristic property and become the highly differentiated neural cells of the retina.

In similar fashion, the iris of certain urodeles regenerates a new lens after removal of the original (Reyer, 1954). Here, pigmented cells of the iris—usually at the dorsal margin of the pupil but elsewhere under suitable conditions—depigment, undergo proliferation to form a spherical

mass, and transform into lens cells and ultimately into lens fibers. The exact mechanism of initiation of the process is not clear, but the evidence suggests that the amount of some factor present in the intraocular environment is controlling. The lens almost certainly affects the level of this factor, and the retina may play a role as well. The two appear to operate in opposite directions, the lens inhibiting and the retina promoting the transformation process.

The depigmentation which precedes both lens and retinal regeneration recalls the behavior of iris cells in culture, and the accumulation of depigmented cells prior to differentiation can be likened to blastema formation in limb regeneration. In all cases it appears that on removal of some controlling factor, reorganization with accompanying overt dedifferentiation occurs. In the culture case the cells repigment and redifferentiate when nutritional conditions are somehow limiting; in limb regeneration it is not clear whether they simply redifferentiate or undergo metaplasia. In retinal regeneration the transformation replaces normally intimately associated cell types even in the presence of these elsewhere in the eye; in lens regeneration the transformation is to a cell type not normally intimately associated, and it is inhibited by lens present elsewhere in the eye chamber. Pigmented retina can regenerate both iris and lens (Stone, 1955).

There is evidence that transformations comparable to these in urodeles may occur in special circumstances in higher vertebrate tissues. Formation of pigmented tapetum from retina, and vice versa, have been described in grafted or cultured embryonic chick eyes (Dorris, 1938). Some years ago Toro (1931) reported that cultures of chick or rat embryonic iris, on transplantation into a lentectomized eye, would produce lens. The claim recalls the earlier experiments of Fischer (1924), who described cornification of iris epithelium in culture and illustrated it with a figure which strongly suggests lentoids. An attempt to confirm the phenomenon was unsuccessful, however, Toro's results being ascribed to failure to remove completely the original lens epithelium (Zalokar, 1955). Very recently, Moscona (1957b) has described formation of lentoids by dissociated and reaggregated embryonic retinal cells of the chick *in vitro*. The reaggregating retinal cells first formed rosettes or cell clusters. If these were cultured under standard conditions, they formed sensory tissue. But if they were heaped together and "overcrowded" for several days, each rosette transformed into a lentoid. This occurred in the presence of many cytolized retinal cells. As expected from earlier results, lens fibers were not formed in culture, and reimplantation was not attempted. It is not clear, therefore, whether fully typical lens was produced. Moscona suggests that the modified pattern

of reorganization following disaggregation, and the presence of cytolyzed cells, may be prerequisite to the transformation.

It is to be recalled that three of the four tissue types under discussion—neural retina, iris, and pigmented retina—are derivatives of the optic vesicle. Lens ordinarily arises from superficial ectoderm under the influence of optic vesicle, and the superficial ectoderm and optic vesicle share a common derivation in gastrular ectoderm. Transformations among them, striking though they are, are within a “developmental family.” Moreover, the source of transformation in each case is a relatively simple epithelium, iris, pigmented retina, and superficial ectoderm. It is interesting to note that if overt pigment formation is regarded as something different from, or something less than, most other differentiations—akin to secretion as suggested by Fischer (1938)—then the several transformations described simply constitute renewed differentiation of a germinal layer represented by the iris and pigmented retina. The mechanism of renewal, and the factors that control its course, are of exceptional interest. Intensive experimental analysis of the favorable material provided by these transformations should do much to clarify our conceptions of cytodifferentiation.

A bewildering number of transformations among mesenchymally derived cell types also have been described, both in the animal (Willis, 1953) and *in vitro* (Bloom, 1937). These are not, by any means, to be regarded as all metaplasia of stable types—it seems clear that many represent transitory states of the same cell type in differing environments. But the appearance of bone in rabbit muscle (Levander, 1938), whatever the mechanism of induction, clearly is at least tissue metaplasia. And the origin of macrophages from muscle elements (Chevremont, 1955) is cellular metaplasia in the phenomenological sense in which the term is used here. Indeed, the macrophage transformation reportedly is not limited to the mesenchymal family, occurring as well in epithelial (Chevremont, 1955) and Schwann cells (Weiss, 1944; Weiss and Wang, 1945). Nor is this surprising when it is recalled that mesenchyme normally transforms to epithelium in the kidney, and the Schwann cells are derived from neural crest, which also gives rise to ganglionic cells, pigment cells and cartilage (Horstadius, 1950). Even in postfetal stages and in the adult there are reports of interconversion between epithelial and mesenchymally derived tissues (Politzer, 1955; McCreight and Andrew, 1956).

Available facts on cellular metaplasia, therefore, indicate that transformation between certain classically acceptable cell types certainly occurs, and probably is not at all uncommon. In general, these transformations are either between relatively unspecialized cell types, or from

relatively unspecialized to specialized. There appear to be no well-authenticated cases of direct transformation of one highly specialized cell type into another.

3. Propagability

Two main questions concern us in the relation between cellular propagation and differentiation. Does the differentiative state of a cell affect its capacity to divide? Under what conditions, and for how many generations, does a dividing cell transmit its differentiated properties to its offspring?

There has long been an impression that differentiation and propagation are reciprocal in some degree, that increase of one implies decrease of the other (Weiss, 1939; Willmer, 1954). Confining ourselves to cyto-differentiation and omitting the complexities of tissue behavior, it is clear that the overtly more specialized cell types *in vivo* either show no mitotic activity or a very low rate (fixed postmitotic cells of Cowdry). Mitoses are very rare in brain and spinal cord, cardiac and striated muscle, and in free blood cells. In continuously proliferating tissues mitotic activity is usually confined to a zone of lesser differentiation. Thorell (1953) finds that most formation of hemoglobin occurs in the later stages of erythropoiesis, when growth and division of the erythroblasts has been completed. In the skin division occurs in the basal layer, which includes relatively undifferentiated germinal cells capable of forming epidermis, hair follicles, sebaceous cells, etc. (Montagna, 1956). Mitotic activity is rare above the basal layer in the epidermis, in the secretory cells of sweat glands, above the matrix in a hair follicle, and at the centers of sebaceous glands. In the hair follicle the cells which concentrate radio-cystine and synthesize keratin lie well above the zone of active proliferation, and there appears to be no specific uptake of cystine by the basal cells of the keratinizing, vaginal epithelium (Bern *et al.*, 1955b). In human skin, stripped of its cornified layer and cultured under conditions which inhibit proliferation, Matoltsy and Sinesi (1957) find no sulfhydryl or disulfide groups in the basal layer which "either lack or are not yet capable of developing a keratin-producing system." Sulfhydryl groups, indicating unstabilized keratin, first appear in the suprabasal layer and are converted to disulfide groups of the oriented keratin molecules in the distal layer. The impression in all of these sites is that in a particular cell mitotic activity ceases with, or close to, the beginning of differentiation. On the other hand, renewed mitotic activity, as seen in wound healing, regeneration, or tumorigenesis, frequently accompanies, or is the sequel to, overt dedifferentiation.

The case of mammalian liver, however, cautions against too simple

assumptions. Here, mitotic activity normally is quite low but increases markedly in regeneration following partial hepatectomy. Do liver parenchyma cells dedifferentiate to some degree before undergoing mitosis? Or is their type of differentiation, presumably more marked chemically than morphologically, not such as to preclude division? Much more information is needed on the precise relationship between specific differentiative properties and mitotic activity. For the moment, we cannot generalize beyond saying that differentiations of certain kinds, in unknown ways, definitely reduce the probability of mitotic division—in some cases to the vanishing point.

This statement provides part of the answer to the second question. To the extent that differentiation precludes cell division, it cannot be indefinitely propagable. But what of cells which can continue to divide? Here we return to the data which already have been considered with reference to stability of differentiated cell type. It was noted, on the basis of available *tissue* culture data, that although overt dedifferentiation of proliferating tissues is the general rule, redifferentiation has been demonstrated for a limited number of tissues under favorable conditions. The most reliable cases involve generalized epithelial behavior, pigmentation of the iris, and bone formation. But redifferentiation is demonstrated most readily and typically when tissues are cultured for relatively short periods with a minimum of disturbance and reorganization, and in none of the experiments recorded were data obtained on the number of generations passed through by particular cells before redifferentiation.

For the questions at issue, therefore, these demonstrations of a modicum of tissue stability are unclear in two respects. They do not tell us through how many divisions various differentiated properties may persist. And they, in general, do not distinguish between system stability set up in an interacting mass of cells and intercellular materials, and cell stability resulting from determinants propagating within the cells. To obtain information on these matters tests must be made on cell cultures rather than tissue cultures, with close observation of generation time and with frequent cloning to ensure that the properties under study are actually transmitted through individual cells. Information of this sort is just beginning to become available (e.g., Puck *et al.*, 1957), and preliminary data suggest only a limited persistence of original differentiative properties, and significant variability of a genotypic kind. The safe conclusion at the moment is that stability of differentiation while propagating within a multicellular, and usually heterogeneous, system, is established. Whether, how long, and for what properties stability persists in isolation from other cells—and hence the degree to which differentiated

properties are transmitted through cell generations by genetic or quasi genetic mechanisms—must be regarded as an open question.

IV. THEORETICAL CONSIDERATIONS

Perhaps the most important conclusion to be drawn from the data which have been reviewed is the negative one reached in the preceding section. What we know of vertebrate cytodifferentiation does not *require* a general assumption of heritable propagation at the cell level. Available information indicates that certain types of differentiation pass the point of reversibility and that interconvertibility of highly differentiated types is, at best, difficult and rare. But since highly differentiated cells show little or no tendency to divide, their irreversibility establishes nothing about propagability. Differentiated cells which do divide, and whose descendants differentiate in the same path as the parental cells, have not been shown to do so under conditions which ensure that transmission is through a *cellular* lineage, comparable to what is observed in micro-organisms. To show this requires the newer techniques of cell culture, with frequent cloning or other assurance of the absence of population effects, and the unequivocal recovery of differentiated type after a known number of cell generations. Until this is accomplished, a general assumption that differentiative properties are transmitted through intrinsic genetic mechanisms is not required.

On the other hand, the data suggest strongly that important aspects of cytodifferentiation are extrinsically dependent in their initiation and progression, and even in their maintenance. Overt dedifferentiation accompanies tissue disaggregation in regeneration and in culture. Though the evidence does not entitle us to regard this as simple return to a more embryonic state, it does not entitle us, either, to regard the phenomenon as superficial and of minor significance. In many instances, most or all of what we can recognize as differentiation disappears, and the impression is that the greater the dissociation and the longer it is maintained, the less likely the cells are to show or subsequently recover overt differentiated characteristics. The persistence of covert differentiation under these circumstances has been demonstrated no more convincingly than its absence—the status of differentiation of long-term suspension cultures is a question which urgently needs answering. But it seems clear that recognizable cytodifferentiation is most easily attained in cell aggregates, and that cells propagating as individuals undergo changes which prohibit easy assumption that they can again differentiate.

Since the data do not require the hypothesis that cellular heredity

is the major mechanism of cytodifferentiation, the theoretical problem is both unnecessarily complicated and viewed too narrowly, when thinking is limited to genetic models. The hereditary system revealed by genetic analysis certainly must be related to differentiative processes, but it need not be forced to provide the basis for either the origin or the stabilization of cytodifferentiation. It may be noted that though cytodifferentiation is part of the broad spectrum of cellular variation, it differs from the classical genetic region in important respects. It is continuous rather than discontinuous change; the change is of appreciable duration; it is predictable on individual rather than a statistical basis, and—above all—it is importantly controlled by and, at the moment, inseparable in important aspects from the cellular environment.

An alternative to the assumption of intrinsic stabilization of differentiative properties via genetic determinants lies in the concept of group or system stability. In these terms stability of differentiated cell type is a function not only of cell properties, but of system properties as they reflect in the microenvironment supplied to the cell. This microenvironment involves both other cells (Weiss, 1947, 1950, 1953) and the intercellular materials (Weiss, 1933; Huzella, 1941). Since the intercellular materials are subject to local cellular as well as general systemic influence, and in turn control the cells both as substrate and by influencing exchange, they are an important element in cellular systems. To the extent that a common and relatively stable microenvironment is provided within a cellular aggregate or group, new members arising by division will continue their type. Stability of type, in these terms, may be expected to depend upon the existence of a cellular mass sufficient to establish a stable microenvironment set off from the general environment—which is in accord with the minimum mass requirement referred to above. When the mass becomes too large to allow maintenance of a common microenvironment throughout (see Weiss, 1953), diversification will occur. When two cell masses of differing properties come into intimate association, their interaction may lead to alterations in the microenvironments of both and new differentiative behavior will result (Grobstein, 1954).

It is to be noted that the stabilization provided by the group conceivably may extend beyond the properties which we regard as immediately differentiative. The variations which have been recognized in increasing number in long-term cell cultures, and which have not been so obvious in the organism or in earlier forms of culture, have resemblances to microbial genetic variation (cf. Puck, 1957). It is too early to say whether variation of this kind is enhanced under culture conditions,

or whether variants only have better opportunities for survival. If the former, the group environment—or some element in it—must be conceived as antimutagenic for individual cells. But, even if only the latter, the group would still stabilize its own population properties by not allowing individual variants to proliferate and become a significant proportion of the total. The group, in this sense, would be a genetic stabilizer as well as a developmental one. It seems fairly clear that, in a long culture period permitting genetic variation, changes might occur which would preclude not only differentiation, but reaggregation (cf. "aggregateless" slime molds, Sussman and Sussman, 1956); the cells might "deteriorate" genetically from their metazoan toward a microbial state. In this event, duration of the culture period would be critical if cells were to be found which had dedifferentiated developmentally, but were not so altered genetically that redifferentiation was still possible. It is also clear that to the extent that the rate of developmental change is lower than the genetic one, overtly dedifferentiated cultured cells may be found to be "something different" from their origin and not able to re-establish their properties as member of a cellular community.

The alternatives of group and intrinsic stability, of course, are not exclusive. The first may be more important in some instances, the second in others. Or, what seems quite likely, stabilization may begin by group mechanisms but be increasingly supplemented by intrinsic stability of individual cells. When individual cell stability appears it may or may not affect the ability to divide (this is an open empirical question), and it may or may not be transmissible. Rigorous data are needed for individual instances before generalization will be meaningful. Only for those instances of unequivocally demonstrated transmissibility through a significant number of cell generations in the absence of group effects, do we require a genetic explanation. In the case of pigment cells, the evidence (I. Fischer, 1938) comes closest to indicating such propagability. It is lacking in finality, however, because the cells were carried as a tissue and data are not available on number of cell generations. But, assuming propagability, there is evidence suggesting that group factors ("crowding") also are important (Foster *et al.*, 1956), that pigmentation may be induced by a metabolic substrate (Wilde, 1955), and that depigmentation can be followed by alternative differentiation (Stone, 1950b, 1955). Clearly, this is a case for further analysis, though with the caution that its characteristics may not prove to be general ones.

The distinction between intrinsic and group stability, it should be noted, is not identical with, though it may be found to be closely related to, that drawn by Weiss between modulation and differentiation. Re-

gardless whether the stabilization is of the cell or of the group, it is conceived as persisting beyond any initiating stimulus which may act from outside the group (e.g., a hormone). But it is true that in a completely group-stabilized system individual cells would show characteristic properties only so long as they are in the specialized microenvironment of the group, and would generalize in properties as soon as they separate from it. This is what is frequently seen overtly in the behavior of cultured cells. To the extent that this is true, individual cell behavior is modulatory *with respect to the microenvironment* within the group. In this language our present need is for more information on the relative contribution of what might be called modulatory stabilization and intrinsic stabilization in the differentiation of various cell types—particularly those still capable of propagation.

These considerations are very general, and they emphasize background rather than the details of specific differentiative mechanisms. The dearth of rigorous data relating to mechanism recently has encouraged an abundance of ingenious theoretical models, mainly based on the genetics of microorganisms. The latter, summarized by Ephrussi (1956), will be helpful for those instances in which cytodifferentiative properties prove to be transmissible. Weiss (1953) has emphasized particularly, in his concept of molecular ecology, the importance of the cell periphery in cytodifferentiation and the intimate causal relation between the properties of periphery and interior on the one hand, and periphery and microenvironment on the other. His model has the heuristic advantage of directing attention to the relation between cell and microenvironment and to the importance of macromolecular interactions in these relations—an emphasis also placed by Schmitt (1956) and Tyler (1947).

There is a general consensus that the mechanisms of differentiation must be closely tied to protein synthesis, and the relationship of ribose nucleic acids to such synthesis implicates these materials as well. It has been suggested that differentiation may be induced by direct transfer of ribose nucleoproteins from cell to cell on a viroid model (Brachet, 1949; Medawar, 1947), or by presentation of metabolic substrates on the enzyme induction model afforded by microorganisms (Spiegelman, 1948; Monod, 1947, 1956). The latter is rendered particularly attractive by findings of "memory," i.e., residual enzyme synthesis requiring a number of generations to dissipate following withdrawal of substrate (Cohn, 1956). Combined with sequential induction (Stanier, 1954) such mechanisms conceivably could lead to complex, and irreversible, changes of the sort seen in some cytodifferentiations—and without involving genetic replication. On the other hand, it must be kept in mind that the microbial

data relate to small-molecular substrates, which do not appear generally to be significant in controlling cytodifferentiation as seen in developmental induction (Grobstein, 1956).

In this connection, it is worth repeating that though comparisons between microorganisms and metazoan cells are stimulating, and sometimes illuminating, they must be made guardedly—particularly with respect to differentiation. It is clear that intrinsic and extrinsic determinants must interplay in both, but that the relative importance of the two may well be different. A free-living organism in a variable environment places emphasis on interiorized controls if it is to preserve its properties, and it must not sacrifice the capacity for division. The accent is on insularity, and the periphery accordingly will be sharply bounding and relatively insensitive and stable. If specialization is to become irreversible it must be reproducible. The organization and behavior of the ciliate cortex, with its propagating infraciliature, conforms to these requirements.

Differentiation of a metazoan cell, however, is subject to very different requirements. Here the premium is on exterior controls, since independent behavior threatens the integrity of the whole organism. The environment is highly complex and homeostatically stable, and is characterized by the presence of large molecules whose marked specificities much enhance the possible delicacy of control. In the metazoan cell, particularly early in development, the accent is on plasticity and close integration of the periphery with the microenvironment. Indeed, the question frequently has been raised whether there is a true boundary at all. Thus, differentiation of metazoan cells and microorganisms both represent specialization of fundamental cell organization. But since one specializes the cell to behave as a whole organism, and the other as a part, they well may proceed by entirely dissimilar mechanisms.

Returning to the issues raised in the introduction, we may state the assumptions of fact which appear to be warranted by the evidence reviewed. (1) There are important similarities in the several instances of cytodifferentiation which justify considering them as a group. There are, however, important differences as well, and the nature of these is not yet clear enough to assume a single generally applicable mechanism of cytodifferentiation. (2) A number of cytodifferentiations clearly are irreversible. Others are reversible, as judged by overt criteria. Reversion to an earlier embryonic state has not been demonstrated, but has not been excluded. (3) States of differentiative bias can exist in the absence of other currently detectable signs, but evidence is lacking that this relates to properties of individual cells rather than to those of groups. Cryptic intrinsic determination of cytodifferentiated states has not been demonstrated. (4) Typical differentiated states so far appear to be exclu-

sive; intermediate or mosaic differentiations have not been demonstrated within vertebrate cells. However, all properties of a particular differentiated type are not concordant, either in genesis or in the special circumstances of tissue culture and tumorigenesis. Differentiated states, therefore, may not be integral. Interconvertibility between highly differentiated states has not been demonstrated, but interconvertibility between lesser differentiated states has. (5) Differentiated states have not been demonstrated unequivocally to be transmitted through cell generations in the absence of possible group stabilization. Cellular heredity, therefore, need not be invoked as a general characteristic, or explanation, of cytodifferentiation. (6) The nucleus may be significantly altered, at least in certain differentiative processes (see Chapter 13).

Clearly, this is a far less certain statement of facts than given in the introduction. Clearly, too, it indicates broad gaps in our empirical knowledge which must be filled before theoretical generalization can be profitable. With the more powerful and precise methods now available, it should be possible to establish the beginnings of identifiable materials or processes associated with particular differentiations, to determine their relation to material or processes earlier present, and to sort out the conditions—both intrinsic and extrinsic—under which the new materials or processes appear, increase, and are stabilized. It is to be expected that such studies will lead inward, through the mechanism of synthesis and interaction of large molecules, to the replicative mechanisms of the cell; and outward, through the intricacies of cell and microenvironmental interaction, to the homeostatic mechanisms of the organism. The resulting theory of cytodifferentiation will then portray the changing properties of the cell—and their increasing stabilization—as the progressive, functional adaptation of its conservative heredity to a communally controlled and changing local environment.

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CHAPTER 12

Patterns of Cell Growth and Differentiation in Plants

By RALPH O. ERICKSON

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I. INTRODUCTION

Perhaps the most arresting feature of cellular structure in most plants is the more or less rigid wall of cellulose and other materials which surrounds each cell. The cell walls usually stand out clearly in a thin section, even when unstained, and their differential reactions to stains such as safranine and light green are familiar. It was, of course, the walls of a slice of cork which led Robert Hooke to refer to cells, and it was the delicate and regular pattern of the secondary wall thickenings of wood which inspired Nehemiah Grew's beautiful woodcuts and engaging descriptions. Even today, the greater part of our knowledge of (microscopic) plant anatomy has to do with the walls of cells. The criteria

which are used to distinguish the principal cell types in the higher plants: parenchyma, collenchyma, sclereids, fibers, tracheary elements, sieve cells, etc., are primarily wall criteria (Esau, 1953). Parenchyma cells are defined as more or less isodiametric and thin walled, and collenchyma cells as having thick walls of cellulose which is laid down in characteristic patterns. Sclereids, which are roughly isodiametric, and fibers, which are elongate, have very thick walls in which the cellulose is impregnated with lignin. The tracheary elements, which constitute the xylem, are highly diverse, and, since these water-conducting cells are devoid of protoplasm when mature, it is a truism to say that they are characterized by features of the wall. In annular, spiral, or scalariform tracheids conspicuous ribs of lignified cellulose are laid down on the inner surface of the wall in rings, spirals, or in ladderlike bars. Other tracheids, and vessel elements, can be characterized in a most detailed way by the nature of their pits, or perforations through an otherwise uniformly thick secondary wall of lignified cellulose. The sieve-tube elements of the phloem, which are responsible for transport of organic materials, are perhaps primarily characterized, and distinguished from their companion cells, by the peculiar sieve plates of their end walls, though there has been a good deal of study and discussion of the nature of the cytoplasm and slime contained in these cells as well, and of the developmental origin of this material from the original protoplasmic contents of the cell (Esau, 1953). It may be noted that comparative studies of the detailed structure of xylem (Bailey, 1954) and phloem (Esau, 1950) cells in the angiosperms have yielded information of considerable interest for angiosperm phylogeny. In a more general sense, the comparative anatomy of xylem (and to a lesser extent of the phloem) has become increasingly important in understanding phylogenetic relationships in all groups of vascular plants.

In addition to its thickness, chemical composition, and sculpturing, the rigid wall gives the plant cell a well-defined shape, and cell shapes have been a subject of some geometric investigation. Haberlandt (1914) made his primary subdivision of cell types on this basis, distinguishing parenchymatous, more or less isodiametric, cells, from prosenchymatous, or markedly elongated, cells. In thin sections of parenchymatous tissue the cell walls frequently form a fairly regular pattern of polygons, and in three dimensions they appear as polyhedra. If one considers the mathematical question of subdividing a plane surface into contiguous regular polygons, one finds that this can be done most economically with regular hexagons. Similarly it was shown by Kelvin (1887) that space can be subdivided into contiguous equal volumes by orthic 14-hedrons, with the greatest economy of partitioning material. The orthic 14-hedron

is not one of the five regular polyhedrons, but has 6 square and 8 hexagonal faces. Many authors have observed that parenchymatous cells are roughly hexagonal as they appear in thin sections. There have been a considerable number of studies of the three-dimensional shape of parenchymatous cells (e.g., Marvin, 1939; Macior and Matzke, 1951) from which it appears that Kelvin's 14-hedron is not closely approximated, but that parenchyma cells have various numbers of faces, ranging from about 9 to 20. However, the fact that the average number of faces is very near to 14, and comparisons with compressed lead shot (Marvin, 1939) and soap bubbles in foam (Matzke, 1946; Dodd, 1955) can perhaps be taken to indicate that packing or surface forces play a significant role in determining these shapes. There are, however, a great variety of plant cells which are not even approximately similar to the orthic 14-hedron and which have scarcely been studied from this geometric point of view.

Many other examples could be cited to illustrate the prominent place which the cell wall occupies in plant morphology. It should not be thought, however, that there is nothing more to a plant cell than its wall. The wall is, of course, laid down by the cytoplasm on its inner surface, and the subject of the development and fine structure of the wall is a subject discussed in Volume II. Our considerable knowledge of the cell nucleus and its chromosomes and of plastid structure and development is discussed in Volume II.

Just as descriptive plant anatomy is largely concerned with wall arrangements and characteristics, so our present knowledge of plant development has largely to do with the wall, with planes of division, and with cell lineages as inferred from these planes of division. To illustrate this point, I shall discuss a limited number of examples of patterns of apical development of the shoot and the root. In some cases the geometric pattern in which new cell walls are laid down, that is to say the cell lineage, is almost diagrammatically clear; in other cases, the interpretation to be put on the cell pattern seen in sections is not obvious and is the subject of current controversy. It is not my purpose to present a comprehensive review of developmental patterns. In the case of apical development, the books of Esau (1953), Foster (1949), Wardlaw (1952a, b), and Schüepp (1926) are more inclusive than the present chapter, and they also include other topics, such as the marginal development of a leaf, and cambial activity, which I shall omit. In the development of plant embryos, minute study has been devoted to patterns of cell division, and this subject has been treated by Wardlaw (1955), Maheshwari (1950), and Johansen (1950). Cellular developmental patterns, such as those involved in the formation of archegonia,

antheridia, sporangia, and in the germination of spores of the bryophytes, and pteridophytes in the broad sense, are frequently quite striking, as are a number of cases in the algae and fungi.

There are, of course, well-defined developmental patterns in coenocytic organisms or tissues which lack cross-walls, as in the coenocytic algae and fungi, the megagametophyte of the heterosporous ferns and lycopods and of the gymnosperms, and in the embryo sac and endosperm of the angiosperms. Frequently cross-walls appear after an initial coenocytic stage. Such patterns will not be discussed here.

The prominence of the cell wall in plant tissues is in some ways a disadvantage, and in other ways an advantage, to the student of plant development. The fact that the cells are encased in cellulose and are effectively cemented together appears to render them inaccessible to the grafting and transplant techniques which have been so powerful in the experimental embryology of various animals. Very little information is available on this subject, but it would appear that the injury done to the cells by cutting through the wall is the obstacle. The grafting of shoots, which is practiced in horticulture, is not a simple phenomenon but involves the formation of callus tissue, as in the healing of a wound, and the subsequent formation of vascular connections through the callus (Sharples and Gunnery, 1933). Recently techniques for dissociating cells of plant organs and tissue cultures have been described, but it appears to be too early to assess their importance in the study of developmental problems.

The advantage which the cell wall offers to the student of development is the very fact that cellular patterns can be interpreted in developmental terms with greater or lesser precision. Coupled with the relative rigidity of the cell walls, is the usual lack of anything like the cell migrations and rearrangements which are so characteristic of vertebrate development. There are, of course, conspicuous changes in size and form of plant organs in their development. In the nineteenth century several botanists assumed that sliding growth was a factor in these changes, that is that the wall or a part of the wall of one cell literally slides over the wall of an adjacent cell during development. Perhaps the strongest argument against this notion is the existence of pits. A pit is a perforation through the secondary wall, and is typically opposite a pit in the wall of an adjacent cell, the two forming a pit-pair. The fact that the pits in one cell are regularly found to be in register with those of the adjacent cell in immature as well as mature tissue is certainly not consistent with the shearing which would occur in sliding growth. Intrusive growth, in which typically the growing end of a pointed cell thrusts its way between the walls of two adjacent cells, however, is

well documented (see references in Esau, 1953), though its mechanism does not seem to be completely understood. Intrusive growth, on the other hand, does not lead to great changes in the spatial relationships between cells, as sliding growth presumably would.

In short, the nature of plant cells makes for some degree of maintenance of their relative spatial relationships during growth and development. In a sense, a plant leaves a record of its development in the geometric pattern of its cells and tissues. I believe that a proper interpretation of this record on the basis of appropriate morphological and growth data can add much to a developmental study. Except in the most obvious cases, I feel that botanists have not yet taken full advantage of this circumstance.

II. SHOOT DEVELOPMENT FROM A SINGLE APICAL CELL

A. *Nitella* and *Chara*

In a number of plants, the origin of all of the tissues of the shoot or root can be traced back to a single apical cell. One of the simplest of these, which can be said to have a shoot, is *Nitella*. *Nitella* was at one time considered to be a green alga, but its peculiarities of vegetative structure and reproduction are such that it is now often considered to belong to a separate phylum, the Charophyta, which also includes *Chara* and one or two other similar genera. The long internodal cells of *Nitella* and *Chara*, which may be as long as 5 cm., have provided the material for many studies of the composition of the vacuole contents, permeability of the vacuole membrane, electrical stimulation and conduction of impulses similar to nerve impulses, streaming of the cytoplasm, etc. It is unfortunate that there seems to be no agreement as to the nuclear life cycle in *Nitella* and *Chara*, despite a number of cytological studies.

The shoot of *Nitella* is organized into internodes and nodes as is the shoot of a higher plant. Each internode consists of a single elongate cell, while a node consists of a number of much smaller cells. A number of "leaves," or lateral shoots of limited growth, are attached in a whorl at each node. These lateral branches are similarly organized into nodes and internodes.

The cell lineage involved in formation of the shoot of *Nitella* has been known since the middle of the last century. Giesenhangen's (1897) study, from which Fig. 1 is reproduced, appears to be definitive. The apical cell (*v* in Fig. 1, A) divides transversely to form a new apical cell and a segment cell (*v* and *g* in Fig. 1, B). The segment cell, *g*, divides transversely to form two daughter cells, the more apical of

which, k , undergoes a number of divisions to form a node, and the more basal, i , elongates without further division to form the internode. The daughter apical cell, v in Fig. 1, B and C, again divides as did the original apical cell. This process occurs repeatedly in the formation of the succession of nodes and internodes which make up a shoot. Giesenhagen symbolized the three stages in this process, which are illustrated in the figure, with the equation

$$V = v + g = v + (k + i)$$

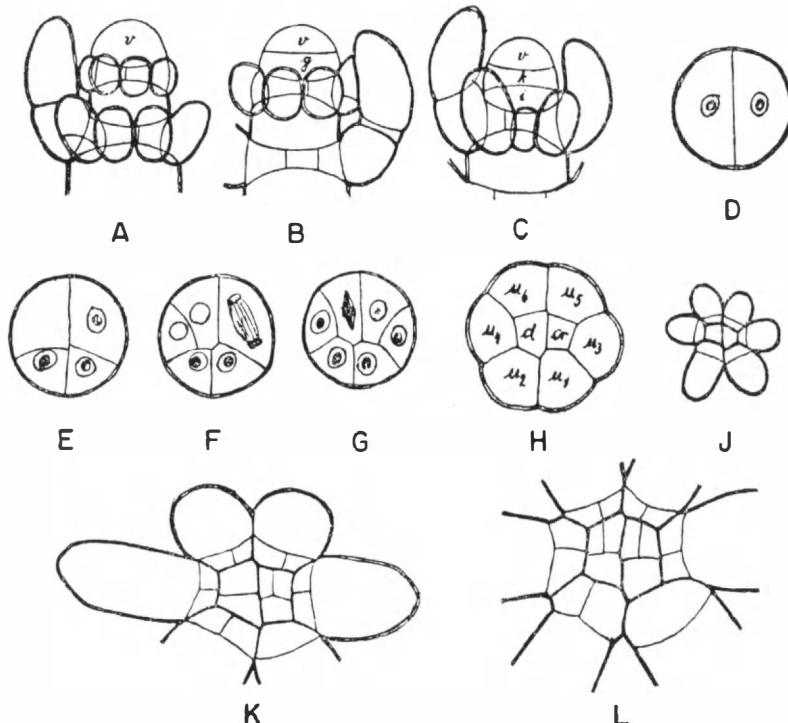


FIG. 1. Stages in the formation of internodal and nodal cells in *Nitella gracilis* (A-C and J-L) and *Chara aspera* (D-H). A-C, magnification $\times 172$; D-H, $\times 270$; J-L, $\times 161$. From Giesenhagen (1897).

in which V represents an apical cell about to divide and v a newly formed daughter apical cell. He then says that v grows to the original size V and new divisions ensue, to give $v + g_1 = v + (k_1 + i_1)$, so that the formation of an entire shoot from an original apical cell can be represented as

$$V = v + (k_n + i_n) + (k_{n-1} + i_{n-1}) + \dots + (k_1 + i_1) + (k + i)$$

Giesenhenagen also illustrates the subdivision of the nodal cell K into six or more peripheral cells u which are forerunners of the lateral branches, and central cells c (Fig. 1, D-H), pertaining, however, to *Chara*. Slightly later stages in development of the node and the laterals are illustrated in Fig. 1, J-L (this time for *Nitella*). Giesenhenagen has written similar equations for the divisions involved in development of the node, and he and others have made similar cell lineage studies of the formation of rhizoids, of the origin from the node of the cortical cells which surround the internodal cell in *Chara*, and of the antheridial and oögonial structures.

Giesenhenagen's figures have been reproduced or redrawn by many authors of manuals and textbooks and, while there have been other studies of cell lineage in the Charophyta, it is my opinion that little has been added to his account until the recent work of Green (1954, 1958). In my judgment, such cell lineage studies in which "successive stages" are illustrated fall far short of giving an adequate description of the developing *Nitella* shoot. Nothing is said about the frequency of division of the apical cell or segment cell, about rates of growth of the large internodal cells as compared with the nodal cells, or of the contrast in developmental pattern between the laterals of limited growth and of the primary shoot which can grow more or less indefinitely.

Another early paper dealing with *Nitella* is that of Askenasy (1880). He presented measurements of length of successive internodal cells of several shoots of *Nitella flexilis*. Some of his data are plotted in Fig. 2. The length of each internode is plotted against its serial number, counting from the apical younger internodes to the basal mature ones. Askenasy reasoned that since internodal cells are cut out from the segment cells periodically, and presumably at equal intervals of time, the lengths of successive internodes plotted against their serial numbers would be equivalent to a plot of the lengths against time and would represent the "grand period of growth" of such a cell or, in more modern terms, its sigmoid growth curve. This procedure involves the implicit assumption, which Askenasy did not state, that the pattern of internode elongation does not change with time. He termed the interval of time involved in cutting out two successive internode cells a *plastochron* or *Formungszeit*, and the former term has come to be quite widely used by plant morphologists. In Fig. 2, it appears that the relationship between internode length and node number, i.e., plastochrons, has exponential characteristics at the earlier stages, even though the semilogarithmic plot is not linear throughout. As Askenasy points out, the factor by which an internode increases in length during each plastochron should therefore be of interest, even though it is not a constant as in simple exponential

growth, and he terms it the *Wachstumsintensität*. The natural logarithm of this factor is also of interest, and Askenasy calculated a value a , or the *Wachstumsgeschwindigkeit*, by taking the natural logarithm of the ratio of lengths of two successive internodes. His constant, a ($= \ln L_2 - \ln L_1$) is comparable to the relative rate (Briggs *et al.*, 1920) or specific rate (Richards and Kavanagh, 1943) of growth with respect to time, but I would prefer to call it a "relative plastochron rate" of elongation to

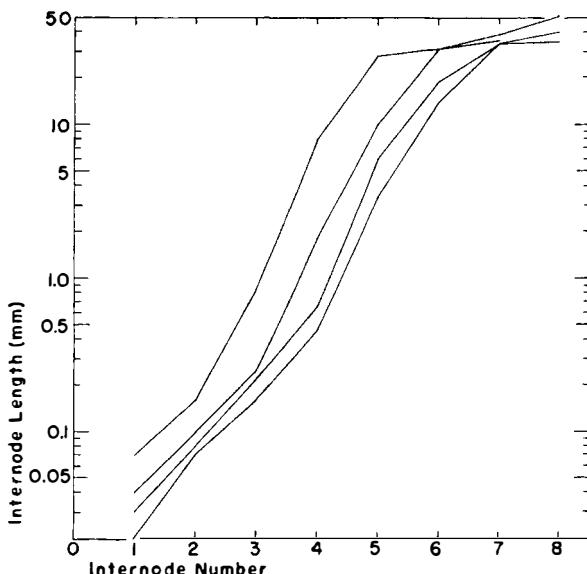


FIG. 2. Lengths of internodal cells of *Nitella flexilis* plotted logarithmically against their serial numbers, to represent the growth of an internode. Data from Askenasy (1880).

distinguish it from a time rate.¹ Askenasy reports maximum values of the ratio of lengths of successive internodes [which might be termed "plastochron ratios," following Richards (1951)] of 6 to 8. These cor-

¹ The simple exponential equation $Y = Y_0 e^{rt}$, or $\ln Y = \ln Y_0 + rt$, arises from the differential equation $dY = Y dt$, or $d\ln Y = r dt$, where Y is the variable being measured, t is time, and r is a constant, the relative rate. It is customary to estimate the relative rate in exponential growth, $r = \frac{1}{Y} \cdot \frac{dY}{dt} = \frac{d\ln Y}{dt}$, by finding the increment in $\ln Y$ over an interval of time, i.e., $r = \frac{\ln Y_2 - \ln Y_1}{t_2 - t_1} = \frac{\ln(Y_2/Y_1)}{t_2 - t_1}$ (Briggs *et al.*, 1920, and other authors). This formula for estimating the relative rate has sometimes been used erroneously, when growth is demonstrably not exponential and when the time interval used is not negligible. The relative rate of growth, $d\ln Y/dt$, is a valuable statistic, however, whether it is constant or not.

respond to values of the relative plastochron rate of elongation of 1.8 to 2.1 per plastochron. Clearly it should be possible to convert relative plastochron rates into relative time rates of elongation by multiplying by the duration of a plastochron in days or hours. Askenasy apparently made no growth studies, in the sense of measuring the same shoot from time to time. He stated that the absolute duration of the plastochron in *Nitella* was unknown to him and that it was of no significance in defining the "grand period of growth."

Since the apical initial cell divides once per plastochron, one can also arrange Giesenhausen's figures (Fig. 1, A, B, and C) on the presumed time axis of plastochrons. By making the assumption that the basal wall of each internodal cell is displaced from the tip of the apical cell in a smooth exponential manner and tracing the drawings to represent repeated divisions of the apical cell, the graph of Fig. 3 results. If Fig. 1, B is taken to mark the beginning of a plastochron, it appears that

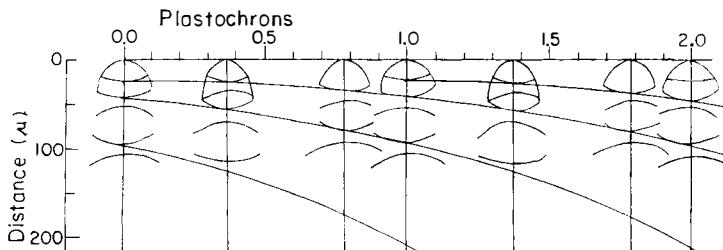


FIG. 3. *Nitella gracilis*. Tracings of Fig. 1, A-C, plotted against a scale of plastochrons, to represent the time course of apical development.

C and A are not spaced at equal thirds of a plastochron thereafter, but at 0.36 and 0.78 plastochrons, respectively. There is little point in elaborating the discussion of such speculative curves, since investigation may well show them to be erroneous. If such data were at hand, however, they would add a great deal to the understanding of the developing system. In descriptive cytology and histology one could specify precisely the developmental status of the cells in question. Similarly, in experimental studies of development, one could make a more precise choice of material and relate experimental results to the normal developmental pattern more precisely.

In his studies of the structure and development of the cell wall in *Nitella axillaris*, Green (1954, 1958; Green and Chapman, 1955) has made consistent use of growth data in specifying the age of material subjected to light and electron microscopic study and in selecting cells of known developmental stage for experimental study. Under his cul-

tural conditions, internodal cells are produced at about 3-day intervals, so that successive internodes differ in age by about 3 days (Green, 1958). Instead of simply numbering the internodes consecutively from apex to base as Askenasy did, he has estimated the age of each cell to a half-plastochron by observing the pattern of cells at the apex. If the youngest internode cell of a shoot is surmounted by a pronode cell, k , and apical cell, v , as in Fig. 1, A and C, it is designated 1, and the successive internodes basal to it are designated 2, 3, 4, etc. If the youngest internode has a pronode cell, a segment cell, and the apical cell above it, as in Fig. 1, B, it is designated 1.5, and successively older internodes as 2.5, 3.5, 4.5, etc., plastochrons old. Green has plotted logarithms of internode cell lengths against these estimated plastochron ages to obtain graphs quite similar in form to those of Askenasy (Fig. 2). From these

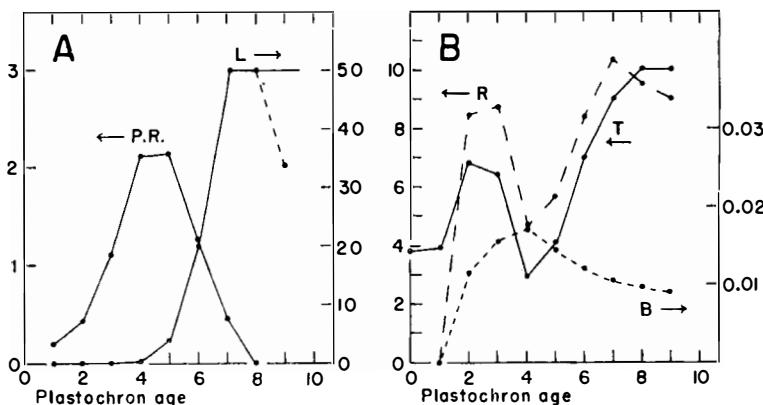


FIG. 4. *Nitella axillaris*. A. Lengths in millimeters of internodes (L) and relative plastochron rates of elongation (P.R.) of internodes of a representative shoot. B. Optical measurements of the walls of internode cells of the same shoot. (T), optical thickness in 0.1μ units; (R), retardation in $m\mu$; (B), birefringence, R/T . Redrawn from Green (1958).

length measurements, he has estimated the relative plastochron rate of elongation, as a function of plastochron age (Fig. 4, A). This rate has a maximum of about 2.1 per plastochron when the internode is 4 to 5 plastochrons old, and falls to zero at a plastochron age of 8. Since the plastochron is about 3 days the maximum relative time rate is about 0.7 per day.

Green has also determined the thickness of the wall, its birefringence and retardation, etc., for internode cells whose plastochron ages were estimated in this way. From Fig. 4, B, for instance, it can be seen that the thickness of the wall increases during the first two plastochrons

(6 days), then decreases to a minimum at 4 plastochrons and increases rapidly to its mature thickness at 8 plastochrons. Rates of change of thickness, and of other properties, of the wall can be estimated in much the same way as was the relative rate of cell elongation. In my opinion this interpretation of the morphology of the *Nitella* shoot in terms of growth processes has added much to the study of the development of the wall.

B. *Equisetum*

In *Equisetum*, the only living genus of the once larger phylum Sphenophyta, the shoot has a nodal organization with a whorl of leaves, and in some species a whorl of branches, at each node. In this respect it resembles *Nitella* superficially, but it is considerably more complex in

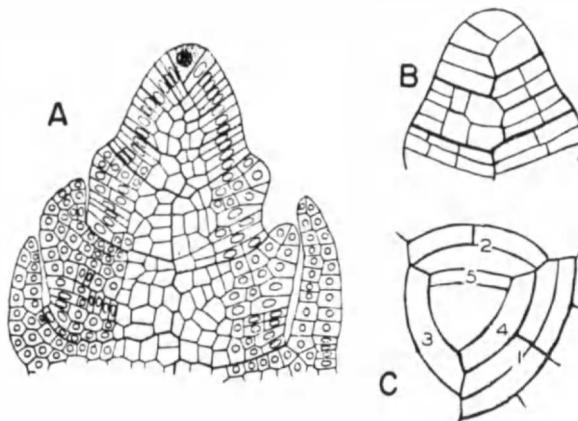


FIG. 5. Shoot apex of *Equisetum* in longitudinal (A, B) and transverse section (C), to show tetrahedral apical cell. A, magnification $\times 96$; from Smith (1955); B and C, from de Bary (1884).

its histology, having a well-developed vascular system and other specializations of structure which are lacking in the Charophyta. The development of the shoot from a single apical initial cell has been described by a number of authors, of whom Golub and Wetmore (1948) may be cited. They refer to earlier literature. The apical initial cell has somewhat the shape of a tetrahedron, or three-sided pyramid. One face of the tetrahedron is convex and forms the apical surface of the shoot meristem. The other three faces of the initial cell are in contact with cells derived from it (Fig. 5, A and B). In a transverse section through the apical cell, the arrangement of the most recently formed walls implies that each successive plane of division of the apical cell is

parallel to each of its basally oriented faces in turn, so that the two daughter cells are in each case a new tetrahedral initial and a 5-faced segment cell. Successive segment cells are cut out in a spiral succession which is indicated in Fig. 5, C by numbering the segments from older to younger. The first division of a segment cell is again parallel to the wall which separated it from the initial cell, and it is subsequently subdivided into many cells to form the various tissues of the shoot. In favorable longitudinal sections, the outlines of the segments can be distinguished for some distance back from the apex, as shown in Fig. 5, A, so that the cell lineage can be inferred to some degree. It is apparent that the successive segments seen in a longitudinal section can be taken to represent successive stages in the development of such a segment, each stage being separated from the next by one plastochron, on the assumption that the growth pattern remains constant with time. There are three tiers of segments constituting the apical cone, as Golub and Wetmore show, and it seems clear that the apical initial cell divides three times during a plastochron, to cut out three segment cells, and that three successive segment cells, constituting a so-called segment-ring, eventually give rise to a node and internode. Furthermore, it appears that a node, with its whorl of leaves and branches, can be traced back to the apical, and the internode to the basal, daughter cell cut out from each of the three segment cells at its first division.

It has been alleged that the three segment cells constituting a segment-ring are cut out in fairly rapid succession, and that after a pause another ring of three segment cells is cut out (Reess, 1867). Golub and Wetmore (1948) and other authors are of the opinion that the apical cell divides continuously, without any appreciable pause between sequences of three divisions. In my opinion, no conclusive evidence on this point has been presented. Another point which has been discussed at length is the mechanism whereby the whorled arrangement of leaves at a node arises from the original helical arrangement of the segment cells. Inequalities in the longitudinal growth of the segment tiers, and the existence of a prospective intercalary meristem have been cited, as Golub and Wetmore say. It is my feeling that data from growth studies are required for such questions. Direct or photographic observation of a living apex through one or more plastochrons, for instance, should be feasible and would leave no question as to its division schedule. To my knowledge, no growth data of this sort exist for *Equisetum*.

In the absence of growth studies, interpretations of the development of *Equisetum* in terms of plastochrons are more dubious than those made for *Nitella* above. Nevertheless, it is possible to state, for instance, that the appearance of the leaf primordia as recognizable protuberances occurs 4 or 5 plastochrons after the original cutting out of a segment-

ring. It should also be possible, for example, to determine the volumes of successive segment-rings by measurement or reconstruction of longitudinal sections, and from these to estimate the relative plastochron rate of increase in volume. Similar interpretations of later stages of development of the leaves, branches, internodes, the vascular system, etc., would be relatively simple. The development of the procambium and vascular strands could be put in like terms. Golub and Wetmore (1948) describe these processes for *Equisetum*. I shall refer to procambial development and vascular differentiation in other plants below.

I feel that much would be added to one's understanding of development by procedures such as those outlined above. For the simplest interpretation of the relative plastochron rate, the plastochrons should be equal in length, and the morphological pattern of the shoot apex should remain constant from time to time (except for the cyclical changes during a plastochron). Whether this is the case in *Equisetum* remains to be seen. *E. arvense*, at any rate, is markedly seasonal in its growth in the field.

C. Ferns

In most of the Filicinae, both the shoot and the root develop from a single apical cell succession, similar to that of *Equisetum*. Fig. 6, A is a classical illustration of the root apex of *Pteris cretica*. In this case the tetrahedral initial cell cuts off segment cells and new initial cells at each of its four faces. The lenticular segments which are cut off at the apical surface develop into portions of the root cap, while those from the three basal faces of the apical cell develop into the root proper. Just as in the *Equisetum* shoot, the original segment cell walls are identifiable for several plastochrons. In Fig. 6, A four successive segments can be identified on either side of the drawing. If each of these is considered to represent a 120° sector of a figure of revolution, the volume of the sector can be calculated from measurements of the drawing. Assuming again, in the absence of any growth data, that the segments are cut out at equal intervals of time, their growth in volume appears to be roughly exponential over the first four plastochrons, and the relative plastochron rate of increase in volume is about 1.28. This may perhaps be compared with the values calculated by Askenasy for the segments of the *Nitella* shoot (cf. page 505). We have made similar analyses of a number of sections of *Aspidium* roots, with similar results. There are, of course, no nodes and internodes in the root, and hence no such correlation with the segment-rings. Furthermore, no growth data are at hand, so that the plastochron rate cannot be converted to a time rate.

The fern shoot apex, particularly that of *Dryopteris aristata*, has been

the subject of extensive experimental investigation, principally by Wardlaw. In Fig. 6, C, from Wardlaw (1949) the apical cell is clearly evident. Whether an orderly series of segments can be identified, as in the root, and whether there is a clear relationship of segments to initiation of leaves, are not apparent from the published figures which I have seen.

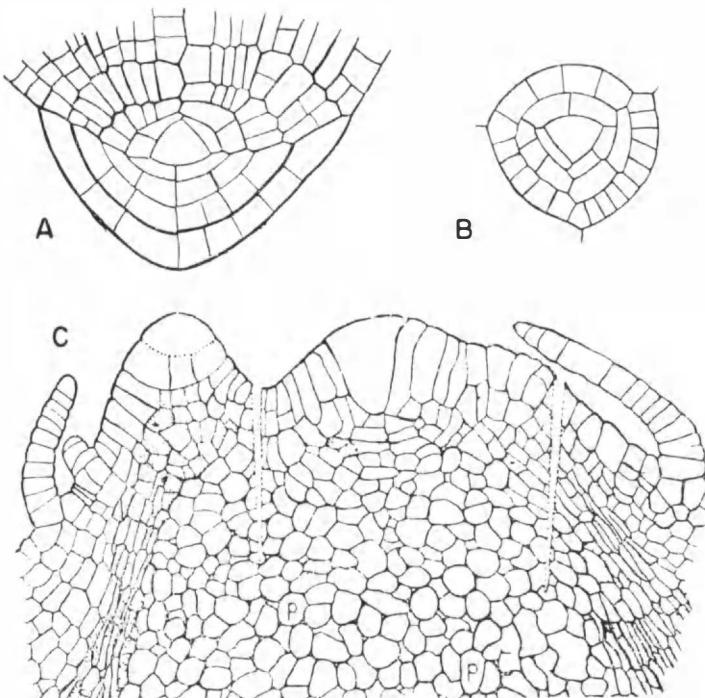


FIG. 6. Fern apices. Root apex of *Pteris hastata* in longitudinal (A) and transverse section (B). Shoot apex of *Dryopteris aristata* in longitudinal section (C). A and B, magnification $\times 188$; from de Bary (1884). C, $\times 120$; from Wardlaw (1949).

D. Other Plants and Organs

In many other species of Bryophyta, Psilophyta, Lycophyta, and true ferns, the development of shoots, roots, leaves, prothallia, reproductive organs, and other structures proceeds from a single apical initial cell. This fact is usually stated in texts of comparative morphology such as those of Smith (1955) and Eames (1936).

III. APICAL DEVELOPMENT FROM A GROUP OF INITIAL CELLS

A. The Shoot of Angiosperms

As several authors have summarized (Esau, 1953; Foster, 1949; Schüepp, 1926), the single apical cell which is so conspicuous in the

apical development of the lower vascular plants led the earliest investigators, such as Nägeli (Foster, 1939), to assume that the gymnosperms and angiosperms had a similar mode of apical development, despite the fact that sections of apices of these plants showed no such striking pattern as in the lower plants (Figs. 7 and 8). Presumably it was felt that

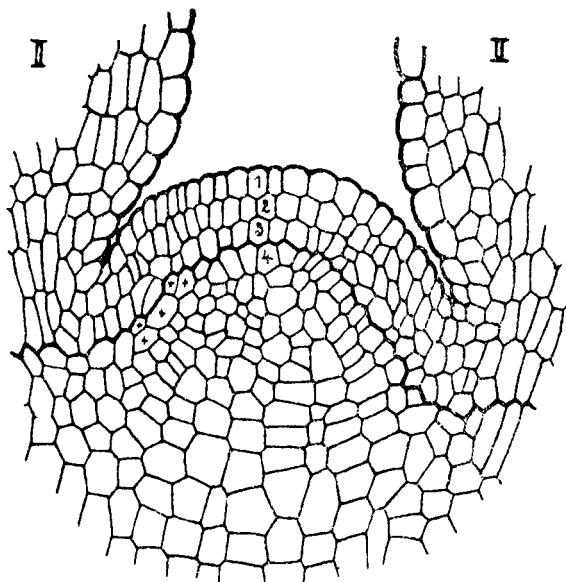


FIG. 7. Shoot apex of *Vinca minor* in longitudinal section. The three cell layers of the tunica are separated from the corpus by a heavy line. Magnification $\times 335$; from Schmidt (1924).

the apical cell would be revealed by sufficiently careful study, even though it was not as diagrammatically clear as in more primitive forms. Although von Guttenberg (1948) has identified a single *Zentralzelle* in the apices of roots of many dicotyledon species, most morphologists are in agreement that the seed plants have a group of initial cells at the root and shoot apex, rather than a single initial cell. The feature which is quite clear in many sectioned shoot apices of higher plants is a concentric, or *periclinal*, arrangement of cell layers in the apical region. This is paralleled by the concentric zonation of the tissue systems of the mature parts of primary stems and roots, the epidermis, the cortex, and the stele or central cylinder. Hanstein (1868) was presumably led by this parallel to propose that the shoot apex consists of three *histogens*, the *dermatogen*, the *periblem*, and the *plerome*, which give rise respectively to the epidermis, cortex, and vascular cylinder of the stem, and corresponding tissues of the leaf. There is little reason to doubt, even today, that the epidermis, the outermost layer of cells of leaves, stem,

and floral parts, is derived from the outermost cell layer of the apex and that the dermatogen is therefore a histogen in Hanstein's sense. It is probably also true that this outermost layer is derived from a small group of cells at the very apex, which serve as initials by virtue of their position (Haberlandt, 1914). These initial cells and all the rest of the dermatogen cells appear to divide exclusively in anticlinal directions (with the new wall approximately perpendicular to the surface). The absence of periclinal divisions (parallel to the surface) is, of course, responsible for maintaining the dermatogen and epidermis as a single layer of cells.

With regard to the periblem and plerome, however, later investigators than Hanstein encountered difficulties in applying his histogenetic scheme. The presumed boundary between these two regions of the apex is not always self-evident, and the problem of determining the histogenetic relationships between meristematic and mature tissues does not yield to a simple study of sectioned material. Only when there are clear geometric clues, as in the *Nitella* and *Equisetum* shoot, can the cell lineage be made out from fixed material for a few plastochrons. Some method of marking initial or meristematic cells, following the marked cells as they develop, and identifying them in mature tissue, would be helpful.

Fortunately a method of study approximately like this has been available for a number of years, in the periclinal and sectorial chimeras which have been found or experimentally produced in a number of plants (Jones, 1934). The earliest chimeras (dating back to the seventeenth century) arose from grafts between two varieties, species, or genera, and have been referred to as graft hybrids. It was believed by the earlier investigators, and more recently by Lysenko (1954), that the grafting operation leads to a combination of the hereditary characteristics of the stock and scion, in much the same sense as the crossing of two parents by sexual means does. Winkler (1907) produced chimeras experimentally between the black nightshade and the tomato by making a graft and, after it had taken, severing the stem through the graft union. Some of the buds arising from the callus which developed at the cut surface were chimeras which combined the characteristics of the stock and scion. Winkler regarded these chimeras as graft hybrids, but other investigators such as Bauer (1910) and Buder (1911) interpreted them as plants which consisted of two genetically distinct tissues, those of the stock and scion. Apparently no conclusive evidence has ever been presented for the formation of a *burdo* by fusion of vegetative cells or nuclei in the chimera, as was hypothesized by Winkler. Plasmodesmata, however, undoubtedly occur between the cells of the two components of the chimera (Hume, 1913).

That many of the presumed graft hybrids were periclinal chimeras was evident, since it was frequently the case that only the epidermis was of one species and the remainder of the tissues of the other. In other cases, the epidermis and two or more layers of cells just below it were of one species, and the remainder of the other. It was concluded, quite correctly, even before careful histological work was done, that these facts implied a periclinal organization of the shoot apex. In other words, the apex must consist of a few concentric layers of cells, each with its own independent set of initial cells. The continued existence and relative stability of the periclinal chimeras imply exclusive anticlinal division of these initial cells and their derivatives for several generations, so as to maintain the concentric cell layers. It also became evident that there was some variability in the relationships between mature tissue types and the meristematic cell layers from which they stemmed.

In 1924 Schmidt, who was a student of Buder, provided clear histological evidence of the periclinal organization of the shoot apex in several plants having the decussate leaf arrangement, and proposed that Hanstein's histogenetic terminology be replaced by the two descriptive terms *tunica* and *corpus*. The tunica was defined as the outer portion of the apex in which the cells are arranged in easily recognized periclinal layers, and divide anticlinally, at least near the apex (Fig. 7). The number of tunica layers varies, as later investigators have shown (Gifford, 1954), from one to four in various species, and each layer originates, by anticlinal division, from its own initial region, comprising perhaps from four to six cells.² The corpus derives from a further initial region. The corpus initials and the rest of the corpus divide in various planes, leading to an irregular cell pattern. Although the tunica and corpus were intended by Schmidt (1924) to be simply descriptive terms, and have been used by most authors in this way, it is possible to relate at least the earlier stages of differentiation of the characteristic tissues of the shoot to these portions of the promeristem. Schmidt (1924) described

² The cells of the initial region do not differ greatly in appearance from their immediate neighbors, and can be defined only by the fact that they maintain their position at the apex (Haberlandt, 1914; Esau, 1953). Except for the paper of Newman (1956), there seem to have been no observations of cell division in living apices. It seems reasonable to suppose, however, that any daughter cell of an initial which is not in contact with other initials will be displaced from the initial region by subsequent growth and division. Consequently only four to six cells which are actually in contact with each other can be considered as the initials of a periclinal layer. It also seems reasonable to suppose that the actual configuration of this small initial region changes continually as its cells divide and grow. The tunica and corpus can perhaps be regarded as constituting the *promeristem* in Haberlandt's (1914) terminology. Other parts of the primary meristem which are derived from it would then be the *protoderm*, *procambium*, and *ground meristem* (Esau, 1953).

the formation of procambial strands, the earliest stages of leaf initiation, and the derivation of the tunica and corpus of a lateral bud from that of the primary apex, in the several species of plants with decussate leaves which he studied, and similar histological studies have been carried out by a number of authors.

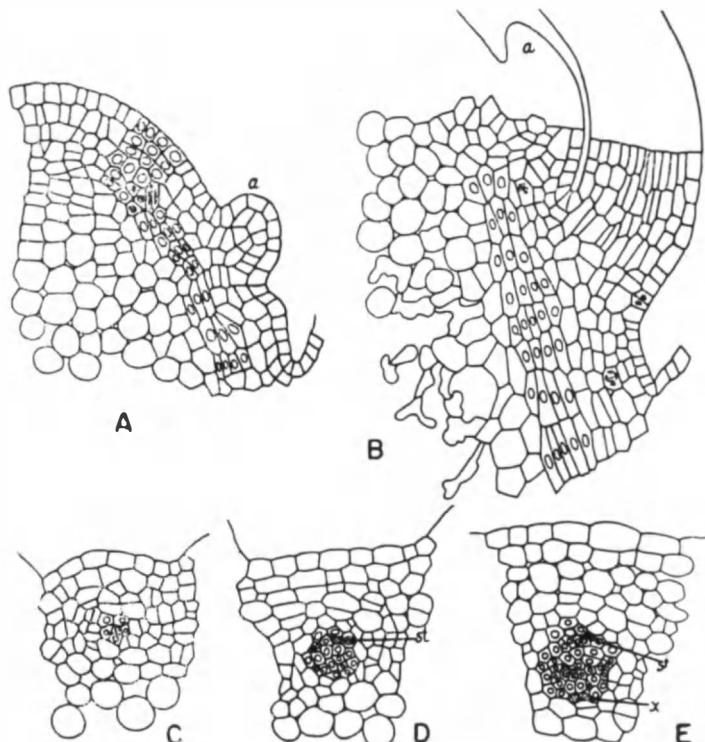


FIG. 8. *Linum perenne*. A. Longitudinal section through shoot apex to illustrate an early stage of initiation of a leaf and a procambial strand. B. Another section of same specimen as A, to show basal continuation of procambial strand. C-E. Transverse sections, 0.098, 0.245, and 0.385 mm. basal to apex, to show increase in diameter of procambial strand. Magnification $\times 300$; from Esau (1942).

Esau's (1942, 1943) meticulous study of flax, *Linum perenne*, may be taken as illustrative of the histological processes involved in leaf initiation and procambial development. In *Linum*, the tunica consists of only one layer. The earliest indication of leaf initiation appears to be the occurrence of periclinal walls in the outer layer of the corpus which in its apical part divides anticlinally (Fig. 8, A). Later the second and third corpus layers also contribute to the young primordium. The tunica layer, which might be termed the protoderm, divides only anticlinally, to form

the epidermis of the leaf. In other species the contributions of the tunica and corpus to leaf initiation are quite variable, and no general statements can be made (Esau, 1953). The procambium, from which the xylem, phloem, and cambium later develop, makes its first appearance in the division of cells of the corpus, with walls placed more or less parallel to the axis, so that the resulting daughter cells are more elongate and slender than their neighbors (Fig. 8, A, E). The procambium cells are also characterized by more densely staining contents than surrounding cells. In the further development of a procambial strand, its cells continue to divide with longitudinal walls, and surrounding cells apparently also contribute to the strand by dividing in this manner (Fig. 8, C-E).

Again, the relation of the procambial strands to the tunica and corpus appears to vary in the several species. The cell pattern is such that it would appear difficult, on the basis of histological studies alone, to make conclusive correlations of the procambium with cell layers of the apex.

In Fig. 8, A it is apparent that the first stages of leaf initiation and of procambial formation are both present. The question of which precedes, whether the procambium is differentiated first and "induces" the formation of a leaf primordium, or vice versa, is one of considerable interest. However, there appears to be no clear basis on which one can choose between these alternatives (Esau, 1953) in *Linum* or in other species.

The progressive development of the procambium, and the maturation of the procambium cells into functional xylem and phloem (usually referred to as xylem and phloem differentiation) follow the phyllotactic pattern of the shoot. Esau (1942, 1943) described *Linum* shoots with the (5 + 8) and (8 + 13) patterns of orthogonal parastichies.³ The simpler (5 + 8) pattern is illustrated in Fig. 9. Figure 9, A is a transverse section of a *Linum* shoot just below the shoot apex, showing the

³ Phyllotactic patterns are usually described in terms of *parastichies*, which are assumed to be equiangular spirals in the plane model, helices in the cylindrical model, and in general could be considered loxodromes (Church, 1904; van Iterson, 1907; Richards, 1951). In a given pattern, a number of sets of intersecting parastichies can usually be traced which connect leaves differing in serial number, respectively, by 1, 2, 3, 5, 8, 13, These numbers constitute the Fibonacci series. Other series, and multiples of these, are known. It is customary to designate a given apical pattern by citing the two sets of parastichies which intersect most nearly orthogonally, e.g., (2 + 3), or which connect leaves which are in proximity or "contact." The orthogonal parastichies and the contact parastichies need not be the same ones, though it is sometimes assumed that they are. See also the discussion on page 531, and Fig. 16.

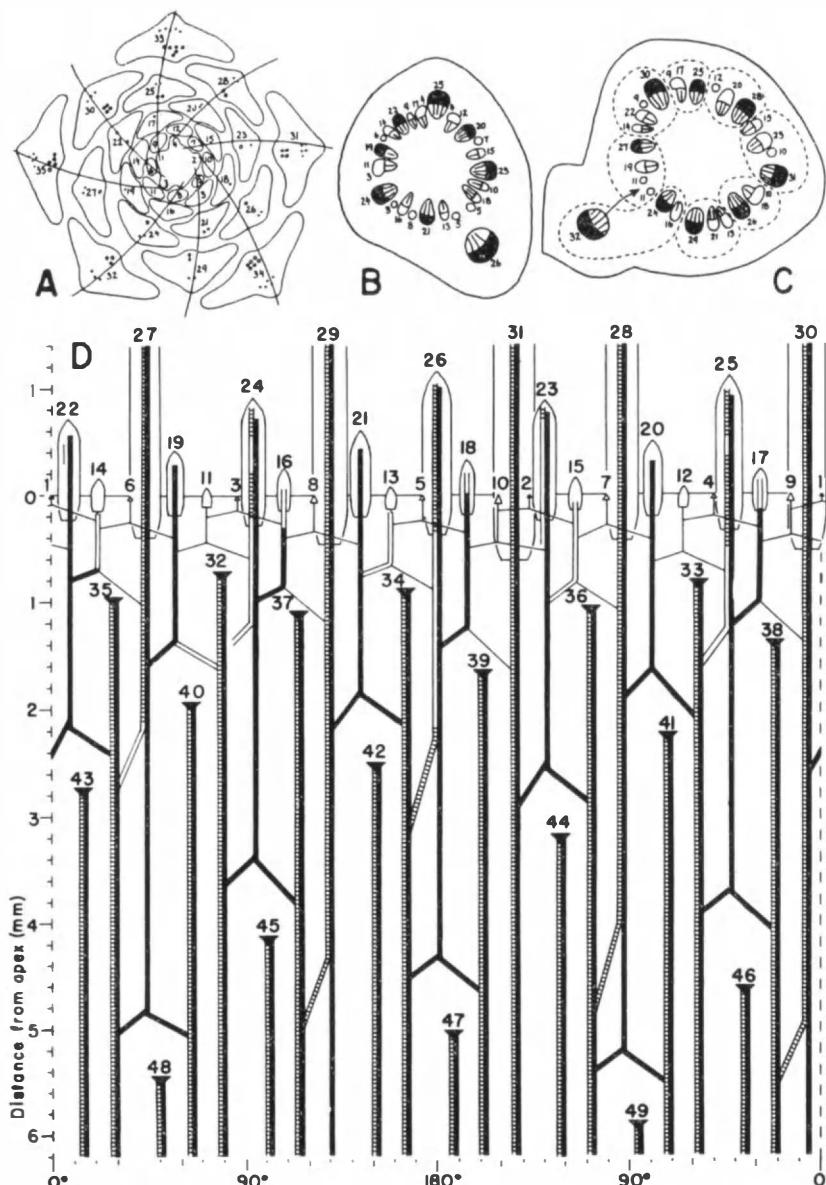


FIG. 9. *Linum perenne*. A. Transverse section of apical bud, just below apex, to show phyllotactic pattern. The 8-parastichies, which are drawn, intersect the 5-parastichies nearly orthogonally ($\times 57$). B and C. Transverse sections of stem, 0.22 and 0.53 mm. below the apex. Numbers placed beside procambial or vascular strands correspond to those in A and D. ($\times 56$). D. Reconstruction as an unrolled cylinder of procambial and vascular tissue of the shoot illustrated in A-C.

phyllotactic arrangement of the young leaves, which have been numbered consecutively from younger to older. The 8-parastichies, which connect leaves differing in number by 8, are drawn. The 5-parastichies, spirals which run in the opposite direction and intersect the 8-parastichies nearly at right angles, can be visualized. Figure 9, B is a transverse section through the stem of this shoot, near the insertion of leaf 26, and Fig. 9, C, near the insertion of leaf 32. The vascular bundles or strands appearing in Fig. 9, B and C are each in continuity with the vascular strands of leaves inserted nearer the apex.

If the interval of time between initiation of two successive leaves is again defined as a plastochron, and it is assumed that the growth pattern does not change with time, it is clear that the successive leaves differ in developmental age by one plastochron each, and that the same is true of the procambial and vascular strands associated with each leaf. The successive leaves on a single shoot can then be construed as representing successive stages in the development of a typical leaf. By careful study of serial sections, Esau has reconstructed the complete vascular pattern of the young shoot, and presents a diagram similar to Fig. 9, D, in which procambial strands, immature or mature sieve tubes, and immature or mature xylem elements are indicated. If a strand is traced basally, it will be seen to branch, and the two branches to connect with the vascular strands of the two leaves which are respectively 8 and 13 plastochrons older than it is.* This pattern applies to the procambial strands as well as to the older vascular tissue.

Procambial strands are shown in Fig. 9, D for the youngest primordia which can be identified, and these strands are in unbroken continuity with more mature procambial and vascular strands basal to them. This is usually said to imply that procambial differentiation is acropetal, that is that it proceeds toward the apex. Actually, the appearance of new procambium seems simply to keep pace with the apical growth of the shoot as a whole and with the initiation of successive new leaf primordia.

The differentiation of phloem tissue involves division of each pro-

The horizontal dimension is exaggerated, but the angular positions of the strands correspond to A. Procambium is indicated by slender lines; strands containing one or more mature phloem or xylem elements, by heavy solid or crosshatched lines respectively; immature but recognizable phloem or xylem are unshaded. A-C, from Esau (1943); D, redrawn from Esau (1943).

* It is interesting to note in Fig. 9, A that the closest contacts between the leaves are along the 3- and 5-parastichies, that the 5- and 8-parastichies intersect at an average angle of 89°, and that the vascular connections are along the 8- and 13-parastichies. All of these spirals can be drawn easily. Clearly one must specify the basis on which one designates a phyllotactic pattern.

cambial or meristematic phloem cell to produce a sieve-tube cell and one or more companion cells, followed by a striking series of protoplasmic changes in each sieve-tube member. Slime bodies appear in the cytoplasm and fuse into a slime mass which fills the greater part of the cell. The nucleus disappears and characteristic sieve plates develop at the end walls. These cellular changes are described and illustrated by Esau (1953).

In development of the *Linum* shoot, immature phloem can first be recognized in leaf 13, that is 13 plastochrons after leaf initiation, and it is noteworthy that it is in continuity with the mature phloem of leaf 21, which is 8 plastochrons older. The connection to the phloem of leaf 26, 13 plastochrons older, however, is still procambial. Subsequent maturation of the phloem, as illustrated by leaves 14, 15, etc., is acropetal into the blade of the leaf, in the sense that the wave of maturation of procambial cells into sieve tubes proceeds more rapidly than the leaf elongates.

Esau (1953) similarly describes the cellular changes involved in xylem differentiation which, in the case of vessels, include laying down of the characteristic secondary thickenings of the wall, disappearance of the cytoplasm and nucleus, and disappearance of the end walls. The first immature xylem of the *Linum* shoot appears in leaf 22 as an isolated strand somewhat above the center of the leaf. During the next 6 plastochrons procambial cells rapidly differentiate into xylem elements in a somewhat irregular wave which proceeds both acropetally and basipetally. The fact that a xylem strand is not initially connected to older xylem basal to it, and that this connection is only established later, forms a significant contrast to the differentiation of phloem.

It should be apparent that the notion of a plastochron permits a dynamic analysis of the development of the angiosperm shoot, just as in *Nitella* and other cases which have been discussed. Plastochron rates of leaf and stem growth, of phloem and xylem differentiation, could be calculated easily from measurements of sectioned shoots. Although Esau (1943) speaks of short plastochrons in *Linum*, I do not know of any actual estimate of the duration of a plastochron in this plant. If it were known, the plastochron rates could easily be interpreted as time rates.

B. Modern Studies of Periclinal Chimeras

In addition to many detailed histological studies of shoot development such as that just described, several modern studies of periclinal chimeras, in which the component cell layers of the apex are of the same species, but differ in chromosome number, have confirmed and extended the older observations (Satina and Blakeslee, 1941; Blaser and Einset, 1948; Dermen, 1953) and tended to support the concept of the

organization of the apex into a tunica and corpus. These polyploid chimeras have usually been produced by colchicine treatment, which sometimes polyploidizes one or more of the outer periclinal layers of the apex selectively. In the peach, for instance, Dermen (1953) analyzed a number of chimeras in which the periclinal layers of the apex were diploid or tetraploid. In one case, the outermost layer was diploid, the second layer tetraploid, and the third layer together with the remainder of the corpus diploid, an arrangement which he designated 2-4-2. In a transverse section of the stem derived from such an apex (Fig. 10, A),

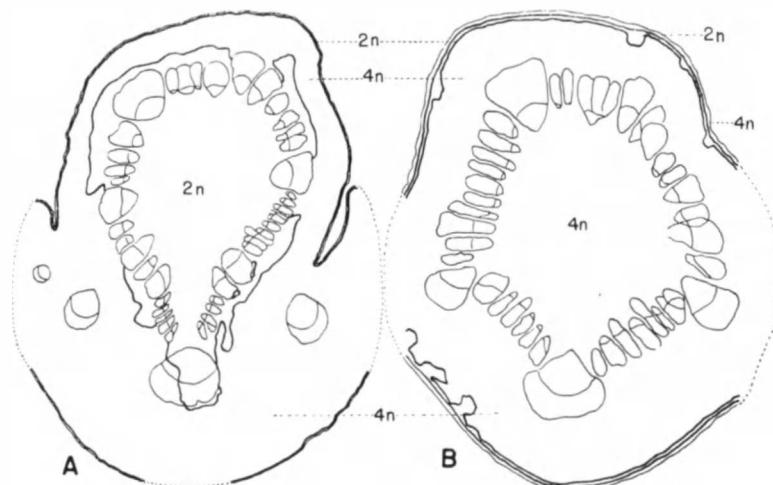


FIG. 10. Transverse sections of stems of periclinal chimeras of peach, showing diploid, $2n$, and tetraploid, $4n$, tissue. Magnification $\times 24$; traced from Dermen (1953).

the epidermis was diploid, and tetraploid tissue derived from the second layer of the apex comprised practically all of the cortex and parts of the vascular tissue of the stele. The third and inner layers of the apex, which must be interpreted as the corpus, gave rise to the pith and the greater part of the vascular tissue. In a 4-2-4 plant (Fig. 10, B), the epidermis, the greater part of the cortex, and the entire stele were tetraploid, while diploid cells occurred only in the single layer of cells just below the epidermis and irregular patches of cells extending into the cortex a short distance. The contrast between these two cases in the amount of tissue derived from the second tunica layer is perhaps consistent with the frequent observation that tetraploid plants are more vigorous than diploids of the same genotype.

Dermen also described leaves of a 4-2-4 plant as seen in a transverse section of a terminal bud. The epidermis was tetraploid in all cases,

indicating its derivation from the outermost cell layer of the apex. Tetraploid tissue derived from the inner layers of the apex appeared only in the central and basal portions of the midrib of the leaves. It should be noted that the entire leaf mesophyll, including the veins, was diploid, and therefore derived from the second layer of the tunica.

Studies such as this provide information on the histogenetic relationships of mature portions of the shoot to portions of the apical meristem which is less equivocal than that derived from purely histological studies. It appears that, while there is a general correspondence between the outer tissues of the mature shoot and the tunica, and between the central tissues and the corpus, there are not obligate cell lineage relationships in the rigid sense which the earlier investigators visualized.

C. The méristème d'attente

Recently Buvat (1952) and his collaborators have advanced another interpretation of the shoot apex in flowering plants, according to which the apical region of the vegetative shoot apex, far from being the initial region, or promeristem, from which all the tissues of the shoot are derived, is regarded as being inactive and playing no role in vegetative development. These histogenetic functions are ascribed to the peripheral region of the tunica and the basal part of the corpus. The apical region, however, becomes active at the inception of flowering, and develops into the flower or inflorescence. Buvat (1952) has summarized these ideas in diagrammatic representations of the apex of several species, of which Fig. 11, A is an example, and in the following terminology:

<i>Tunica</i>	{	<i>Anneau initial</i>		
		<i>Proméristème sporogène</i>		
<i>Corpus</i>	{	<i>Proméristème réceptaculaire</i>	}	<i>Méristème floral d'attente</i>
		<i>Méristème médullaire</i>		

The *tunica* and *corpus* are retained as useful descriptive terms, but each is subdivided into two regions on the basis of supposed function. The *anneau initial* is the peripheral portion of the tunica, and the site of leaf initiation. The conception of this initial ring is closely associated with a theory of spiral phyllotaxis by Plantefol (1946, 1947), according to which leaves are laid down along two or more "foliar helices," rather than along a single "generative spiral."⁵ The *anneau* is said to be the

⁵ It is clear that Plantefol's foliar helices are a set of parastichies (see footnote 3) and that the generative spiral is the 1-parastichy. The basis on which he chooses to designate one, rather than some other set of parastichies, as the foliar helices is not clear to me. It may be noted, however, that his theory appears to be based primarily on consideration of the patterns seen on mature stems, rather than those seen at the apex.

site of initial cells, from which new leaf primordia are formed at the end of the appropriate foliar helices. It is recognized that the association between leaf development and that of the rest of the shoot is close, and hence that no sharp demarcation can be made between the *anneau* and the medullary meristem, which gives rise largely to the pith. This region of the meristem certainly corresponds to what other authors have termed the ground meristem, or (inappropriately, in my opinion) the rib meristem, and it is characterized by a pronounced transverse layering of the cells and hence by a predominance of cell divisions transverse to the axis. The appearance of the medullary meristem is reminiscent of the vascular cambium and its immature derivatives, of an old stem and it is assumed to have its own initials in the same sense that the cambium does.

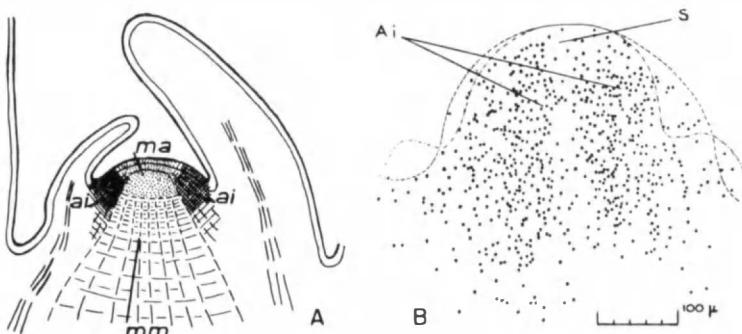


FIG. 11. A. Diagram of longitudinal section of shoot apex of *Cheiranthus cheiri*, to illustrate functional regions. From Buvat (1952). B. Diagram of longitudinal section of shoot apex of *Vicia faba*. Mitotic figures seen in 16 sections have been shown as points. From Lance (1952).

The *méristème d'attente* is conceived as playing no part in vegetative development, and its cells are described as *inactives* or *peu actives*. They are somewhat larger and more vacuolate than those of the *anneau* or the medullary meristem, and in other characteristics of the nucleolus, chromocenters, chondriome, etc., are regarded as lacking the pronounced meristematic characteristics of the cells of the youngest leaf primordia. I am not able to evaluate these cytological observations. Data on the frequency of mitotic figures in transverse and longitudinal sections of shoot apices are also presented by Buvat (1952) and Lance (1952). In Fig. 11, B, for instance, sixteen longitudinal sections from eight plants have been superimposed, and the location of each mitosis has been represented by a dot. Although Lance concludes that the apical region, S, is "entirely devoid of mitoses," I do not believe that such a categorical statement is justified. No estimate of the total number of cells repre-

sented by these sections is given, so that the data cannot be put in the form of a mitotic index (Erickson and Sax, 1956). Even if mitotic indices were available, no conclusions about time rates of division would be possible. A mitotic index can be interpreted as an estimate of the proportion of the entire duration of the mitotic cycle occupied by the specified stages. Only if this duration is known, and it can be shown that all the cells in question were dividing with the same average duration at the time of fixation, can the mitotic index be interpreted as a time rate of cell division.

Buvat (1952) has also described the development of floral structures from the *méristème d'attente*. In very brief outline, this central portion of the meristem first becomes markedly elevated. The resulting *méristème reproducteur* develops into the terminal flower or inflorescence, the *proméristème sporogène* giving rise to the floral parts, and the *proméristème réceptaculaire* to the receptacle of the flower (or inflorescence).

Buvat's concepts of the organization and function of the apical meristem have been the subject of some controversial discussion (Ball, 1955; Gifford, 1954; Wardlaw, 1957). In my opinion, the questions posed by this new interpretation cannot be answered by histological study of fixed material alone; indeed, the very proposal of such a divergent interpretation illustrates the limitations of purely histological study. The difficulty lies in the fact that the shoot apex of the angiosperms provides only somewhat indefinite geometric clues to the cell lineages involved in development. The crucial question is, of course, whether the cells at the very apex of the meristem function as initials in the vegetative development of the shoot, or whether they are as Buvat says "inactive." The existence and behavior of periclinal chimeras, referred to above, strongly implies the existence of a bona fide initial region at the center of the apex, and this evidence has been cited in opposition to Buvat's concept. It is interesting to note, however, that Buvat also appeals to the data from periclinal chimeras in support of his argument. It appears to me that decisive information can be provided only by making observations of growing apices, with sufficient resolution so that cell outlines can be identified, and over a sufficient period of time so that cell divisions could be observed. The techniques which Snow and Snow (1948), Wardlaw (1949), Ball (1948) and others have developed for exposing apices, carrying out microsurgery, and later growing and observing them, together with the elegant photographic techniques which Goodwin and Avers (1956) have developed for observing cell division and growth in the epidermis of the *Phleum* root should make such a study practicable. With the exception of an unsatisfactory preliminary series of

camera lucida drawings of the *Tropaeolum* apex by Newman (1956), there appear to have been no growth studies of this sort. Other more indirect ways of making growth interpretations of the shoot apex will be suggested below.

D. The Shoot of Gymnosperms

In the cycads, conifers, and *Ginkgo*, a number of authors have described the histological structure of the shoot apex in terms of so-called "growth zones." *Pseudotsuga taxifolia*, the Douglas fir, may be taken as an example (Fig. 12, A; from Allen, 1947). At the very apex is

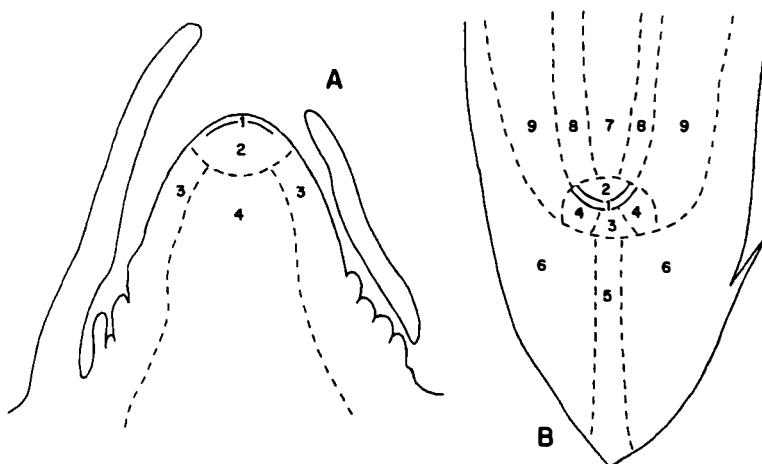


FIG. 12. Diagrams of shoot apex (A) and root apex (B) of *Pseudotsuga taxifolia* in longitudinal section to illustrate the "growth zones." In A (1) is the zone of apical initials, (2) the zone of central mother cells, (3) the zone of peripheral tissue, and (4) the rib meristem. In B, (1) is the root initial zone, (2) the stellar mother-cell zone, (3) the column mother-cell zone, (4) the cortical mother-cell zone, etc. Magnification $\times 48$; redrawn from Allen (1947).

a group of cells which are regarded as initials. These, however, are not arranged in clearly discernible periclinal layers, as in the angiosperms, and instead of being exclusively anticlinal, divisions are largely in periclinal orientation. The apical initial group (1) therefore contributes cells to a zone below it, termed the central mother-cell zone (2), as well as to one or more surface layers, which are peripheral to the initial zone. Basal to the central mother-cell zone is a so-called rib meristem (4), and surrounding this are peripheral subsurface layers of cells. In general, the cells of the apex are arranged in fairly well-marked files radiating from the initial region, implying that cell division is pre-

dominantly perpendicular to the radiating lines marked out by these files.

It has sometimes been stated that periclinal chimeras are impossible in the gymnosperms and that this is consistent with the organization of the shoot apex. Recently, however, Hejnowicz (1956) has interpreted the horticultural variety *Juniperus sabina variegata* as a chimera, in which the outer cell layer of the apex gives rise to white tissue, lacking chloroplasts, and the central portion produces green tissue. The white sectors and branches which are occasionally produced result from occasional periclinal divisions in the outermost layer of the apex, which give rise to entirely white sectors or apices.

Careful studies have also been made of procambial and vascular development in several gymnosperms (Esau, 1953).

E. The Root of Angiosperms and Gymnosperms

As compared with the shoot apex, relatively few studies have been made of the histological organization of the root apex. It is clear, however, that there is at least as great a diversity of types of organization of root apices as of shoot apices. Several differences between the root apex and that of the stem can be cited in general terms. The root possesses a root cap, and its apex often has a well-defined calyptrogen, with its own initial region. The root lacks the nodal organization of the shoot, and hence its apical development does not involve leaf initiation and associated processes. The vascular structure of roots, and hence procambial differentiation in the root apex, differs from that of stems. The formation of branch roots is not a function of the apex, but occurs at some distance basal to the apex and by quite a different mechanism. The root apex, however, is usually interpreted as having a group of initial cells, just as does the shoot apex, and in some species these initials appear to be periclinally arranged. Von Guttenberg (1948) on the other hand speaks of a *Zentralzelle*, from which the remainder of the meristem is derived.

In the gymnosperms, most of the recent descriptions of the root apex are couched in terms of "growth zones," similar to those of the shoot apex. In *Pseudotsuga taxifolia*, for instance, Allen (1947) has described a single initial zone and eight growth zones derived from it.

In some angiosperms, such as the grasses and several dicotyledonous families, three distinct tiers of initials exist, those of the root cap, of the epidermis and cortex, and of the central vascular cylinder. These can be seen in Fig. 13, A of barley, and the arrangement of cells in definite diverging files leaves little doubt of the histogenetic interpretation. As a matter of fact, one is tempted to use Hanstein's terminology in describing this apex and to refer to the three histogens as the plerome, peri-

blem, and calyptrogen, despite the strictures of students of the shoot apex.

In other angiosperms there are apparently only one or two tiers of initials, and in other cases the histological pattern is quite unclear. In Fig. 13, B, for example, a longitudinal section of a pea root, there is a transverse band of cells which suggests the cambium of an older stem. Several classifications of root apex types have been presented (Esau, 1953), but there appears to be no very clear consensus of opinion on this subject.

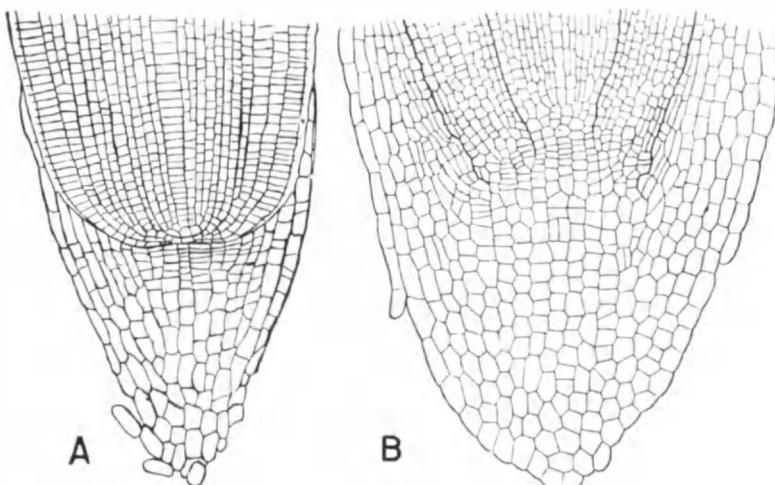


FIG. 13. Longitudinal sections of root apices of *Hordeum vulgare* (A) and *Pisum sativum* (B). A, magnification $\times 97$, from Strasburger and Hillhouse (1900). B, $\times 114$ from de Bary (1884).

Although the apical organization of some roots, such as that of barley (Fig. 13, A), is periclinal in nature, there appear to be no records of periclinal root chimeras in any species. This is probably to be explained by the fact that branch roots arise from the pericycle of the central cylinder at some distance from the apex. Hence, even if a periclinal arrangement of two tissue types were established at the apex of the root, only the inner, and not the outer, periclinal component would appear in branches of this root.

Brumfield (1943), however, has produced sectorial chimeras in roots of *Crepis* and *Vicia* by radiation and has failed to obtain periclinal arrangements in either species, even though the apices were examined shortly after treatment so that the question of branching was not involved. Although he presents no specific information about the histo-

genic organization of the root of *Crepis*, studies of other composites would suggest that the organization is much like that of Fig. 13, A, so that one might expect that periclinal chimeras could occur. In *Vicia*, the organization is similar to Fig. 13, B and it is difficult to visualize a periclinal arrangement. Clearly more investigation would be required to clarify this matter.

F. Growth of the Root

While the histology of root apices has not been studied as exhaustively as that of shoot apices, growth studies of the root have been car-

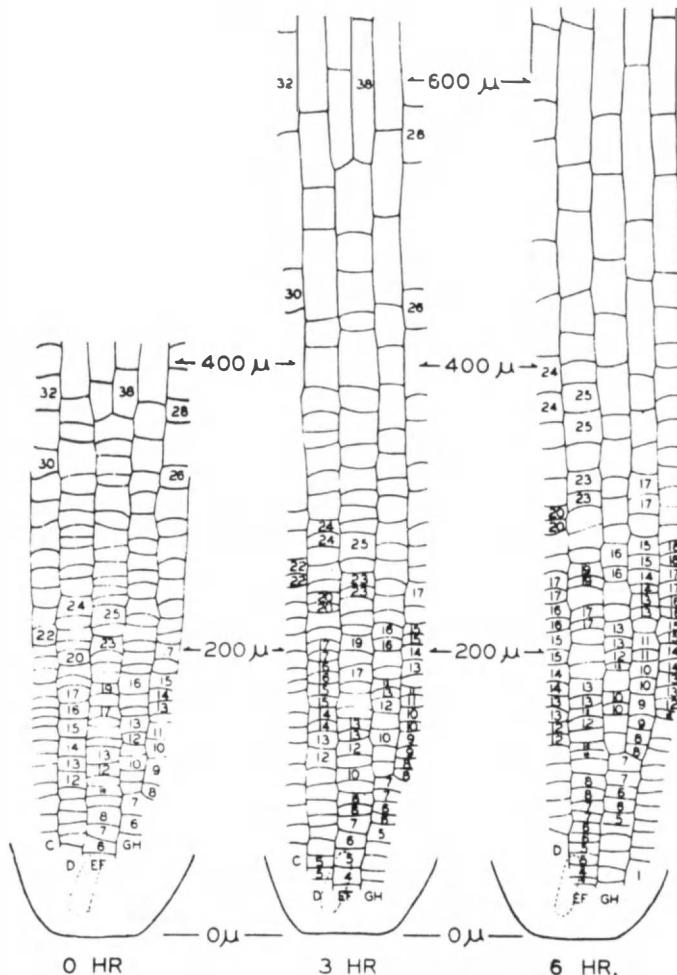


FIG. 14. Tracings of epidermal cells of a growing root of *Phleum pratense*. From Goodwin and Avers (1956).

ried considerably farther than for the shoot. The growth pattern has been worked out in sufficient cellular detail as to have some meaning in interpreting meristematic processes by Goodwin and Stepka (1945), Erickson and Goddard (1951), Erickson and Sax (1956), Hejnowicz (1956), and Goodwin and Avers (1956). For their study of the root of *Phleum pratense*, Goodwin and Avers developed a photomicrographic technique for scanning the entire growing region of the living root, about 1.2 mm., with a series of overlapping photographs having sufficient resolution for the recognition of the epidermal cell walls. Successive photographs were matched, as in photogrammetry, and a continuous tracing was made which showed each cell of several epidermal files at a given time. Such tracings of a single root could be made for each half-hour or hour interval during a run as long as 18 hours (Fig. 14).

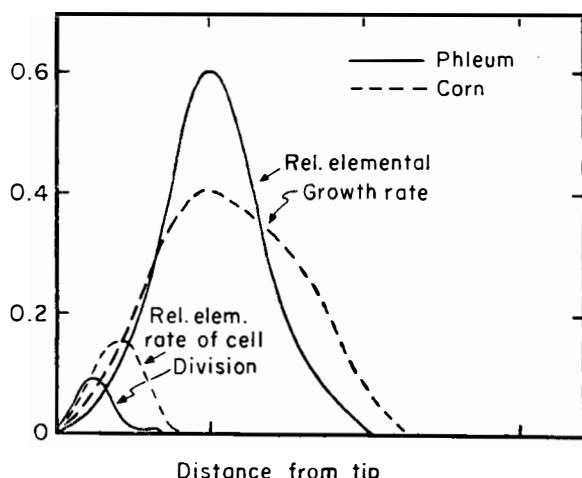


FIG. 15. Relative elemental rates of elongation and of cell division in growing roots of *Phleum pratense* and *Zea mays*. Distances from the tip have been adjusted so that the rate curves for the two species correspond. From Goodwin and Avers (1956).

Since the cells were individually identifiable, the appearance of new cell walls could be recognized unequivocally and the number of cell divisions per hour could be determined directly. The tracings were also analyzed for rates of displacement of given walls from the meristematic apex. These rates, dX/dt , were regarded as a function of distance from the tip, X , and derived with respect to X to give values of $d(dX/dt)/dX$, which are estimates of the relative rate of elongation of infinitesimal portions or elements of length (dX) of the root (Erickson and Sax, 1956). We have called this expression the relative elemental rate of

elongation. The cell division data were analyzed similarly, to yield estimates of the relative elemental rate of cell division at various points along the root. These rates are plotted against distance from the tip of the meristematic apex in Fig. 15, together with the data which we have obtained by a somewhat different method for *Zea mays*. The maximum elongation rate, 0.6 hr.^{-1} , occurs at 0.6 mm. from the tip in *Phleum*, well behind the region where cell division is most rapid. The maximum relative elemental rate of cell division is 0.12 hr.^{-1} at about 0.2 mm. from the meristematic apex. It may be noted that this distance, 200μ , is relatively great in terms of cellular dimensions. Hejnowicz's (1956) results with *Phleum pratense* roots are essentially in agreement. In the much larger roots of *Zea*, the relative elemental rate of elongation is at a maximum of 0.4 hr.^{-1} at about 4 mm. from the tip of the root cap, and of cell division, 0.16 hr.^{-1} at about 1.5 mm.

The rate of cell division at the meristematic apex itself in both *Phleum* and *Zea* is very low, so low in fact that it would be difficult to quote a precise value. It should not be supposed, however, that the cells in the initial region are inactive. The small roots of *Phleum* are advantageous for this sort of study, since the root cap is quite small and the epidermal cells of the root proper can be identified to within four or five cells of the apex. While Goodwin and Avers did not observe the cells at the very apex, it is apparent that cells 4 and 5 in Fig. 14 are in fact being displaced, although at a slow rate. There appears to be no reason to doubt that the cells at the very apex are growing and dividing and are in fact functioning as initials.

G. Growth of the Shoot Apex

No growth studies of the shoot apex have been made which are at all comparable to those which Goodwin and Avers have made of the root. The problems involved in observing a living shoot apex are perhaps more difficult than for the root, but, as I have suggested above, they should be feasible and should at the least throw some light on the questions raised by Buvat's concept of the *méristème d'attente*. There have, of course, been many studies of growing plants, mainly by physiologists, in which the height of a growing shoot has been measured from time to time either with a rule or with some sort of auxanometer, but these data are too crude to have any pertinence to the questions associated with the structure of the shoot apex. The same can be said of many studies of leaf growth, bud dormancy, seed germination, etc. The microsurgical experiments of the Snows, Wardlaw, Ball, and others are of course closely associated with problems of growth of the shoot apex. For the most part, however, the apices have been allowed to grow for

several days or weeks, before being sacrificed for study. While these experiments have illuminated some aspects of the problems of leaf and bud initiation and vascular development, the information they have yielded about the growth pattern of the apex has been largely inferential. The experiment which Wardlaw (1949) reported, that of painting the apex of *Dryopteris* with India ink and photographing it after a time, is suggestive, as is Ball's (1955) histological study of the *Lupinus* apex shortly after making a vertical cut. Direct observations such as Newman's (1956) on the apex of *Tropaeolum* should be repeated and refined.

The lack of pertinent growth data, however, has not inhibited morphologists in making growth interpretations of fixed and sectioned material. As examples, I may mention again the "growth zones" of gymnosperm apices and Buvat's intention to describe the angiosperm apex in functional terms. Many other examples could be cited, of conclusions that one region of the apex is growing more or less rapidly than another, based entirely on examination of fixed material. There is nothing wrong in principle with such attempts to interpret histological pictures in dynamic terms. It should be the goal of developmental morphology to describe the developing system, rather than the killed preparations with which one works. The difficulty with most of the growth interpretations which have been placed on the shoot apex of higher plants is that they are intuitive and qualitative, and therefore controversial, rather than rigorous and quantitative, as I believe they could be.

I have attempted above to show that in *Nitella*, *Equisetum*, and the fern root, where the cell lineage is geometrically clear, at least in part, this cellular pattern can be interpreted with some precision as a growth pattern. Even though the geometry of the apices of higher plants is not so regular, this possibility of interpretation is still present because of the fact that the cells, with their rather rigid walls, maintain their relative positions to a large degree during development. Just as in the examples cited above, the plastochron may be used as the unit of time for such interpretations. The plastochron, customarily defined as the interval of time between initiation of successive leaves, has been used by many morphologists in a descriptive sense. For example, Schmidt (1924), Zimmermann (1928), and others have described the "plastochronic" changes of dimensions of the apex associated with leaf initiation. Esau (1943, 1945) has compared the state of differentiation of the procambium, xylem, phloem, and other tissues in successive leaves on the same shoot, using *Linum* and other species, with the clear understanding that successive leaves differ by one plastochron in developmental status. Other authors have similarly used this device.

We have recently proposed a plastochron index of the developmental status of a shoot, and a related leaf plastochron index, for studies of leaf development based upon studies of vegetative growth in *Xanthium* (Erickson and Michelini, 1957). For this purpose, we have defined the plastochron in terms of an arbitrary leaf length (10 mm.) rather than in terms of the moment of leaf initiation, and have proposed to regard a shoot as having a plastochron age (P.I.) of n , when leaf n has reached the arbitrary reference length. When leaf n has exceeded 10 mm. in length, but leaf $n+1$ has not yet reached that length, the plant is regarded as between n and $n+1$ plastochrons old, and interpolation is needed to specify the plastochron age precisely. The interpolation can be based on a simple geometrical relationship between the length growth curves to yield the following formula for the plastochron index

$$\text{P.I.} = n + (\log L_n - \log 10) / (\log L_n - \log L_{n+1})$$

where n is the serial number in order of appearance in time of the leaf which is just longer than the reference length, L_n is the length of that leaf, and L_{n+1} is the length of the next leaf. The leaf plastochron index, L.P.I., of a given leaf is simply the plastochron index of the plant minus the serial number of that leaf. In other words, the L.P.I. scale is such that a leaf has a plastochron age of zero when it is 10 mm. long (or other arbitrary reference length), has a negative plastochron age when it is shorter than the reference length, and a positive plastochron age when it is longer. Both the P.I. and the L.P.I. are linearly related to time in the vegetative growth of *Xanthium*. Statistical evidence can be presented to show that the index specifies the developmental age of a plant or a leaf with a standard error of a few hundredths of a plastochron. Although our work has been with *Xanthium*, this procedure should be adaptable to the study of vegetative development of other plants as well.

I feel that much would be added to morphological studies of apical development, such as those reviewed above, if the plastochron age of each histological preparation were cited. Various developmental processes could then be described in terms of the plastochron scale, that is of a relative time scale, and various measurements could be plotted against P.I. or L.P.I. For instance, in describing the changes in size and shape of the apex during a plastochron, as Zimmerman (1928) and others have done, one could specify precisely the stage of each apex studied rather than merely arranging them subjectively in a series of "successive stages." As another example, one could plot the development of procambial strands, and of phloem and xylem, against this substitute time scale of plastochrons rather than simply presenting illustrations from which one can form only an impression of the processes involved.

Unfortunately, it does not seem possible to recast any of the voluminous published information on apical development into this form, since the requisite data are not provided, at least in the papers with which I am familiar.

It is possible, however, to make a simple growth interpretation of the angiosperm shoot apex, much like that attempted above for *Nitella* and *Equisetum*, on the basis of phyllotactic theory. In the references to phyllotaxis above (page 515 and footnote 3) it was pointed out that a given pattern is usually specified by citing two sets of intersecting parastichies. In the case of apical patterns, such as Fig. 9, A and Fig. 16, it has been customary since the time of Church (1904) and van Iterson (1907) to refer the pattern to polar coordinates and regard the parastichies as equiangular spirals for the purposes of mathematical discussion. If this is done, the formula for the 1-parastichy or generative spiral which passes through all the leaves in succession could be written

$$R_n = R_o e^{r\psi} \quad \text{or} \quad \ln R_n = \ln R_o + r\psi$$

where R_n is the radial distance from the apex to the center of a primordium n , and ψ is the angular divergence between two successive leaf primordia. The significant property of this equiangular spiral is that the radial distances, R , to successive primordia are in a constant ratio, r . Other parastichies, such as the 3- and 5-parastichies, would be represented by equiangular spirals in which the constant ratio would be $3r$ and $5r$ respectively.

Richards (1951) proposes that phyllotactic patterns be described by citing the divergence angle, ψ , and the ratio, r , which he terms the plastochron ratio, rather than a pair of intersecting parastichies.⁶ He makes the point that these two constants can be estimated from measurements of a plan or section of an apical bud by well-established statistical methods. Since standard errors of these estimates can also be calculated, his method is certainly preferable to the subjective judgment of orthogonal or contact parastichies.

For mathematical and statistical reasons, Richards also deals with the natural logarithm of the plastochron ratio, and he points out in discussion that this can be construed as a relative growth rate. If R_n is taken as the radius vector, or distance from the origin, of leaf primordium n , and R_{n+1} that of the next leaf, the expression $\ln(R_n/R_{n+1}) = \ln R_n - \ln R_{n+1}$ will be seen to be in the same form as the denominator of the plastochron index, and of the expression used above to estimate the relative plastochron rate of internode elongation in *Nitella*. It is also

⁶ It may be noted that van Iterson (1907), in his elegant mathematical study, also considered the divergence angle, α , and a ratio, a , which is the reciprocal of Richard's plastochron ratio, as two important parameters of a phyllotactic pattern.

similar to the formula usually used to estimate the relative time rate of growth, as pointed out in footnote 1. I would propose, therefore, that it might be symbolized by p , just as in the discussion of the plastochron index (Erickson and Michelini, 1957), and be referred to as the relative plastochron rate of radial displacement of the leaf primordia.

It is interesting to consider the question of the *méristème d'attente* in this light. Buvat (1952) has published figures of transverse sections of the apical bud of *Cheiranthus*, one of which is reproduced in Fig. 16. This apex is also diagrammed in longitudinal section in Fig. 11, A. In the case of Fig. 16 we might assume for discussion purposes, as Buvat does

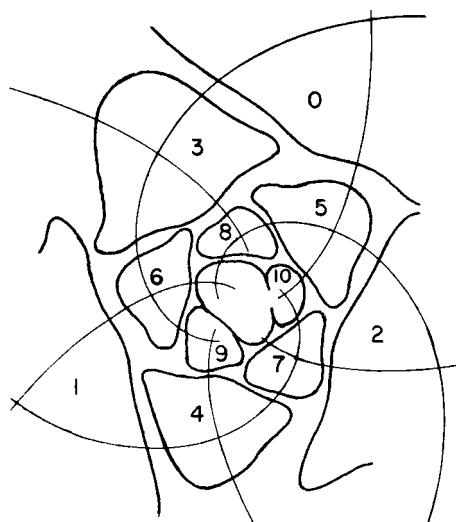


FIG. 16. Diagram of transverse section of apical bud of *Cheiranthus cheiri*. Equiangular spirals have been drawn to represent the 3- and 5-parastichies. Traced from Buvat (1952).

not, that the entire apical region and the young primordia are growing radially at a constant relative rate. This is the assumption basic to the mathematical treatments of phyllotaxis of Church, van Iterson, and Richards, and is strongly implied by the regularity of apical phyllotactic patterns. By a least-square method of solution similar to that outlined by Richards, the mean divergence angle, $138.7^\circ \pm 0.35^\circ$, and relative rate of radial displacement of 0.198 ± 0.016 per plastochron can be calculated. This corresponds to a plastochron ratio of 1.22.⁷

⁷ This does not differ significantly from the theoretical plastochron ratio, 1.19, calculated for a divergence angle of 138.7° and orthogonal intersection of the 3- and 5-parastichies.

It is apparent from this calculation that the apex is growing slowly, at about 20% per plastochron in radial dimension, even on the assumption that its growth is uniform with that of the young primordia. This rate can be put in absolute, rather than relative, terms if the scale of the drawing is introduced. Although Buvat does not cite the magnification of this figure, it can be guessed from a statement in the text, and from other figures, that the apex may be about 170 μ in diameter and that the cells at the apex are about 10 μ in diameter. Assuming a group of four or five initial cells, the radius of this group might be put at about 12.5 μ . The relative rate of 0.198, multiplied by this dimension, then gives an absolute rate of displacement from the initial group of 2.5 μ per plastochron. If the plastochron is taken as 3 days, this is indeed a slow rate. However, I see no reason to assume that this slow rate of expansion is inconsistent with the hypothesis that there are functional initial cells at the apex. Obviously, direct observation of the apex would be preferable to this hypothetical reasoning.

The similarity in form between the logarithm of the plastochron ratio and the relative plastochron rate of leaf elongation, p , which we derived in consideration of the plastochron index, suggests comparison of these two statistics in the same plant. We have done this in a most preliminary way in *Xanthium*. The relative plastochron rate of radial displacement (ln P.R.) has been estimated from a number of transverse sections of buds to be about 0.5. It is not surprising that the relative plastochron rate of elongation of the young leaves, estimated from the same sections, is considerably higher, about 0.8. This is simply a restatement of the obvious fact that the leaves arise as protuberances above the meristematic surface. However, an analysis of apical growth in this manner may reveal significant relationships.

In summary, I feel that much can be done to put our knowledge of apical development, and other modes of development, in plants into more dynamic and quantitative terms. It may be that the striking cellular patterns which are frequently encountered in meristematic tissues have led morphologists to devote too much attention to cell patterns per se. While it is true that certain of these geometrical patterns can be interpreted very easily and directly as developmental patterns, there is also a considerable diversity of apical patterns in various plants and a considerable number of apparently conflicting interpretations which have been made of similar patterns by different authors. These facts together with the evidence provided by periclinal chimeras, that histogenesis at least in the angiosperms does not follow rigidly defined cellular lines, suggest that developmental morphology should not be so preoccupied as it has been with cellular patterns. The real significance

of the organization of the plant into rigid-walled cells is that the plant leaves a record of its development which can be interpreted, with some degree of precision, in chronological terms. Developmental morphology has much to gain in making these interpretations.

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CHAPTER 13

Nucleocytoplasmic Interactions In Eggs and Embryos

By ROBERT BRIGGS and THOMAS J. KING

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I. INTRODUCTION

It is difficult, but necessary, to begin this chapter by admitting that the main part of cellular embryology as we know it today is based on discoveries made more than fifty years ago. A simple list of some of the achievements of the nineteenth century embryologists is impressive. By their efforts the cellular nature of the sperm and egg was established, the events of fertilization worked out, and the development of the egg through a long series of cell divisions and morphological transformations was described. In addition they discovered all of the microscopically visible cell organelles we know today and immediately recognized that the problems of development and heredity could be posed in terms of the functions of these subcellular elements. Of the organelles which could be studied the chromosomes were the most striking in both appearance and behavior. The fact that the chromosomes of the zygote are derived in equal numbers from the egg and sperm and are distributed by mitosis to all cells of the embryo during development suggested that they are the principal agents of heredity and differentiation (Roux, 1883). This led, in turn, to the elaboration by Weismann (1892) of a detailed theory of heredity—a theory which occupied a central position in the subsequent development of cell biology and is the starting point for this chapter.

For our purposes we will consider briefly only the parts of Weismann's theory relating to development and heredity; i.e., to the differentiation of the various body structures during the development of individuals in each generation, and the transmission of their specific character from generation to generation. The theory accounts for both types of phenomena in the following way. It was supposed that at the beginning of development the zygote contains in its chromosomes a large number of determinants, corresponding to the number of different cell types later to emerge. Heredity was accounted for by stipulating that one of these cell types, the germ cell, retains the full complement of determinants, which is passed on intact and unchanged from generation to generation via the germ line. This hypothesis of "continuity of the germ plasm" provided for the first time an intelligible and useful conception of heredity, and in modified and highly refined terms is the central fact of genetics today.

Turning to the differentiation of somatic cells, Weismann supposed that during development the different kinds of cell type determinants are segregated at each ontogenetic stage according to a precisely ordered pattern—a pattern inherent in the architecture of the germ plasm and expressed through its control of the cell division process. The segregation was supposed to result finally in only one kind of determinant re-

maining in each cell type, although provision was made for the existence of inactive determinants in cells capable of more than one expression. Recognizing that the individual cell is a complex of many structures Weismann stipulated that each cell-type determinant itself consists of many "biophors." The determinant was thought to control the cell by liberating the biophors, which migrate through pores in the nuclear membrane into the cytoplasm, where they determine the parts of the cell, each biophor being responsible for a different intracellular structure. Weismann thus conceived of biophors as the ultimate units of heredity, existing as organized chromosomal constituents, capable of reproducing themselves, extremely specific in their action, and extremely small—in the order of 50 Å. in diameter according to his rough estimation (Weismann, 1892, see p. 86). And embryonic differentiation he conceived of as an orderly segregation of groups of biophors acting as cell-type determinants.

The part of Weismann's theory dealing with heredity, based as it was on the most fragmentary evidence, has been substantiated to a surprising degree by the genetical work of the succeeding years. However, his somatic segregation hypothesis of development has experienced a different fate. Since it was so explicitly stated it could be subjected to clear-cut experimental tests. These were soon carried out by the great embryologists of the time and gave a clear result. Briefly, the tests were made by constricting or flattening eggs during the first few cleavages, thus forcing the nuclei into different cytoplasmic regions than they would normally occupy. Contrary to what would be expected on the basis of Weismann's hypothesis, these eggs developed into complete embryos which displayed a normal arrangement of their parts. This result was really conclusive in the experiments performed on "mosaic" eggs and showed that the different developmental values of the blastomeres could not be due to a segregation of nuclear determinants. Although it was not supported by experiment the additional conclusion was made that the nuclei remain identical following their distribution to the various cytoplasmic regions and that this identity probably persists throughout development (Wilson, 1925, see pp. 1061-1062).

Following the disproof of Weismann's theory of differentiation, at least in its original form, the attention of embryologists shifted to the egg cytoplasm. A long series of studies demonstrated the presence in the egg of cytoplasmic materials which sooner or later assume a specific distribution and subsequently determine the pattern for the formation of the main organ areas of the embryo. As will appear later in this chapter, fundamental problems concerning these cytoplasmic materials remain to be effectively attacked, particularly in regard to the nucleocytoplasmic interactions leading to their formation during oogenesis. But

that they exist in the egg cytoplasm and provide the initial stimulus for organ formation has been established beyond doubt.

Growing out of the work on cytoplasmic localizations came the discovery that different regions of the embryo interact in necessary and specific ways during development. This is the phenomenon of induction, in which one region depends upon a specific stimulus from another in order to carry out its own differentiation. Since the time of Spemann's original work on the subject (Spemann and Mangold, 1924; Spemann, 1938) a large proportion of embryological research has been devoted to problems of induction, most of it having been done at the "tissue level" by the familiar grafting and explantation techniques. Among other things this work has revealed that development, at least in the well-analyzed forms, involves a series of inductions, each dependent on the one preceding it. This discovery has provided a convincing demonstration of the inadequacy of any theory of development which depends entirely upon a segregation of pre-existing cytoplasmic (or nuclear) determinants and has made it necessary to reconsider the problem of nucleocytoplasmic interactions in development. A general statement of this problem, in cytological and genetical terms, may now be attempted.

(1) During oogenesis the various cytoplasmic materials are formed which later control the initial steps in organogenesis. Our first problem then concerns the nature of these materials, their location in the cytoplasm, and the nature of the nucleocytoplasmic interactions leading to their formation during the growth of the oocyte.

(2) At some point early in development the cytoplasmic materials become localized in the different regions of the egg, thus establishing a pattern for the formation of the main organ areas. The zygote nuclei play no specific role in this localization, but the material derived from the germinal vesicle is involved in it, in a manner as yet poorly understood.

(3) While the pattern for the main organ areas is initially established by the cytoplasmic localizations, these localizations are not capable by themselves of promoting gastrulation and the inductive interactions leading subsequently to organ formation. For these events the nuclei are absolutely essential, and the nature of the nuclear contribution in relation to that of the cytoplasm poses a major problem.

II. ORGANIZATION OF THE EGG

A. *The Cytoplasmic Localizations*

The original experimental method for studying the organization of the egg was in principle a very simple one. It involved destroying or removing a known portion of the egg and observing the developmental

potentialities of what was left. This method was first used by Roux (1888), who destroyed one of the first two blastomeres of the frog's egg and observed that the remaining blastomere developed into a half embryo, as if there had been a segregation of developmental potentialities at the first cleavage. Unfortunately, this result was not as clear-cut as it appeared to be at first. The complications arose from the fact that the killed blastomere was allowed to remain in contact with the surviving one and exerted a suppressing effect on its development. This particular confusion was not cleared up until considerably later when the problem of the organization of the amphibian egg was attacked by Spemann and others (see page 542). However, the influence of Roux's work was far-reaching. His method of analysis was refined and applied to eggs of numerous other species, including those of echinoderms, mollusks, ascidians, ctenophores, amphibians, and others. A particularly clear result was obtained very early with the ctenophore egg (Driesch and Morgan, 1895a, b; Fischel, 1898a, b, 1903; Yatsu, 1911-1913). This egg normally develops into a larva displaying eight rows of swimming plates, which experiment showed are derived from portions of the cytoplasm already localized before the first cleavage. The evidence for this conclusion was obtained by excising various regions of the egg cytoplasm. Removals of portions of the vegetal cytoplasm had no influence on the development of the swimming plates, but when portions of the peripheral cytoplasm of the animal hemisphere were excised the corresponding part of the larva failed to develop the plates.

Another classical example of early localization of morphogenetic materials is provided by the work of Wilson (1904) and others on the eggs of mollusks and annelids. Thus, in the mollusk *Dentalium* the unfertilized egg displays a broad equatorial pigment band and two white polar regions. At the time of the first cleavage the white material of the vegetal pole region protrudes as a rather large lobe, the well-known polar lobe. The material of this lobe is subsequently incorporated entirely into one of the first two blastomeres (the CD blastomere of cell lineage studies). At the second cleavage the polar lobe is again formed, and when the cleavage is completed it passes into one of the four blastomeres, the D blastomere. In this fashion the material of the polar lobe is eventually localized in the 2d and 4d somatoblasts, known from cell lineage studies to give rise to practically all of the larval post-trochal region. That the polar lobe is in fact acting as a morphogenetic region was demonstrated by experiments in which it was removed. Removal at the two-cell stage resulted in embryos lacking the post-trochal region. Removal of the white material of the vegetal pole region of uncleaved eggs resulted in the absence of the posttrochal region as before, but in addition the apical organ of the resulting larva was also

missing. Thus it appears that already in the uncleaved egg a visibly recognizable cytoplasmic region, the vegetal "white" region, contains materials required for the development of both the apical organ, and the posttrochal region (later to produce the coelomoblast, foot, shell and mantle folds).

Perhaps the most detailed and elegant demonstration of morphogenetic regions in the egg cytoplasm is that of Conklin, performed on eggs of the ascidian, *Styela partita* (Conklin, 1905, 1931). Following maturation and fertilization these eggs show an extensive regrouping of cytoplasmic materials, which can be followed accurately because the regions involved contain granules of characteristic appearance. Prior to first cleavage at least three different kinds of cytoplasm could be recognized by Conklin, as follows: (1) A yellow material, possessing pigment granules or mitochondria, and destined to form the mesenchyme and muscles of the larva. (2) A slate-gray material, containing yolk granules, and later to give rise to endoderm, notochord, and neural plate. (3) Clear material containing nucleoplasm from the germinal vesicle and destined to form the ectoblast. Slightly later, at the time of completion of the first cleavage, the above regions are further differentiated into a total of six cytoplasmic zones, each possessing a specific morphogenetic potentiality.

While the evidence for the existence of localized morphogenetic regions in the egg cytoplasm is most definite and precise for species like those mentioned above, the eggs of other species also display cytoplasmic localizations. In the amphibian egg the gray crescent material is localized shortly after fertilization on the dorsal side of the egg where it later leads to the formation of the chorda-mesoderm. Since the chorda-mesoderm can induce adjacent tissues to participate in the formation of a complete array of axial structures, any egg fragment or isolated blastomere which contains a supply of gray crescent material can develop into a complete embryo (Fig. 1). Contrariwise, egg fragments or blastomeres lacking gray crescent material fail to gastrulate and form no differentiated embryonic structures (Spemann, 1901; Fankhauser, 1930, 1948). In the sea urchin egg cytoplasmic materials concentrated in the vegetal blastomeres are essential for gastrulation and skeleton formation, while those concentrated in the animal blastomeres are required for the formation of ectodermal structures (Hörstadius, 1939). Here there is no precisely fixed localization, in the sense that each blastomere contains morphogenetic potentialities not present in other blastomeres. Rather it appears that both animalizing and vegetalizing materials exist throughout the egg in the form of a double gradient. [For a much more thorough discussion of the organization of various types of

eggs, see Dalcq (1938), F. E. Lehmann (1945, 1956), Costello (1948), Fankhauser (1948), Raven (1958), Watterson (1955) and others.]

From this brief survey we may take it as a general rule that the egg cytoplasm contains morphogenetic substances and that for each type of egg these substances assume a characteristic distribution which directly or indirectly determines the positions of the main organ areas of the embryo. We turn now to the problem of the nature of these substances. The first attack on this problem was made long ago by Lyon (1906), who introduced the method of centrifuging eggs to dislocate their particulate elements. This method was subsequently applied to a variety of eggs and gave a consistent result (Wilson, 1925; Conklin, 1931; Harvey, 1946; and others). In short it was found that the particulates that were known at the time (yolk, pigment, mitochondria) could

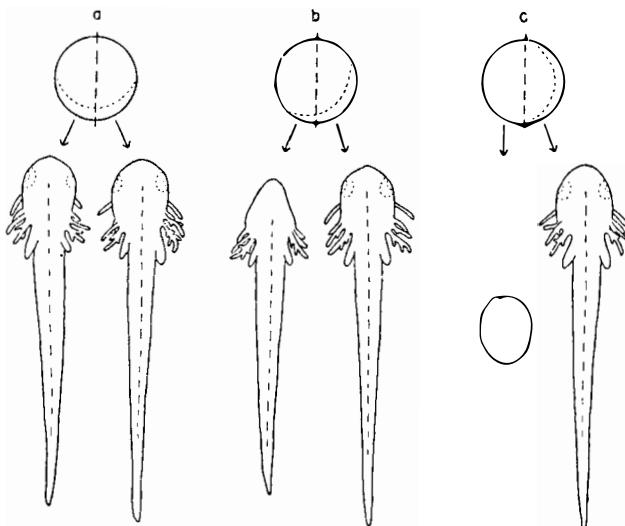


FIG. 1. Diagram illustrating the role of the "gray crescent" region of the amphibian egg in the formation of axial structures (Fankhauser, 1955).

be redistributed at will without effect on development; therefore they could not represent the morphogenetic materials. This does not mean that they play no role in differentiation. Recent work has demonstrated specific cytoplasmic distributions of mitochondria (or mitochondrial activities?) in a variety of eggs (Reverberi, 1957a-c; Berg, 1956, 1957; Lehmann and Wahli, 1954; Gustafson and Lenique, 1952; Shaver, 1956, 1957). It may be assumed that the mitochondria normally carry on essential and perhaps specific enzymatic activities in the different parts of these eggs. However, the control of these activities, insofar as they

are specifically concerned in differentiation, must reside in the "ground substance" of the cytoplasm. As to what it is in the ground cytoplasm which sets the pattern for differentiation we really have no direct evidence. However, the remarkable developments in electron microscopy and cytochemistry of the past few years should lead soon to important new studies on morphogenetic materials, and it would be appropriate at this time to attempt an appraisal of the new situation.

For our present discussion the most significant recent advances have to do with the fine structure of the cytoplasm in various types of somatic cells. This part of the cytoplasm is now known to contain an extensive system of membrane-enclosed spaces, the endoplasmic reticulum (see review by Porter, 1957). This reticulum shows connections with both nuclear and cell membranes, and has associated with it varying numbers of small (100–150 Å.) RNA-rich particles (Palade, 1955). In different cell types it shows characteristically different dispositions. Finally, this composite structure (membranes plus particles) is known to be the principal site of protein synthesis in the cytoplasm (Zamecnik and Keller, 1954; Palade and Siekewitz, 1956). Taken together these properties suggest that the endoplasmic reticulum is intimately involved in cell differentiation. In addition to the endoplasmic reticulum there is the Golgi apparatus to consider. This is now known to consist of flattened vesicles or double membranes, spherical vesicles of various sizes, and "Golgi" granules (Dalton and Felix, 1954; Sjöstrand and Hanzon, 1954). Again, the morphology of the components and their distribution in the cytoplasm varies from one cell type to another. Another interesting group of structures consists of centrioles and basal bodies, which appear to serve as centers for the formation of extensive fibrillar systems (see pages 554–5).

It would be of great interest to know the detailed form of these and other submicroscopic structures in the different cytoplasmic regions, particularly in mosaic eggs like those of annelids, ascidians, etc. However, as yet there appears to be very little information available, possibly because eggs may pose difficult technical problems in electron microscopical work. Lehmann and Wahli (1954) studied smear preparations of the 2d (neuroblast) and 4d (mesoblast) cells of *Tubifex* embryos and were able to detect differences in the populations of particles present. In the 2d cell there were numerous microsomes and spindle-shaped particles of intermediate size, but few mitochondria, while the 4d cell contained numerous mitochondria but few of the other particles.¹ In addition to this investigation there have been several studies of grow-

¹ A more recent note, by Lehmann and Mancuso (1957), reports on differences in the structure of asters and nuclear membranes in the blastomeres of *Tubifex*.

ing oöcytes showing that they contain the basic structures present in cells in general, plus some structures which appear to be peculiar to eggs. These we will take up in the appropriate part of the next section, on oögenesis.

B. Oögenesis

While it is customary to speak of the determining factors in early development as cytoplasmic localizations, etc., it is understood that this is merely a matter of convenience—to denote their location in the mature egg. That the nucleus contributes to the production of cytoplasmic materials during oögenesis was appreciated at least as long ago as 1892 when Rückert described the remarkable growth of the chromosomes in dogfish oöcytes. Rückert observed that the chromosomes become extremely long in growing oöcytes and are then reduced to a small fraction of their maximum size when growth is completed—indicating that they give off large amounts of their substance to the cell body. Weismann (1892) attached great significance to Rückert's observations and made a remarkably modern speculation concerning the nature of the chromosomal materials and their mode of transfer to the cytoplasm. He proposed (Weismann, 1892, see pp. 50–51) that "minute, specific, vital particles, and not merely nutritive substances, are produced by the chromosomes during the growth of the egg, and are then emitted through the nuclear membrane into the cell body"; and in a more general consideration of the same subject (pp. 75–76) he was more specific concerning the mode of transfer, suggesting that the particles pass through pores of the nuclear membrane. In this section we shall to some extent be examining Weismann's proposal in the light of evidence collected fifty years or more after it was made.

During oögenesis the egg cytoplasm may be assumed to interact with its own nucleus and with the nuclei (and cytoplasm) of follicle cells and nurse cells. Let us consider first the interactions between the egg cytoplasm and its own nucleus. The outstanding feature of these interactions is that noted by Rückert (1892) and since confirmed for a variety of egg types—namely, the formation of extended lampbrush chromosomes during the active growth period. These chromosomes are now known to have a structure like that illustrated in Figure 2, consisting of a very long, thin, axial fiber with numerous chromomeres along its length from which the paired lateral loops take origin (Duryee, 1950; Ris, 1954; Callan, 1955; Gall, 1955, 1956). Detailed studies of these chromosomes have been made during the past several years, and it now appears quite definite that they produce numerous particulate products which eventually find their way to the cytoplasm. The most striking

of these are the nucleoli. According to Gall (1955) nucleoli are produced repeatedly at a single locus (identical with the nucleolar-forming locus of somatic chromosomes) and liberated into the nucleoplasm where they accumulate in large numbers during oogenesis. Other observations, by Duryee (1950), Wittek (1952), and others, indicate that whole nucleoli, or contents thereof, pass through the nuclear membrane into the cytoplasm. In addition to the nucleoli thousands of smaller granules of various sizes are found in the nuclear sap. Although the evidence is not absolutely decisive it appears extremely probable that these originate as products of the loops. The loops vary greatly in size and appearance, some being fuzzy and possessing very small granules while others clearly display coarse granules approaching the nucleoli

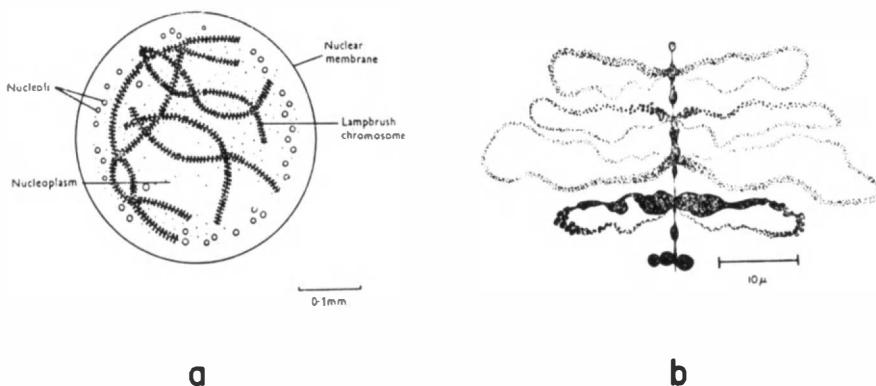


FIG. 2. a. Diagram of nucleus from a medium-size, rapidly growing amphibian oocyte (Gall, 1955). b. Semidiagrammatic representation of the detailed structure of lampbrush chromosomes (Gall, 1956).

in size. The presence of granules of apparently identical type in the nucleoplasm in the vicinity of the loops suggests that these granules are repeatedly produced and detached by the loops during oocyte growth.

The question of specificity of chromosomal products during oogenesis is, of course, an extremely important one. We refer here to the problem of whether the different chromosomal loci give rise to distinctly different products, with different functions in development. So far there is only a partial answer to this question, based on the observation that particular loci regularly give rise to loops which are distinctive as to loop size and type of particulate produced. Differences between loops associated with different loci are sufficiently characteristic and uniformly expressed to serve as a basis for mapping (Gall, 1955). That these differences are

expressions of differences in gene activity has been assumed on general grounds. This assumption is now given some support by Callan and Lloyd's (1956) recent observation of allelic differences in the form of certain loops in *Triturus* chromosomes. Thus, on a morphological basis at least it can be said that the different loci give rise to products of specific type and that in all probability these products are gene-controlled.

Concerning the function of the chromosomal products in development we know practically nothing aside from the fact that they are essential (see page 560-1). Cytochemical studies have shown that the chromomeres contain DNA (deoxyribonucleic acid), while the loop granules and nucleoli contain RNA (ribonucleic acid) and protein. (See reviews by Gall, 1955; Brachet, 1957). Generalizing on the biochemical studies of isolated organelles of mammalian somatic cells, we would expect the chromomeric DNA to be essential for the RNA and protein synthesis in the nucleoli and loops (Allfrey *et al.*, 1957), and the RNA to be essential in turn for specific types of protein or other syntheses following its transfer to the cytoplasm (Zamecnik and Keller, 1954; Goldstein and Plaut, 1955; and others). While we have no proof of such interdependencies between DNA, RNA, and various synthetic processes in the oocyte itself, there are at least strong indications that part of such a system, the transfer of RNA from nucleus to cytoplasm, does occur. Several years ago Caspersson and Schultz (1940) and Brachet (1944a) observed an accumulation of RNA in the perinuclear cytoplasm of growing oocytes. More recently Ficq (1955) has obtained evidence indicating that this perinuclear RNA is in fact derived from the nucleus. Her experiment involved injection of adenine-C¹⁴ into female frogs and at intervals thereafter observation of the distribution of the label within growing oocytes by means of radioautography. The label appeared first in the nucleus; then as the amount of labeled material later decreased in the nucleus it began to appear in the perinuclear cytoplasm. That the labeled material was RNA was demonstrated by the fact that all of it could be removed by RNA-ase. And that while still within the nucleus it was located on the chromosomes and other sedimentable structures was shown by the fact that it could be thrown down with these structures by centrifugation (Brachet and Ficq, 1956). Considering this along with other evidence concerning the location of RNA in the nucleus and its transfer from nucleus to cytoplasm (see review by Brachet, 1957), there can be little doubt that RNA is first formed in the nucleus, is located there on loop granules and nucleoli, and in some form or other is transferred to the cytoplasm. If such a transfer involved the particulates themselves it might occur through the

pores in the nuclear membrane, as suggested by Watson (1955) and others. Or it could be accomplished by a process of budding off of portions of the nuclear membrane (Rebhun, 1956a; Afzelius, 1957; Gay, 1956). However, it is also possible that RNA and other substances are transferred across the nuclear membrane in nonparticulate form, in which case the transfer could occur by diffusion through the membrane either into the endoplasmic reticulum or directly into the nonreticular cytoplasm, as well as by the other routes mentioned. This is a matter of conjecture so far and is mentioned only to point out that several possible routes of intercommunication between nucleus and cytoplasm are known which could accommodate not only large molecules but also formed organelles.

Cytoplasmic structures which may be especially concerned in nucleo-cytoplasmic interactions have recently been described by Afzelius (1956a, 1957), Rebhun (1956a, b) and others. Of greatest interest is the occurrence in the egg cytoplasm of membrane systems having properties very much like those of the nuclear membrane. In the sea urchin oöcyte each of these membranes is a double structure and displays pores of the same size and distribution as those seen in the nuclear membrane (Afzelius, 1955, 1957). As viewed in sections, the membranes enclose lens-shaped or triangular spaces packed with small (150 Å), dense particles, similar to the small particles described for other cell types by Palade (1955) and others. The origin of these membranes from the nuclear membrane is indicated not only by the close similarity in structure, but also by the fact that the nuclear membrane itself shows what appear to be stages in their formation. These range from simple granule-filled outpocketings of the nuclear membrane, to membranes which appear to be completely separated from the nucleus but still lie very close to it. While it is difficult to be sure of the nature of a dynamic process on the basis of static electron microscope images, yet the case appears quite strong for supposing that the outpocketing and detachment of granule-filled segments of the nuclear membrane is an important means of transferring material from nucleus to cytoplasm.

A phenomenon similar to the one described by Afzelius for the sea urchin oöcytes has been reported by Rebhun (1956a, b) for oöcytes of the snail, *Otala*, and the clam, *Spisula*. Here also there is evidence for the formation of cytoplasmic membranes by detachment of portions of the nuclear membrane. However, the systems formed by these membranes appear to differ in mollusk and sea urchin. In the mollusk oöcyte the membranes display small particles on their outer surfaces and are arranged in the form of stacked hollow vesicles within which yolk granules can sometimes be seen.

This leads us to a brief consideration of the so-called yolk nuclei, which take various forms in different eggs and may or may not be visibly concerned with yolk formation. In the mollusk, as mentioned above, membranous vesicles, derived from the nuclear membrane, are combined to form the yolk nucleus (Rebhun, 1956b). In the sea urchin, particle-coated cytomembranes, with no apparent relationship to the nuclear membrane, are arranged concentrically to form the yolk nuclei within which yolk granules can be seen (Afzelius, 1957). In the spider a very prominent yolk nucleus of complex structure is formed (Sotelo and Trujillo-Cenóz, 1957). This is now known to consist of a cortex, made up of many concentrically arranged double membranes with small particles on their outer surfaces, and mitochondria and Golgi elements included in the spaces between the membranes; and a medulla containing small vesicles and larger "geminated masses" of unknown function. This imposing structure shows no definite relationship to yolk formation. Still other variations in the form of yolk nuclei have been described, all of which makes the interpretation of these structures quite uncertain.

Another cytoplasmic structure which bears a relationship to the nucleus and shows interesting changes during oogenesis is the dictyosome (Golgi body). According to a recent study of Afzelius (1956a) the dictyosome in sea urchin oocytes consists of three components: (1) a stack of flattened vesicles, (2) swollen vesicles of various sizes, and (3) "Golgi" granules. In these respects it is similar to the Golgi apparatus of other cell types. The interesting feature concerns the changes in number and distribution of dictyosomes during oogenesis and early development. In very young oocytes the dictyosome is present in the usual juxtanuclear position. As the oocytes enter the active growth phase the dictyosomes become very numerous and are distributed throughout the cytoplasm. The increase in number apparently occurs by a process of division—suggesting the possibility of genetic continuity among the dictyosomes. As the oocytes reach full size the number of dictyosomes appears to decrease so that in the mature egg relatively few are present, still dispersed in the cytoplasm. In the developing zygote the dictyosomes increase in number but remain dispersed until gastrulation when they again assume a juxtanuclear position. It is of considerable interest that this reassociation of dictyosome and nucleus occurs just at the time (gastrulation) when the zygote nucleus begins to have a specific influence on developmental events (cf. pages 568, 579).

So far we have been dealing only with interactions among the various organelles within the oocyte; but there are also interactions between the oocyte and the adjacent follicle cells and nurse cells which may be important in the elaboration and organization of morphogenetic sub-

stances in the egg cytoplasm. The importance of these interactions is obvious in those cases in which the polarity of the egg is determined by its relationship to follicle or nurse cells. A particularly clear case of this sort is provided by the oöcyte of *Myzostoma*, the main axis of which is established by the position of two nurse cells, located on opposite sides of the egg (Wheeler, 1896, 1898). Other instances are known in which polarity is determined in this general fashion (Wilson, 1925, see p. 331). However, many eggs fail to show any such simple relationship between their polarity and their position in the ovary, so no general rule bearing on the matter can be laid down.

A more important question concerns the nature of the materials which we know must pass from the ovarian tissues into the oöcyte during its growth. The evidence on this problem is mainly cytological. First, there are various observations indicating quite clearly that considerable masses of material can be transferred into the egg from the adjacent cells. A recent observation of Kemp (1956) on the frog oöcyte is of interest here, although it does not provide direct evidence of material transfer. In electron microscope studies Kemp noted the presence of large numbers of microvilli projecting from the surface of growing oöcytes. These interdigitate with microvilli of the surrounding follicle cells, providing what must be a tremendous surface for exchange between the two types of cells. Microvilli have also been noted on sea urchin (Afzelius, 1956b), annelid, and enteropneust eggs (Colwin *et al.*, 1957), and may represent a common specialization of the oöcyte cell membrane, the function of which would be to maintain an adequate surface of exchange as the cell grows to very large sizes.

Convincing evidence of transfer of material from follicle cells or nurse cells into the oöcyte is available from cytological studies of insect oögenesis. In the forms being considered here the egg is associated with a number of nurse cells. During the first two-thirds or so of the growth period both the oöcyte and the nurse cells increase in size. The nurse cell nuclei also increase in size, and in *Drosophila* are known to contain polyploid amounts of DNA (Freed and Schultz, 1956), and to develop lateral loops on the chromosomes giving them a lampbrush appearance (R. C. King *et al.*, 1956). These are characteristics of metabolically active nuclei, and it may be supposed that the nurse cells are contributing something to the growth of the oöcyte. Cytological evidence of such a contribution has been provided by W. S. Hsu (1952, 1953), who traced the origin of oöcyte yolk platelets to precursors derived from the nucleoli of the nurse cells. Another cytological arrangement whereby this general sort of transfer might be accomplished has been described by Nusbaum-Hilarowicz (1917) for the water beetle (*Dytiscus*). In this

form there is a special cytoplasmic region, characterized by the presence of numerous mitochondria, which surrounds the nurse-cell nuclei and extends in continuous streams to a similar cytoplasmic region encircling the oöcyte nucleus (Fig. 3). It would be of great interest to know if material is actually being transferred between the perinuclear regions of nurse cells and oöcyte—as this cytological arrangement would suggest.

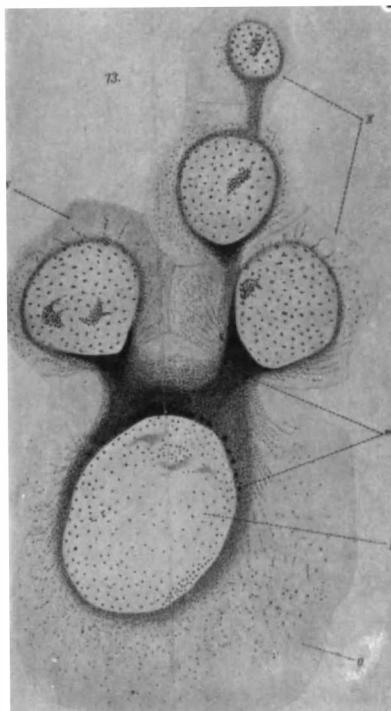


FIG. 3. Cytoplasmic interconnections between oöcyte (*O*) and nurse cell (*N*) nuclei in the water beetle, *Dytiscus* (Nusbaum-Hilarowicz, 1917).

During the latter part of the oöcyte growth period the nurse cells in some forms pass entire into the egg where they break down and become incorporated into the egg cytoplasm. This is the case, for example, in the sawfly (Peacock and Gresson, 1928) and in *Habrobracon* (Von Borstel, 1957) (Fig. 4). Here there can be no question concerning the fact that a mass of cell constituents, including several polyploid nuclei, are contributed by the nurse cells to the egg cytoplasm. In other cases (e.g., *Drosophila*) the nurse cells remain separate from the oöcyte, but decrease in size toward the end of oogenesis and eventually

degenerate as the egg completes its growth. (R. C. King *et al.*, 1956). Presumably the substance of the nurse cells may be utilized by the growing egg.

Concerning the nature and morphogenetic function of materials contributed by follicle or nurse cells to the oöcyte we know very little. Purines (adenosine) and amino acids (Ficq, 1955), and even proteins (Telfer, 1954) are known to pass from the body fluids into the oöcyte,

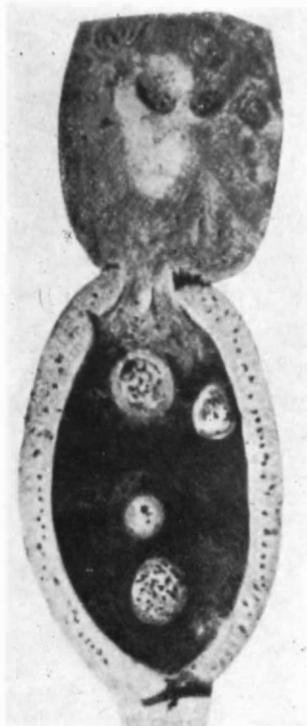


FIG. 4. Photograph illustrating incorporation of nurse cells into the oöcyte of the sawfly, *Pristiphora*. The nurse cells are located above the oöcyte in the figure (Peacock and Gresson, 1928).

but the role played by the accessory cells in the transfer is unknown. In the case of insect oöcytes which indirectly or directly absorb polyploid nurse cells one would expect the mature egg to contain a complex of substances so derived, including nucleic acids or breakdown products thereof. Analyses of unfertilized *Drosophila* eggs give results consistent with this expectation, although it should be pointed out that it is not actually known to what extent the substances in question are derived from

the accessory cells and to what extent from synthetic processes within the oocyte itself. In any case *Drosophila* eggs have been shown to contain large amounts of purine and pyrimidine bases, nucleosides, and nucleotides, including DNA fragments such as deoxyribosides and thymidine, which must be located in the cytoplasm since the amounts present could not be contained in the egg nucleus. It appears further that the relative amounts of certain of these nucleic acid components (e.g., adenine, cytosine, thymine) are different in eggs from XXY females compared with eggs from XX females, indicating a nuclear control of their synthesis (Levenbook *et al.*, 1958; Travaglini *et al.*, 1958; Schultz, 1956). The developmental function of these substances, particularly the DNA fragments, is still an unsolved problem. They occur in frog and sea urchin as well as in insect eggs (see review by Brachet, 1957). It has been proposed that they represent precursors for the rapid formation of new nuclear DNA during cleavage (Hoff-Jørgensen and Zeuthen, 1952; and others). Recent evidence (Grant, 1955, 1958) indicates that new DNA is formed during cleavage and involves the incorporation of both DNA fragments and smaller components (glycine). Whether this new DNA is actually located in the newly formed nuclei apparently remains to be determined.

A more important question from our immediate point of view concerns the relationship of these substances to the morphogenetic regions of the egg and early embryo. About this nothing at all is known, so far as we are aware. However, we should like to mention in this connection a recent observation of Bieber (1958), as yet unpublished, indicating (1) that two types of DNA are present in the frog embryo, and (2) that the ratio of the two types changes during development. The details of this study may be most pertinent to some of the problems discussed in this section.

C. Summary

The early work in experimental embryology demonstrated the central importance of the cytoplasmic localizations of the egg in development, but left unsolved the problems it raised concerning the nature of the localizations and their mode of origin during oogenesis. For many years these problems received little attention, probably because no new approaches were available to add to the kind of information already obtained by classical cytological and embryological methods. However, during the past ten or fifteen years powerful new techniques have been developed in cell biology. Since these have not yet been applied extensively to the problem of the ooplasmic localizations it seemed desirable to emphasize the importance of the problem at this time and to review

some of the work that has been done with the new approaches. This work has already provided evidence for (1) the formation of particulate products by the lampbrush chromosomes during oöcyte growth, (2) the transfer of particulates and perhaps other materials between nucleus and cytoplasm, (3) transfer of materials between follicle cells or nurse cells and the oöcyte, and (4) the presence of a series of submicroscopic organelles, some of them apparently unique, in the egg cytoplasm. It is not yet possible to assign specific morphogenetic significance to any of the recently discovered materials or interactions, but this is not surprising considering that the new work has barely begun. The important fact is that the area of inquiry into the nature of the morphogenetic localizations has now been greatly extended.

III. NUCLEOCYTOPLASMIC INTERACTIONS IN CLEAVAGE

At the end of its growth period the oöcyte consists of a mass of cytoplasm, with its numerous organelles; and a germinal vesicle containing not only chromosomes but also a large volume of nonchromosomal material, including numerous nucleoli and smaller particulates as well as the nonparticulate nucleoplasm. In preparation for the first maturation division the germinal vesicle breaks down, liberating all the components mentioned above into the cytoplasm. At some point in the maturation process insemination occurs, and there is introduced with the sperm not only the chromosomes but also an array of cytoplasmic organelles including the centrioles, remnants of the endoplasmic reticulum, mitochondria, and the acrosome. Our task in this section will be to review the evidence concerning the roles of these organelles or groups of organelles in cleavage. Their roles in more advanced stages of development will be dealt with in a later section.

A. *The Centrosome*

This structure goes by various names—cell center, cleavage center, central body—all indicative of the importance once attached to it as the center of activities in fertilization and cleavage. Among the early experimental embryologists it was the object of a great deal of study, but unfortunately certain important questions about it could not be answered. Perhaps the most difficult and important of these concerned its mode of origin. The balance of evidence indicated that centrosomes are self-duplicating structures, and thereby may qualify as genetic units. But there were also reports of *de novo* origin of centrosomes in the egg cytoplasm which, if substantiated, would place it in quite a different position. Instead of being a controlling element it would now be one which could be discarded or formed anew, as directed by some other

part of the cell. The difficulty in obtaining decisive information on this and other problems was, of course, a technical one. It had been realized since Boveri's time that the centrosome in some cells can be seen to contain a much smaller body, the centriole, thought to be the actual "center." Since the centriole was barely detectable with the light microscope it was impossible to study its structure or to obtain definite evidence concerning even its presence or absence in many cases. This deficiency is now finally being overcome. Recent electron microscope studies have shown the centriole to be a cylindrical body, possessing a characteristic structure, and varying in diameter from 100 to 200 m μ and in length from 300 to 500 m μ . (de Harven and Bernhard, 1956; Burgos and Fawcett, 1955, 1956; Rebhun, 1957; Porter, 1957). In dividing cells it is the center for the fibrillar structures of the mitotic figure (de Harven and Bernhard, 1956), and in some nondividing cells it acts as a center for the formation of specialized cytoplasmic structures, again fibrillar in nature. The outstanding example of the latter is found in the sperm cell, in which the tail flagellum takes origin from one of the two centrioles present at the base of the nucleus. Interest in the centriole is further increased by its very close similarity to the basal bodies of ciliated somatic cells (see review by Porter, 1957). The two structures appear to be identical morphologically, and give rise to fibrillar structures of the same basic design—indicating that they are very closely related if not actually derivable from one another. Considering these properties and others not mentioned here, it would seem that centrioles and related structures are involved not only in mitosis, but perhaps also in the formation of specialized cytoplasmic structures of differentiated cells. (See Porter, 1957, for a discussion of this subject.) It should be made clear that these new studies have not yet been extended to the egg and that most of the old problems concerning the history of the centrosome in fertilization and cleavage are still unsolved. However, they can now be examined against a new background.

B. Source of the Cleavage Center

1. The Sperm Centrosome

There is a great deal of evidence that the centrosome, or cleavage center, of fertilized eggs is normally derived from the sperm middle piece. In many eggs, for example, those of sea urchins, nemertines, ascidians, or amphibians, the center can be observed to arise in the region of the middle piece and later to divide—thus forming the cleavage amphiaster. The egg pronucleus meanwhile gives no indication that it possesses an active center (see review by Wilson, 1925; Fankhauser,

1948). A more convincing demonstration along these lines is provided by Boveri's (1888) discovery of partial fertilization in sea urchin eggs. Boveri was able to show that occasionally the sperm center outstrips the nucleus in its migration toward the egg nucleus. In such cases the center forms an amphiaster which includes only the egg nucleus, and in the ensuing mitosis the lagging sperm nucleus is left behind and passes entirely into one of the first two blastomeres. Here, it would seem, is a clear demonstration of the source of the center and of its controlling role in cleavage.

2. *The Egg Centrosome*

If the cleavage center is derived from the sperm middle piece, we may well ask what has happened to the egg centrosome. Is it lost, or inactivated, or perhaps both? This matter can probably be settled finally only by studies on the centriole itself, which would have to be done with the electron microscope. Such studies have, of course, demonstrated beautifully the paired centrioles of the sperm, so we now know definitely that these important organelles are introduced into the egg in fertilization. But locating the egg centrioles with the electron microscope would be a laborious job and so far as we are aware has not yet been accomplished. Despite this grave deficiency, we may still derive some information from the older literature. For one thing, it is quite definite that the egg centrosome is not lost—at least in the beginning of development. In some cases, e.g., in *Crepidula* (Conklin, 1904) the egg pronucleus normally has associated with it its own center—and apparently both it and the sperm center participate in setting up the cleavage amphiaster. However, the more pertinent cases are those in which the egg nucleus normally displays no active center of its own. In effect this means that it forms no aster, the observations being such that the chance of finding a centrosome in the absence of an aster are very small indeed. Thus the egg centrosome could easily persist and remain undetected; and apparently this is the case. The most convincing evidence to this effect is provided by experiments in parthenogenesis. Here no sperm is introduced; yet the egg can be induced to develop. The parthenogenetic stimulus somehow activates the egg center to form regular amphiasters and spindles. The activation of the center occurs readily in most marine eggs but much less so in amphibian eggs, which never form normal amphiasters unless some particulate product from older embryonic or adult cells is introduced (Shaver, 1953). And even following the injection of active cleavage-initiating material the proportion of eggs which cleave and develop in regular fashion is small—indicating that the amphibian egg center is quite difficult to stimulate to normal mitotic activity.

From the evidence outlined in the preceding paragraph it appears that a center is present but usually remains quiescent in normally fertilized eggs. While this may be due partly to the fact that the egg center is less readily activated than is that of the sperm, it is also possible that the sperm center exerts a suppressing effect on it. Fitting in with this interpretation is a very pretty demonstration of suppression of centers, which we owe to Fankhauser (1948). In the newt egg, fertilization is normally polyspermic, with up to ten or more sperm cells entering the egg. All of the sperm nuclei develop asters and are indistinguishable from one another until one of them fuses with the egg nucleus. At this point the accessory sperm nuclei begin to be inhibited. Their asters fail to divide, the nuclei begin to degenerate, and eventually the whole complex is lost from view in the cytoplasm. Apparently some suppressing influence from the fusion nucleus (and center) traverses the cytoplasm and inhibits the accessory nuclei—centers and all. Of course, there are differences between this interaction and the one which we must assume occurs between sperm and egg nuclei resulting in the suppression of the egg center. Still, Fankhauser's observations provide a particularly clear case of suppression which, if analyzed further might give important information on the suppressing agent and on the fate of the affected centrioles.

C. Independent Division of Centers

The idea that the centrosome is a self-reproducing structure, exhibiting genetic continuity in successive cell divisions, was most strongly supported by Boveri's (1896) discovery of independent divisions of centrosomes in dispermic sea urchin eggs. During cleavage of these eggs the chromosomes sometimes go entirely to one pole of the mitotic figure, so that one of the daughter blastomeres contains a nucleus plus center, while the other contains a center only. Subsequently the center in the nonnucleated blastomere divides and forms a perfect amphiaster. This process is regularly repeated at each cleavage interval, so that eventually a large number of centers arise through this type of independent division. This result has been confirmed and extended by several investigators, demonstrating conclusively that centers may divide independently of the nuclei.

In Boveri's experiments the regular divisions of the centers were sometimes accompanied by the formation of cleavage furrows, but these soon faded away. Thus it appeared that while centers could divide independently they could not bring about genuine cell division in the complete absence of chromatin (see also Dalcq, 1931). This conclusion has since been revised, largely as a result of the work of Fankhauser (1929, 1934a), who has provided unequivocal evidence of cleavage with-

out chromatin in *Triton* (*Triturus*) eggs. It was mentioned above that normally the accessory sperm nuclei in these eggs are caused to degenerate by some influence proceeding from the fusion nucleus. However, if the egg is constricted into two fragments shortly after fertilization this degeneration is prevented in the fragment lacking the fusion nucleus. The accessory sperm nuclei now become mitotically active, but usually in an abnormal fashion. Fankhauser made a careful study of the irregular mitoses and showed that they resulted in the complete elimination of chromatin from some blastomeres, which none the less displayed asters or amphiasters and divided several times, giving rise to large numbers of nonnucleated cells in the blastula (Fig. 5). Similar results have since

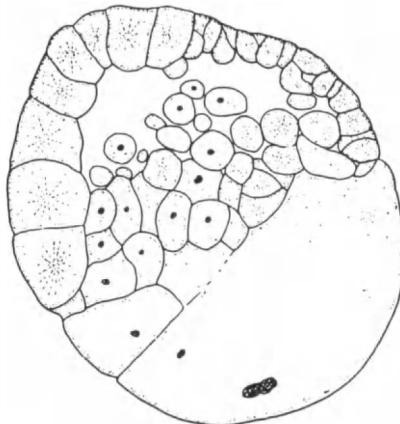


FIG. 5. Cleavage without chromosomes (*Triton*). Section of partial blastula in which majority of cells contains asters but no chromatin (Fankhauser, 1934a).

been obtained by several investigators, establishing firmly the fact that blastomeres completely devoid of chromatin may go through several regular cleavages, if they contain active centers (Harvey, 1936; Stauffer, 1945; Gross, 1936; Tchou-Su, 1931; Briggs *et al.*, 1951). However, there is a definite limit to the extent of the cleavage which can be promoted by the centers in the absence of chromosomes. In sea urchin eggs the nonnucleated blastomeres divide into cells of a size approximating that of nucleated cells of a mid-blastula. No further cleavage and no differentiation occur, even when the nonnucleated blastomeres are in contact with normally differentiating nucleated cells (Lorch *et al.*, 1953). In amphibian eggs the cleavages also stop when the nonnucleated cells reach a size comparable with that of mid- to late-blastula cells of control embryos; and no further division is obtained, even when the non-nucleated cells are grafted to normal embryos (Briggs *et al.*, 1951). This

probably means that the cleavages can only go on until the stage is reached at which interphase cell growth normally begins, the non-nucleated cells being capable of cleavage in the strict sense, but not of divisions that require cell growth in the interphase. The deficiency due to the absence of the chromosomes cannot be made up by diffusible products from normal cells, as the experiments referred to above demonstrate. Whether anything less than an intact nucleus could promote synthesis and further divisions in these cells has not been tested.

D. *De Novo Origin of Centers*

Before going on to a consideration of the relationship between centrosomes and other cell components we should say something on the fundamental and still unresolved problem of *de novo* origin of centrosomes, or more specifically, centrioles. In Wilson's view (1925, see pp. 684-690) the most conclusive of the older work on this problem was that of Yatsu (1905), who cut mature unfertilized eggs of *Cerebratulus* into nucleated and nonnucleated halves and then treated them with a parthenogenetic agent, hypertonic sea water. The nucleated half was thereby stimulated to parthenogenetic development, as anticipated. The half lacking the nucleus and center did not cleave, but it did form numerous cytasters consisting of astral rays and centrosomes within which several tiny granules (centrioles?) could be seen. Before discussing this result we should mention also the more recent and better-known experiments of E. B. Harvey (1936, 1940). These experiments involved centrifuging mature unfertilized sea urchin eggs into nucleated and nonnucleated fragments. Parthenogenetic stimulation applied to the non-nucleated fragments resulted not only in the formation of cytasters, but also in cleavage. In these and other cases (Lorch, 1952) it appears fairly definite that asters arise anew in the egg cytoplasm, and not by descent from the center associated with the egg nucleus. The question that has not yet been resolved is whether the two types of asters are really identical. What evidence we have on this point suggests that they are not. For one thing, it appears that although cytasters may divide they do not form spindles (Harvey, 1940; Yatsu, 1905). In this respect they differ from the amphiasters derived from nuclear centers. These too may lack chromosomes, but nonetheless display spindles (Fankhauser, 1934a; Briggs *et al.*, 1951). A more important question concerns the centrioles. As mentioned previously, centrioles are now known from electron microscope studies to possess a characteristic structure and to be at the center of considerable morphological organization in the cell. It would be a matter of great importance to know if they might arise *de novo* in conjunction with asters in the egg cytoplasm. This was fully

appreciated by the great cytologists of the past, who did a great deal of work on the problem (see review—Wilson, 1925). Because the centriole is so small their results were necessarily equivocal, and the final answer to the problem will have to wait on fine structure studies with the electron microscope.

E. The Nuclear Contribution to Cleavage

Here we have to deal with two main nuclear components, which are: (1) the chromosomes, and (2) the nonchromosomal material of the germinal vesicle. Of the two, the chromosomes have already been discussed to some extent in the preceding section, in which we have presented evidence showing that blastomeres may cleave in the complete absence of chromosomes. To this may be added the fact that, within broad limits, changes in chromosome number or type (e.g., in hybrids) exert no influence on pregastrula development (see following section). However, it would be incorrect to make a general conclusion from these facts that chromosomal material has no influence on cleavage. It is true that in sea urchin egg fragments cleavage occurs to a limited extent in the complete absence of cytologically demonstrable chromatin (Harvey, 1936, 1940). However, in the amphibian egg the situation may be different. For one thing, the achromosomal blastomeres cleave more slowly than do nucleated ones (Briggs *et al.*, 1951), and among the nucleated ones those with abnormally small chromosome numbers appear to cleave less readily than do those with a large number of chromosomes (Dalcq, 1929). So the chromatin has at least a quantitative effect on rate and extent of cleavage. Furthermore, there are some indications that if the egg is completely devoid of chromatin, cleavage may not occur at all. In Fankhauser's (1934a) experiments with newt eggs, the egg fragments displaying achromosomal cleavage always contained, somewhere in the fragment, some chromosomal material. Completely nonnucleated fragments were not observed to divide. The same was true in the experiments of Briggs *et al.* (1951), in which frogs' eggs were inseminated with heavily X-irradiated sperm and then enucleated. The majority of the cleavages occurred without chromatin, but elsewhere in the egg disorganized Feulgen-positive material, derived from the irradiated sperm nucleus, could always be found. An attempt to obtain cleavage in eggs completely lacking chromosomal material was made by injecting cleavage-initiating material (granules from neurulae) into enucleated eggs, but none of the DNA-free eggs cleaved. While these results show that chromatin in some form is always present in cleaving eggs, they still do not provide a definite answer to the question of whether it is essential. Eggs completely devoid of chromatin are also likely to lack

normal cleavage centers, and it may be the absence of the centers which is critical. Thus it appears that a final solution to this problem of the role of chromatin in relation to that of cleavage centers will require experimentation in which the two components can be introduced separately or together into enucleated eggs.

Earlier in this paper we outlined the evidence indicating that the lampbrush chromosomes give off products to the nucleoplasm, and that some of these products are transferred to the cytoplasm during oöcyte growth. However, a great deal of material is accumulated in the germinal vesicle and then dispersed all at once into the cytoplasm when the germinal vesicle breaks down prior to the first meiotic division. An analysis of the role of this material in cleavage was carried out long ago by Delage, Wilson, Yatsu, and others, on eggs of sea urchins and nemertines (review by Wilson, 1925, p. 404). These eggs can be cut into two fragments either before or after dissolution of the germinal vesicle. If the operation is performed *before* germinal vesicle breakdown the half containing the germinal vesicle can be fertilized or parthenogenetically activated, although this happens only after the vesicle in this half breaks down. The other half, lacking germinal vesicle material, cannot be fertilized or activated. On the other hand, when the operation is performed *after* germinal vesicle breakdown both halves can be fertilized and will cleave. If they are parthenogenetically activated the half containing the egg nucleus will cleave; the other half, containing dispersed nonchromosomal germinal vehicle materials, does not cleave but does form numerous cytasters. These experiments show that some nonchromosomal component of the germinal vesicle must be added to the cytoplasm before it becomes fertilizable or activatable. The effect of the absence of this material could be either to prevent the penetration of the sperm cell, or to prevent the formation of asters, etc., from it if it does enter. In the sea urchin egg, at least, the latter would appear to be the case, for immature eggs, with intact germinal vesicles, can be penetrated by sperm cells; but the nuclei of these cells fail to enlarge or form asters (Hertwig and Hertwig, 1887). The evidence thus leads to the important conclusion that some nonchromosomal constituent of the germinal vesicle is required for the formation of asters and spindles, i.e., the mitotic apparatus (Mazia and Dan, 1952). It also appears to be preferentially distributed to certain cytoplasmic localizations in the mature egg—for example the localization giving rise to ectoderm in *Styela* (Conklin, 1905), and possibly the gray crescent region of amphibian eggs (Brachet, 1944a, see p. 359). As to what this material might be we are not quite sure, but there are some very suggestive leads from the more recent literature (see review by

Brachet, 1957, p. 136). Perhaps the most interesting of these concerns the —SH proteins, known for a long time to be essential in cell division (Hammett, 1929; Rapkine, 1931), and now known also to be an important constituent of spindles and asters (Mazia and Dan, 1952). According to Brachet the germinal vesicle is very rich in these proteins. Following its dissolution they would become available for the construction of asters and spindles and might well be the indispensable material contributed by the germinal vesicle. The germinal vesicle in addition contains other substances—including of course RNA—which enter into spindle formation and would also have to be considered in further work on this problem.

F. Other Components of the Fertilized Egg

The centrosomes, chromosomes, and germinal vesicle materials can all be experimentally manipulated so as to reveal something of their roles in cleavage. However, the other constituents of the fertilized egg are more difficult to experiment with, and much less is known about their specific roles in early development. Of the ones mentioned at the beginning of this section, those contributed by the sperm can be disposed of quickly. The acrosome plays an important role in fertilization, discharging the achromosomal filament which penetrates and appears to digest the vitelline membrane and is the part of the sperm which first contacts the egg surface proper (Colwin *et al.*, 1957). Its fate following incorporation into the egg is unknown. The sperm is also known to carry with it mitochondria, remnants of the endoplasmic reticulum, the centrosome and chromosomes of course, and sometimes the whole tail. Except for the centrosome and chromosomes the history and function of these organelles in cleavage is obscure. Since eggs can develop parthenogenetically they cannot be performing indispensable functions in early development. They might conceivably have effects on later phases of differentiation, but this is so far entirely conjectural.

Finally, we should consider briefly what is left of the egg—namely, the main mass of cytoplasm, packed with various organelles, and containing a variety of chemical constituents, including the interesting but still enigmatic cytoplasmic "DNA" (see review by Brachet, 1957). Unfortunately, it has so far been impossible to determine what control this component may exert by itself on cleavage or development, because it must always be mixed with the contents of the germinal vesicle before development can commence. Together these components control the most fundamental features of early development—the type and rate of cleavage and the distribution of organ-forming regions. It would be of great importance to know if the control of these aspects of develop-

ment resides in the oocyte cytoplasm, or in the nucleoplasm of the germinal vesicle, or perhaps requires specific contributions from both. The problem will become more concrete if we describe a specific case, in which an experimental analysis might eventually be feasible. For this purpose we would like to refer to the work of some years ago on the genetic control of cleavage pattern in the snail, *Limnaea* (Boycott *et al.*, 1931). As is well known the spiral cleavage in this form occurs either dextrally or sinistrally, depending on the genetic constitution of the mother. Actually, before the cleavages occur it is already apparent which direction they will follow since the control is first exercised on the spindles, causing them to be tilted one way or the other (Fig. 6). The sperm cell, even though it contributes the cleavage center, and

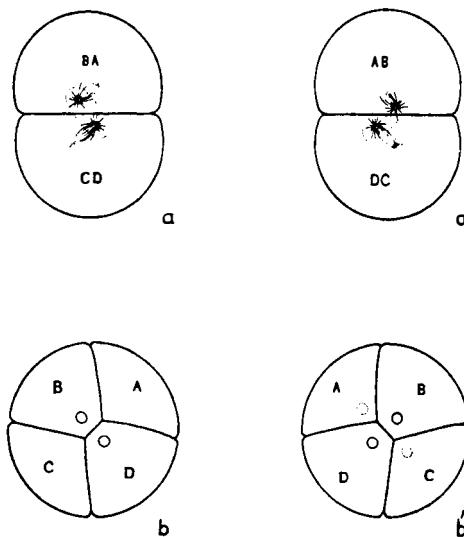


FIG. 6. Genetic control of cleavage in the snail. Orientation of spindles and cleavage furrows in eggs of sinistral (a, b) and dextral (a', b') mothers (Morgan, 1927).

sometimes a genetic make-up differing from that of the egg, exerts no influence on cleavage (except a genetic one to be expressed only in the eggs of the next generation). Thus the cleavage pattern is determined entirely by the egg nucleus through its influence on the egg cytoplasm. How this is accomplished is unknown, but we can recognize two alternatives of general genetical interest. It will be recalled that the oocyte goes through its entire growth without a breakdown of the nuclear membrane. However, there is considerable evidence indicating that chromosomal products may be transferred through the intact membrane

to the cytoplasm, and it is quite possible that the genetic control of cleavage pattern may be effected in this way. Follicle-cell nuclei could also function in this fashion. On the other hand, we have also seen that a massive dispersal of germinal vesicle contents occurs on maturation and that these nuclear products are absolutely essential for the formation of the mitotic apparatus and the initiation of development. It is equally possible that the genetic control of cleavage is mediated at this time rather than earlier, through the intact nuclear membrane. In this particular situation it would seem that an experimental choice might be made between these alternatives—by the theoretically simple expedient of replacing the germinal vesicle material of dextral eggs with that of sinistral ones, and vice versa. Undoubtedly technical problems of some magnitude might be encountered, but the embryological and genetical importance of the problem should be sufficient stimulus for these to be overcome.

G. Summary

In this section an attempt has been made to assess the relative roles of the various cell organelles in cleavage, and in this connection to point out what appear to be the outstanding unsolved problems. The following main points emerge:

(1) Cleavage requires the presence of a normal cleavage center or centrosome, usually derived from the sperm middle piece. Although it cannot be readily seen in the light microscope, the important component of the center is known from electron microscope studies to be the centriole.

(2) The center associated with the egg nucleus appears usually to be suppressed in the presence of the sperm and shows no sign of activity. Its normal fate in cleavage is unknown. In the absence of the sperm it may be activated parthenogenetically to give rise to amphiasters and normal cleavage figures.

(3) Cleavage centers may divide independently of the nucleus and give rise to regular amphiasters. Repeated divisions may be accompanied by regular cleavages of the egg, and the production of blastulae made up wholly or in part of cells completely devoid of chromatin.

(4) While there is much evidence indicating that centers arise by division of pre-existing centers, there are also reports of *de novo* origin of centers in the egg cytoplasm. The status of the *de novo* centers will remain in doubt until it can be demonstrated whether or not they contain centrioles.

(5) The activity of the cleavage centers is absolutely dependent on the presence of some nonchromosomal factor derived from the germinal vesicle during its breakdown at maturation.

(6) Given a normally active center, the rate and pattern of cleavage and the disposition of the organ-forming localizations is determined by a mixture of (a) the oöcyte cytoplasm, plus (b) the dispersed contents of the germinal vesicle. The problem of determining the relative roles of these two components is discussed.

IV. NUCLEOCYTOPLASMIC INTERACTIONS IN DIFFERENTIATION

The early work in experimental embryology soon established the fact that the main organ areas of the embryo are determined by the cytoplasmic localizations, the nuclei in the beginning of development being equivalent with respect to both chromosome make-up and developmental potentialities. But, as we have noted in the preceding section, the nuclei are also known to be essential for the pattern of differentiation to be realized, and it must be assumed that they interact in specific ways with the different cytoplasmic materials during development. The analysis of these interactions, particularly with regard to the role of the chromosomes, dates back to the work of Boveri and includes an extensive series of studies. In what follows we will try to outline the main results of these investigations, referring to various reviews for more detailed and adequate accounts as we go along.

A. Chromosome Studies

1. Chromosomes of Normal Embryos

In reviewing this subject we revert to Weismann and his theory of somatic segregation of chromosomal determinants of differentiation. This theory was disproved by two types of evidence. One of these, already referred to, consisted of the demonstration that the cytoplasmic localizations, and not the nuclei, set the original pattern of differentiation. The second was the cytological demonstration of the individuality of chromosomes, and of the fact that one of each type is distributed to the daughter cells at each mitosis—insuring identical chromosome sets in all the cells of the body (Boveri, 1909; and others). This generalization has stood quite well the test of time. In general, where accurate studies can be made, the numbers and types of chromosomes are found to be the same in a variety of normal somatic cells. Occasional reports of pronounced variations in chromosome numbers (Therman and Timonen, 1951) have not been confirmed on closer study (Hsu and Pomerat, 1953; Hungerford, 1955; Nowell *et al.*, 1958), and deviations from euploidy on the whole are seen only in a small minority of cells. The same situation exists with respect to reductional divisions of somatic cells, such as have been reported for micromeres of sea urchin eggs (Lindahl, 1953), and mesenchyme cells of frog embryos (Green, 1953),

but subsequently refuted. (Freed and Green, 1955; Makino and Alfert, 1954). A more serious question concerning the identity of the chromosome complements arose from reports of variations in the banding pattern of the polytene chromosomes of insects. (Kosswig and Sengün, 1947). But again, detailed studies failed to confirm the existence of such variations, and instead showed that homologous chromosomes in different tissues possess homologous banding patterns. The identity in this case extends to the finest resolvable bands and provides the best evidence we have that the same gene loci are present in the different cell types (see review by Beermann, 1956).

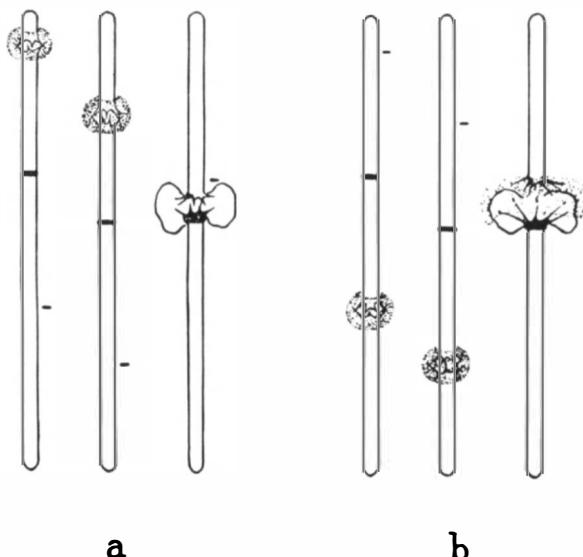


FIG. 7. Chromosomal differentiation in functionally different lobes of the salivary gland of the chironomid, *Acricotopus*. a. Chromosomes of the posterior and lateral lobes. b. Chromosomes of the anterior lobe—the only lobe forming a visible brown secretion. Balbiani rings are shown stippled, the kinetochore regions black, and the single large nucleolus white (Beermann, 1956).

As a result of this recent work the conclusion that differentiation cannot depend on somatic segregation of chromosomal determinants is more firmly established than ever. There are, however, other kinds of chromosomal variations which occur normally and are correlated with differentiation. The best-known of these are cases of polyploidy or polyteny, known for a long time to be characteristic of only certain cell types, e.g., salivary gland or gut cells of insects; liver cells of mammals. We must assume that the presence of multiple sets of chromosomes or chromonemata has some functional significance in these cells, but what

it is unknown. Another type of chromosomal variation, more recently described, would seem to have potentially great significance in cell differentiation. We refer to the discovery that puffs, or Balbiani rings, develop from *different* chromosomal bands in different cell types of chironomid larvae, as illustrated in Figure 7 (Beermann, 1956; Mechelke, 1953). A comparable phenomenon has been described also for sciarid larvae (Breuer and Pavan, 1955), in which there is the additional evidence that puffing is accompanied by a significant increase in the DNA content of the chromosomal regions involved (Rudkin and Corlette, 1957). There is reason to suppose that the puffing of the bands is a reflection of gene activity (Beermann, 1956). If so, the cytological evidence indicates clearly that different genes are called into activity in different tissues.

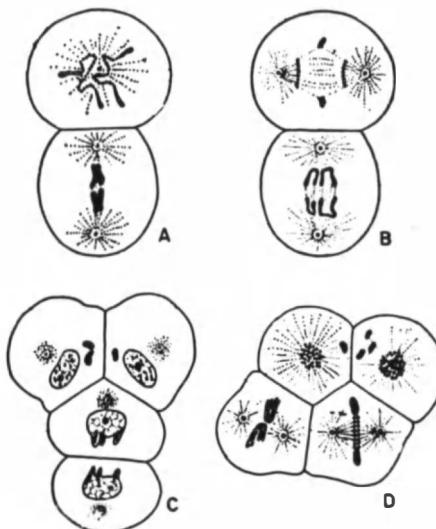


FIG. 8. Chromosome diminution in somatic blastomeres, but not in blastomeres of the germ line, in *Ascaris* (Boveri, 1910; figure from Stern, 1954).

Before closing this brief discussion of chromosomes in normal embryos, we should consider those remarkable cases of chromosome diminution and chromosome elimination (see reviews: White, 1954; Beermann, 1956) which at first sight seem to contradict much of what has been said above. In the classical case (*Ascaris*) the fertilized egg contains two large polycentric chromosomes, which subsequently undergo diminution in blastomeres destined to form somatic cells, but not in those which produce germ cells (Boveri, 1910). Each diminution involves a loss of the larger terminal segments of the chromosomes, and a frag-

mentation of the central regions into a large number of small chromosomes which are then distributed in normal fashion during subsequent cleavages (Fig. 8). This brings about a clear difference in the chromosomes of somatic cells and germ cells and was originally thought to constitute definite evidence of chromosomal segregation correlated with cell differentiation. However, this can be true only in a special and limited sense—limited because the evidence demonstrates differences between germ cells and somatic cells, but *not* between different kinds of somatic cells—and special because of the extraordinary character of the original pair of very large chromosomes. As already noted, these chromosomes retain their large terminal regions only in the germ cells. What function do they perform there, and what is the significance of their elimination from all somatic cells? No answers are available to these questions, but we may venture an opinion that the terminal segments, which appear cytologically to be heterochromatic, are acting as modifiers of gene activity. In *Drosophila*, for example, the heterochromatic Y chromosome, containing itself only a small number of genes mainly affecting fertility, is known to modify the expression of a number of other genes which may be located near it in various translocations (Schultz, 1947a; Hannah, 1951; and others). The simplest interpretation for *Ascaris* would be to suppose that genes concerned with somatic differentiation are inhibited in the germ cells by the heterochromatic chromosome segments, and made available for their various activities by the elimination of these segments in somatic cells. For a much more complete discussion of the role of heterochromatin in differentiation see Schultz (1947b, 1952), Beermann (1956), and others.

2. Effects of Changes in Chromosome Number on Development

a. Variable aneuploidy. The demonstration that the various regions of the embryo not only normally contain equivalent sets of chromosomes, but are also absolutely dependent on this condition for their differentiation, we owe largely to Boveri and Fankhauser. As is true of so much in embryology and cytology, the original contribution was made by Boveri (1902, 1907) in his analysis of the development of dispermic sea urchin eggs. It had been shown previously that the first division of these eggs is multipolar, resulting usually in the formation of four cells. Subsequent cleavages occur in regular fashion, producing blastulae of normal appearance which, however, nearly always display aberrant gastrulation and only rarely form normal plutei. Boveri was able to show that the only factor which can account for the abnormal development is the irregular distribution of chromosomes during the initial multipolar mitosis. The two sperms each contribute a cleavage center, which usually

divides, giving a tetrapolar figure on which the chromosomes are disposed at random. As a consequence the blastomeres resulting from the ensuing division contain extremely variable numbers of chromosomes, and even when the number approximates that of a haploid or diploid set the distribution of types is usually very abnormal with some chromosomes represented several times and others not at all. This can be observed cytologically with respect to the behavior of two or three recognizable chromosomes in the sea urchin (Baltzer, 1908, 1909), but is most elegantly demonstrated in polyspermic merogonic fragments in eggs of *Triton* (*Triturus*) (Fankhauser, 1934b). Here the chromosomes

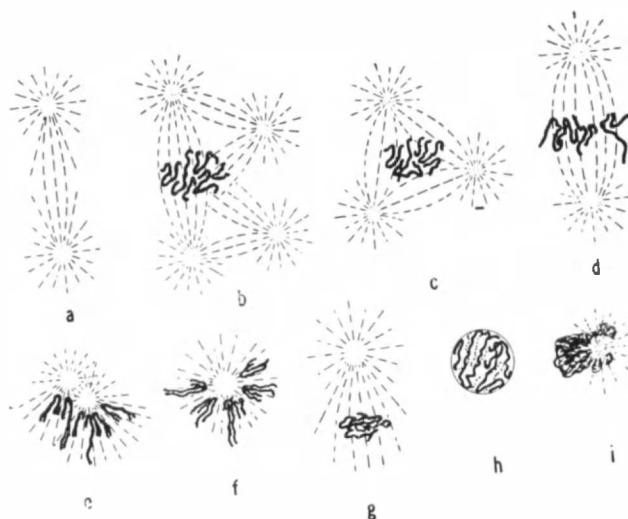


FIG. 9. Types of abnormal mitoses seen in androgenetic fragments of *Triton* eggs. Eggs of this kind may cleave regularly, but contain unbalanced chromosome complements (see Figs. 10, 11) and fail to develop beyond blastula or gastrula stages (Fankhauser, 1948).

are individually recognizable, leaving no doubt about their random distribution during cleavage, leading to the formation of variable and highly unbalanced chromosome sets in the blastomeres (Figs. 9-11). All other factors, even chromosome number per se may be quite normal, and cleavage may proceed in the regular fashion, but the later development of these eggs is almost always severely affected, generally failing in blastula or gastrula stages. From all of this it can be concluded that differentiation depends on the presence of the normal *combination* of chromosomes, with each chromosome making a unique and essential contribution.

b. Uniform aneuploidy. While the analysis of dispermic or polyspermic embryos indicates that each chromosome plays a specific role in development, it does not tell us what the role is. Embryos with different numbers of chromosomes in their constituent cells are too severely abnormal and complicated to permit this. Ideally, for this type of analysis the chromosome complement should be altered in a known way at the

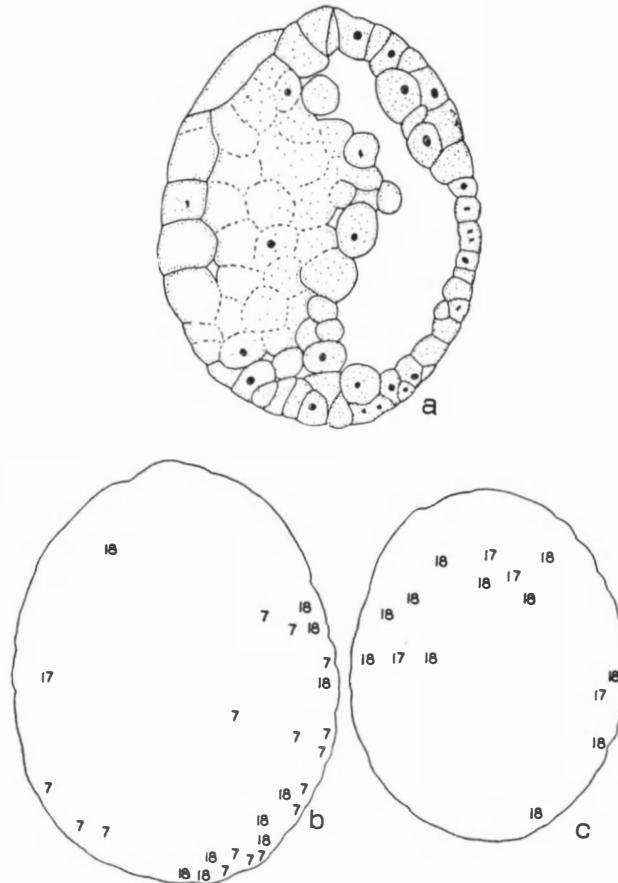


FIG. 10. a. Blastula from androgenetic egg fragment. b., c. Plots of chromosome numbers found in various blastomeres of the blastula shown in (a). The normal haploid number is 12 (Fankhauser, 1934b).

beginning of development and then distributed uniformly to all cells during cleavage thus eliminating the complication of varying chromosome numbers in different parts of the same embryo. Experiments conforming more or less to this set of requirements have been carried out on *Drosophila* and amphibian eggs, with the following results:

(1) Embryos completely lacking one or more chromosomes: In *Drosophila* Poulson (1940, 1945) has produced by genetic techniques eggs which lack the entire X chromosome, or the right or left arms of the X. In the case of the nullo X deficiency the egg arrests in cleavage, prior to blastoderm formation. The X_R deficiency gives a partial blastoderm; the X_L deficiency a complete blastoderm but no separation of germ layers (i.e., no gastrulation). Essentially the same kind of results were

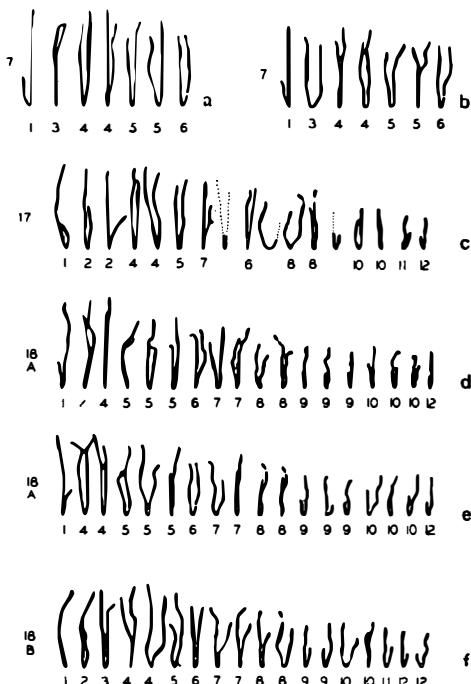


FIG. 11. Abnormal assortments of chromosomes found in various cells of the blastula shown in Fig. 10. On the left of each idiogram the total number of chromosomes is indicated, while the number beneath each chromosome indicates which one of the normal complement of 12 it represents. In each complement some chromosomes are missing entirely; others are represented one to three times (Fankhauser, 1934b).

obtained by Dalcq by the device of fertilizing frogs' eggs with irradiated or dye-treated sperm and then removing the egg nucleus (Dalcq, 1932; Dalcq and Simon, 1932). If the sperm chromosomes are not completely inactivated the eggs cleave with a subhaploid set, of unknown and variable composition in this case. However, the results agree with those mentioned above for *Drosophila*—the subhaploid eggs stop development during cleavage, and apparently never display differentiation.

(2) Hyperhaploid embryos, containing the haploid complement plus one or more additional chromosomes, can be produced by fertilizing normal frogs' eggs with irradiated or dye-treated sperm. These eggs show variable development, presumably because the treated sperms contribute partial complements of unpredictable composition. Development is usually arrested in blastula, gastrula, or early postgastrula stages. It is thus superior to that displayed by subhaploid eggs but is still extremely abnormal (Hertwig, 1924; Dalcq, 1931, 1932; Rugh, 1939; Henshaw, 1943; Briggs, 1952).

(3) Hyperdiploids have recently been obtained in large numbers from matings between triploid and diploid axolotls (Fankhauser and Humphrey, 1950, 1954). As might be expected, these embryos are more successful in development than are the hyperhaploids, but are still far from normal. Some 60–70% of the fertile eggs complete gastrulation, develop axial structures, and survive for varying periods thereafter. While a small number of aneuploids may live for months, the vast majority succumb before reaching a feeding larval stage, and display in varying degrees a complex of deficiencies, mainly in the cardiovascular system. The exact type and the severity of the abnormalities show no correlation with the *number* of extra chromosomes present. Thus, different embryos with the same hyperdiploid number may show wide variations in development. Although the axolotl chromosomes cannot be distinguished from each other cytologically, we would assume that in such cases the extra chromosomes are different individuals, with different effects on development.

The evidence we have outlined briefly above all fits in with Fankhauser's interpretation of the developmental effects of aneuploidy—namely, that the severity of these effects is proportional to the degree of gene imbalance imposed. In subhaploids the imbalance is most marked, and development stops in cleavage, while in hyperdiploids the imbalance must be much less, and development proceeds to advanced embryonic stages. Hyperhaploids are, of course, intermediate. In addition to this general relationship there is also evidence that individual chromosomes differ in their effects on development, but an exact specification of these effects has usually not been possible. No doubt this is due to the fact that each chromosome, with its numerous genes, affects a variety of developmental events, which in turn have secondary influences on additional processes, making the whole complex very difficult to unravel. The developmental effects of single genes are more precisely analyzable, and will be considered in a later section of this paper.

c. *Haploidy and polyploidy.* This subject has been thoroughly reviewed in recent years (Fankhauser, 1945a, 1952, 1954, 1955; von Borstel,

1957; Moore, 1955; and Beatty, 1957) so we will present here only a brief account, with special reference to embryonic (prelarval) development of amphibia.

i. Polyploids. In amphibia the addition of whole sets of chromosomes to the diploid complement has very little effect on embryonic development. Triploids, tetraploids, and pentaploids all proceed normally through gastrulation and the subsequent phases of organ formation. This result is to be expected if the balance of gene activities is maintained, as it apparently is in this series—at least with respect to prelarval development. The fact that development is so normal raises problems

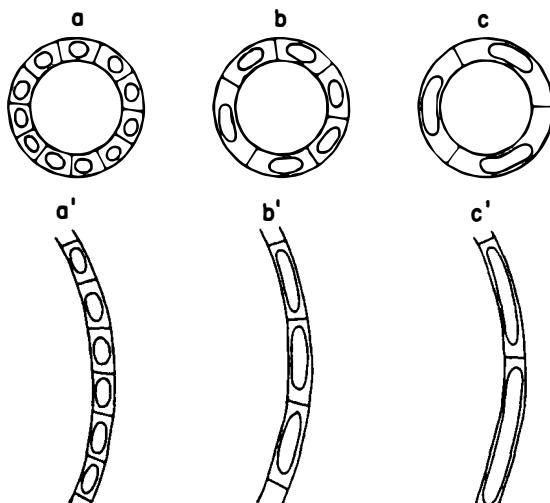


FIG. 12. Pronephric tubules (above) and lens epithelium (below) in haploid (a, a'), diploid (b, b'), and pentaploid (c, c') larvae of *Triturus*. The normal form of the organs is preserved despite large differences in cell size (Fankhauser, 1955).

of a different kind, not connected specifically with gene action. We refer particularly to the fact that the embryos in the polyploid series preserve a normal size and form despite large differences in nuclear and cell size (Fankhauser, 1945b). We know this is accomplished by reduction in cell number, and a molding of the larger polyploid cells to preserve the normal size and form of the embryonic organs—as illustrated in Figure 12. The problem of a mechanism to account for this regulation has been discussed by Fankhauser (1945a, 1952) and by Briggs (1947), but it appears that nothing factual is known about it.

During larval development triploids remain normal in appearance and growth rate. The only organ which is definitely affected by triploidy

is the gonad. The testis differentiates and in some cases produces mature sperms (Fischberg, 1947; Fankhauser and Humphrey, 1954). The ovary is more severely affected, rarely contains growing oocytes, and in one case (*Rana pipiens*), becomes converted into a testis (Humphrey *et al.*, 1950). A partial exception to this is found in the triploid axolotl, in which the ovary eventually produces some fertile eggs (Humphrey and Fankhauser, 1946). Higher polyploids also display deficiencies in the gonads, but in addition are retarded and abnormal to varying degrees in their general larval development.

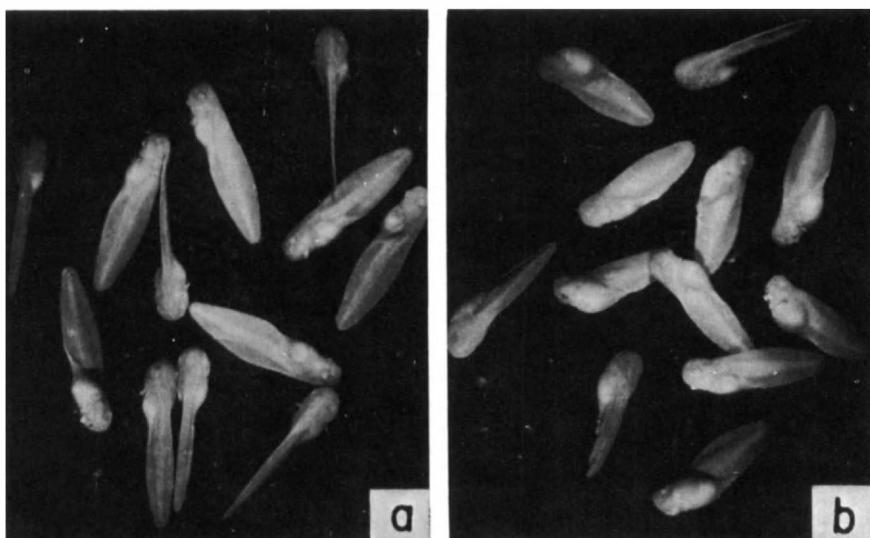


FIG. 13. Diploid (a) and haploid (b) embryos of the frog, *Rana pipiens*. The haploids display a uniform combination of deficiencies; see text (Briggs, 1952).

ii. Haploids. While one or more chromosome sets may be added to the diploid complement without affecting embryonic development, the removal of a complete set has important consequences. Haploids develop normally to the beginning of gastrulation, but thereafter become retarded, form an abnormally short medullary plate, and later display a combination of deficiencies in the gut, central nervous system, sense organs, pronephros, and cardiovascular system. No single one of these abnormalities is unique to haploids. What is unique is the combination, which gives these embryos a characteristic and unmistakable appearance and is so uniformly expressed as to be properly designated a haploid syndrome (Fig. 13). The problem of how a reduction in chromosome number from $2N$ to $1N$ brings about this result is still

unsolved. One theory which has been frequently mentioned can now be disposed of, at least in large part. We refer to the proposal that the abnormalities are caused by recessive lethal genes, which can act in the haploids because of the absence of the normal alleles. On theoretical grounds Moore (1955) has shown that it is extremely unlikely that recessive lethals can account adequately for the haploid syndrome. The actual demonstration of this fact we owe to recent studies by Subtelny (Subtelny, 1958; Subtelny and McLoughlin, 1956). Subtelny's experiments involve transplantation of nuclei from haploid blastulae into

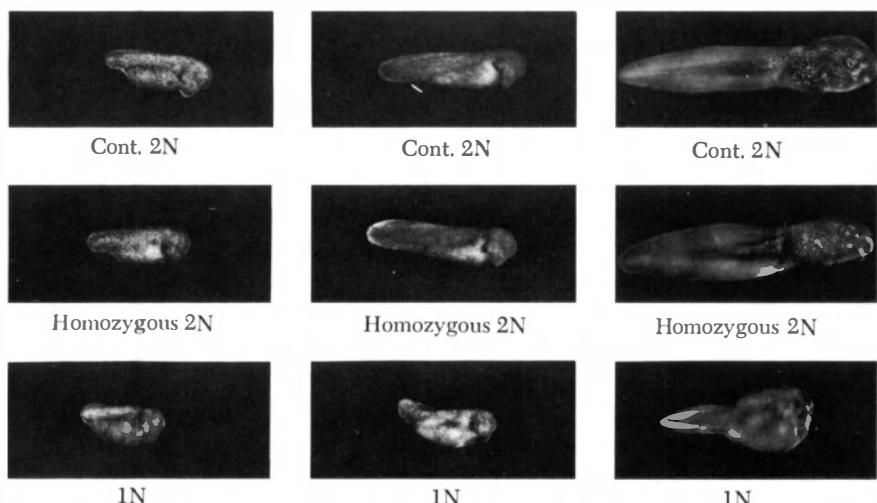


FIG. 14. Comparison of the development of haploids, homozygous diploids, and control diploids of the frog, *Rana pipiens*. The haploids show the characteristic deficiencies in postgastrula embryonic development. In comparable stages the homozygous diploids develop normally. However, in larval stages (not shown above) they become abnormal in various ways and fail to metamorphose. (Courtesy of Dr. S. S. Subtelny.)

enucleated eggs (of *Rana pipiens*). Following the nuclear transfer some of the recipient eggs begin to cleave at the normal time and develop into typical haploids. The remainder are delayed at the beginning by one cleavage interval, during which time the chromosome number is doubled, giving a homozygous diploid. If recessive lethals were responsible for the haploid syndrome, the homozygous diploids should display the same abnormalities. Instead they develop through gastrulation, neurulation, and the early postneurula stages in perfectly normal fashion (Fig. 14). Thus the outstanding feature of the haploid syndrome, the uniformly expressed abnormalities in the earlier stages, can have nothing

to do with lethal factors. It is possible, however, that a genetic basis exists for some of the abnormalities in later stages. Following what appears to be a perfectly normal early development, Subtelny's homozygous diploids later display varying types and degrees of abnormalities. These may appear during late embryonic stages, but more frequently are not apparent until the larval stage is reached. Many of these larvae will feed and survive for weeks or months, but they are always slow growing and eventually die before metamorphosis. Lethal genes could be involved in some of these later abnormalities—but there is no proof of this as yet, and there are arguments against the idea that all of the larvae could be dying from the effects of such genes (see Moore's discussion, 1955, p. 170, of the genetic problems posed by the development of haploids. Some of this would apply also to the homozygous diploids).

The second factor commonly referred to in discussions of haploid development is the nucleocytoplasmic ratio. Since haploids are usually produced by the elimination of one set of chromosomes, without a corresponding reduction in the volume of the egg cytoplasm, the N:C ratio has half its normal value at the beginning of development. In some cell types this abnormally small ratio persists in postgastrula stages and has been proposed as a factor in their deficient differentiation (Hertwig, 1913; Porter, 1939). This idea has been tested by the simple expedient of reducing cytoplasmic volume so as to restore the N:C ratio to its normal value. With *Triton* eggs this is accomplished by producing haploid egg fragments of about half normal size (Baltzer, 1922; Fankhauser, 1938), while in the frog a normal ratio can be obtained by making haploids out of whole eggs of about half the normal volume (Briggs, 1949). These small haploids show an improvement in viability, which is particularly well demonstrated in the case of the frog, as illustrated in Figure 15. However, the characteristic deficiencies of the haploid syndrome persist, despite the fact that the N:C ratio is approximately normal.

As can be gathered from this brief review, the causes of the abnormal development of haploids still elude us. Both recessive lethal genes, and the altered N:C ratio appear to contribute to the syndrome, but its main features are the result of some other causal factor(s). The possibilities left to us appear to be (1) that the syndrome is due to some simple quantitative deficiency in the activity of the haploid nucleus or (2) that the balance of gene activities is altered. This latter could occur if the relationship between gene dosage and activity is different for different genes (see Schultz, 1952). The fact that the homozygous diploids develop normally (in embryonic stages) is consistent with both interpretations, since both of the postulated conditions would be cor-

rected by a simple doubling of the haploid complement. Other features of haploids, such as reduced synthesis of nucleic acids (Brachet, 1944b, 1954), reduced competence to neural induction (Moore and Moore, 1953), and increased sensitivity to an -SH enzyme inhibitor (Briggs, 1946), are also consistent with both interpretations. How to distinguish between them is frankly quite baffling.

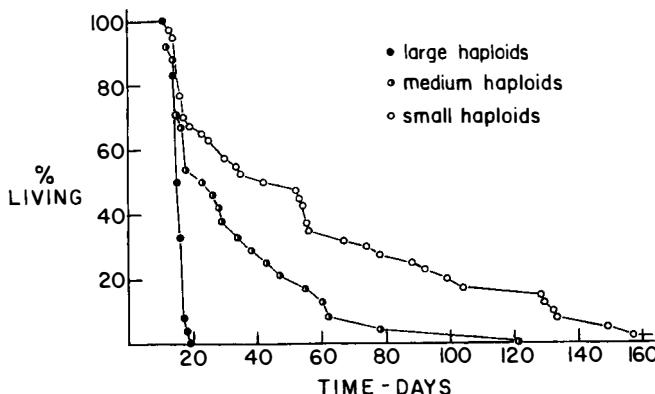


FIG. 15. Influence of egg volume on viability of haploids (*Rana pipiens*). The large haploids are derived from eggs of normal size, with an average volume of 3.4 mm^3 . Corresponding values for medium and small haploids are 2.5 and 1.9 mm^3 , respectively. In all groups the main features of the haploid "syndrome" are apparent. However, during later embryonic stages the small haploids display more nearly normal differentiation, particularly of the gut and vascular system, and live significantly longer than do the large haploids, as illustrated above (Briggs, 1949).

3. Summary

While cleavage can occur with a variety of chromosome complements, or even without chromosomes, the later phases of development depend upon the presence of a complete, balanced set of chromosomes. The evidence for this is as follows:

- (1) The absence of one or more chromosomes results in arrest of development during cleavage. The deficiency cannot be made up by adding extra chromosomes of other types to the complement. Thus, each chromosome is essential.
- (2) The addition of one or more chromosomes to a complete set usually results in abnormalities in early organogenesis, the severity of the effect being roughly proportional to the degree of imbalance, i.e., greater when chromosomes are added to a haploid set, and less severe when the additions are made to a diploid or polyploid set. Therefore, normal differentiation requires not only a complete set, but a balanced one.

(3) In conformity with this experimental evidence, cytological studies on normal individuals show the same number and types of chromosomes to be present in a variety of cell types. However, there are, particularly in insects, differences in the numbers of balanced sets (polyteny), and in the structure of individual chromosome bands, in different tissues.

(4) Changes in "ploidy," since they do not alter the relative numbers of individual chromosomes, are not expected to affect embryonic development. Within certain limits this is true for polyploids, but haploids give a different result, displaying a characteristic syndrome of deficiencies in early organogenesis. Recessive lethal genes, and the abnormally small nucleocytoplasmic ratio of haploids may both contribute to the syndrome, but its main features are due to some other factor(s), as yet undiscovered.

B. Development of Hybrids

The outstanding facts in our account so far are: (1) that the pattern of early organogenesis is inherent in the egg cytoplasm, and (2) that a complete, balanced chromosome set is required in all the cells of the embryo if the pattern is to be realized, i.e., if the different organs are actually to be formed. Of course, this evidence shows only that normal nuclei are essential for morphogenesis and does not indicate whether they exert any control over the process. For a more detailed specification of the role of the nucleus we shall now consider studies of three types, involving respectively: (1) development of hybrids, (2) specific gene effects on development, and (3) analysis of properties of embryonic nuclei by nuclear transplantation. Studies of types 1 and 2 tell us whether nuclei exert any control over morphogenesis, while studies of type 3 indicate whether the nuclei themselves become altered in the process.

In this section we propose to give a brief review of the main facts, and the main conclusions which we think can be drawn from them, concerning the development of hybrids. Much more complete treatments of the genetical and embryological aspects of the subject are given in reviews by P. Hertwig (1936), Hörstadius (1936), A. R. Moore (1949), Baltzer (1952), Fankhauser (1952, 1955), and J. A. Moore (1955). The biochemical researches on hybrids have been summarized recently by Barth and Barth (1954) and by Brachet (1957).

Most of our information on the development of hybrids comes from studies of crosses among various species of echinoderms and amphibians. In both groups the hybrids fall roughly into three classes, according to the type of development displayed.

1. False Hybrids

In crosses of this type the sperm penetrates the egg but the sperm nucleus either fails to fuse with the egg nucleus, or fuses temporarily and is soon eliminated (Baltzer, 1952; Tchou-Su, 1931; and others). Meanwhile the sperm centrosome provides normal cleavage centers which join with the egg nucleus to promote the development of a typical gynogenetic haploid. Crosses of this type cannot tell us anything about the role of the nucleus in morphogenesis, but do illustrate one point very well, namely, the ability of the sperm centrosome to function normally in association with cytoplasm and chromosomes, both of foreign origin. In this connection we might mention an experiment of a different type which again demonstrates the ability of the centrosome to induce cleavage in cytoplasm of a quite foreign species. This experiment involved the transplantation of nuclei (cf. page 602) from *Triturus pyrrhogaster* blastulae into enucleated eggs of *Rana pipiens* (Briggs and King, 1955). The recipient eggs cleaved according to the normal pattern (except in the vegetal hemisphere) and gave rise to partial blastulae in which large areas were uniformly cleaved into small cells. Cytological analysis showed that most of these cells contained no chromatin. However, disorganized masses of chromatin, derived from the injected nucleus, were present here and there in the partial blastulae and appeared definitely to represent a much larger mass than was originally injected as a part of the newt nucleus. This result demonstrates two interesting points, which are (1) that the newt centrosome can induce a series of regular cleavages in frog cytoplasm; and (2) that the newt chromatin, though disorganized, can increase in amount at the expense of substrates provided by the frog cytoplasm.

2. Lethal Hybrids

In crosses of this type the sperm nucleus fuses with the egg nucleus, giving a diploid hybrid which develops normally to the beginning of gastrulation, then becomes arrested, survives for a while in the arrested state, and finally cytolyses. Since the block to development usually occurs at or near the beginning of gastrulation, these hybrids have been extensively studied in the hope that nucleocytoplasmic interactions especially concerned with early morphogenesis might be revealed.

a. *Comparison of diploid and haploid hybrids.* In diploid hybrids two types of incompatibility are possible: (1) Incompatibility between the different types of chromosomes within the nucleus, and (2) incompatibility between the foreign chromosomes of the nucleus and the egg cytoplasm. A choice can be made between these two possibilities by

comparing the development of diploid and haploid hybrids. When this is done it appears that the haploid hybrids always display a more limited development than do the diploids. Thus, the diploid hybrid, *Rana pipiens* ♀ × *Rana sylvatica* ♂, forms a dorsal lip and may display limited invagination before becoming arrested. The comparable haploid, *R. pipiens* (♀)² × *R. sylvatica* ♂, develops only to the late blastula stage and never shows any signs of gastrulation (Moore and Moore, 1953). Several other studies described in the reviews mentioned above give the same type of result, leading to the conclusion that the cessation of development in hybrids of this type is due primarily to an incompatibility between nucleus and cytoplasm.

b. Grafting experiments with hybrid tissues. One method of investigating the nature of the nucleocytoplasmic incompatibility in hybrids involves grafting tissues from the blocked hybrids to normal hosts. Experiments of this type have given the following results:

i. No effect of host on graft. When pieces of the lethal hybrid, *Rana pipiens* ♀ × *Rana catesbeiana* ♂ are grafted to normal *pipiens* hosts the grafts "take" temporarily, but fail to differentiate. Later they undergo cytolytic changes and are cast off by the host (King and Briggs, 1953). The same type of result has been obtained also by Brachet (1954) with grafts of tissues from the lethal hybrid *Bufo vulgaris* ♀ × *Rana esculenta* ♂. In all such cases the deficiencies or abnormalities are apparently intrinsic in the hybrid cells. At least they cannot be corrected by materials such as may be obtained from the contiguous normal cells of the host.

ii. Beneficial effect of normal host on hybrid graft. Many cases are on record in which hybrid tissues which would normally fail to survive or differentiate are somehow enabled to do so following grafting to a normal host. The interpretation of this result varies depending upon whether or not the hybrid in question displays what Baltzer (1952) calls "partial lethality." The clearest case of partial and specific lethality is that described by Hadorn (1932-1937) in the haploid hybrid, *Triton (Triturus) palmatus* (♀) × *Triton cristatus* ♂. This hybrid develops to the neurula stage and then becomes arrested, displaying cell degeneration first in the head mesoderm. When various hybrid tissues are grafted to normal hosts only the head mesoderm is unimproved in its performance (Fig. 18). It still degenerates as it would have done in the intact hybrid, while other tissues survive and display more or less normal differentiation. Thus, the arrest of development in this hybrid is mainly due to the intrinsic lethality in the cells of the head mesoderm. Now,

² It is customary to designate the removal of the maternal chromosomes by putting the female symbol in parenthesis.

with respect to the other cell types there are two explanations of their survival and differentiation following grafting. One is that the hybrid cells are quite normal in their developmental potentialities and need only to be separated from the degenerating head mesoderm to express these potentialities. The second is that the hybrid cells are deficient in some essential substance(s) which they receive from the adjacent normal cells of the host. These possibilities can be tested by explanting the hybrid tissues; i.e., by growing them in salt solutions *in vitro*. When this is done some tissues (epidermis, notochord) are seen to continue their differentiation *in vitro* and are obviously normal in their developmental potentialities. Other tissues (neural tissue and somites), which display some differentiation when grafted to normal hosts, fail to differentiate when isolated *in vitro*. Apparently hybrid cells of this type lack the ability to produce some essential substance(s), and will only differentiate when these substances are provided for them by a normal host.

The *palmatus* × *cristatus* haploid hybrid is perhaps the most instructive one we have in showing that nuclei of a given type (*cristatus*) give quite different results in promoting differentiation, depending upon the cytoplasmic region of the foreign egg in which they happen to lie. In the head mesoderm there appears to be a deep-seated nucleocytoplasmic incompatibility leading to cell death; in somites and neural tissue there is a deficiency in nucleocytoplasmic interactions which can be repaired by diffusible products of normal cells; while in ectoderm and notochord the interactions between *cristatus* nuclei and *palmatus* cytoplasm are apparently adequate for promoting full differentiation. This analysis indicates clearly that different kinds of nuclear functions, as yet unspecified, must be involved in the differentiation of the different regions of the eggs—otherwise we would expect the *cristatus* nuclei to function equally well in all parts of the *palmatus* egg.

The other types of hybrids which have been analyzed in grafting experiments are mainly those which are arrested earlier in development—during gastrulation. Some of these, e.g., the diploid hybrids between *Triton palmatus* ♀ or *T. taeniatus* ♀ and *Salamandra maculosa* ♂ or *S. atra* ♂, also display partial lethality (Baltzer, 1952). In these hybrids some of the cells of the late blastula or early gastrula develop pycnotic nuclei, round up, and are cast off into the blastocoel. Cells of this type cannot be “saved” by grafting to normal hosts. Intermingled with the lethal cells in the hybrid are cells of normal appearance, which do survive and differentiate when grafted. So far as we are aware, there is no adequate explanation of this type of partial lethality. Since “lethal” and “normal” cells occur side by side in the same region of the egg, we cannot call on differential effects of the cytoplasm localizations on

nucleocytoplasmic interactions to explain the lethality in some but not all cells. There is a possibility that random irregularities in mitosis might eliminate foreign chromosomes from some cells—which would thus become viable. However, the fact that the viable cells appear to be diploid (Baltzer, 1952) argues against this interpretation.

Finally, we should mention certain hybrids which give no evidence of containing "lethal" cells, and yet are blocked in gastrula stages. By this we mean that portions of the blocked hybrid resume their development when grafted to normal hosts, and display no cytolysis. A case of this type is provided by the hybrid *Rana esculenta* ♀ × *Rana temporaria* ♂, pieces of which differentiate normally when grafted to normal *Triton* gastrulae (Brachet, 1944b). A similar result is also obtained with grafts of portions of the hybrid *Rana pipiens* ♀ × *Rana sylvatica* ♂ (Moore, 1947, 1948) although in this instance the grafted hybrid dorsal lip shows a lower than normal inductive power, and the grafted ectoderm a reduced competence. The interpretation favored by Brachet for cases of this sort is the one already mentioned above in another connection, namely, that there are certain substances essential for morphogenesis which the hybrid cells cannot produce, but which they can obtain by diffusion from the normal cells of the host. It has also been suggested by Moore (1948) that the deficiencies in the hybrid might result from something akin to metabolic analog formation by the foreign genes. These ideas are most attractive, and will become much more so if it should be possible to make direct tests for either analogs blocking differentiation in hybrids, or for the diffusible substances of normal cells required for overcoming the block.

c. *Nuclear transplantation studies—lethal hybrids.* According to the tissue-grafting experiments reviewed above, we may conclude that certain hybrids are irreversibly blocked in late blastula or early gastrula stages. The question whether the onset of the irreversible block to morphogenesis involves irreversible changes in the nuclei has recently been studied in nuclear transplantation experiments performed by King and Briggs (1953), and by Moore (1958a). In the experiments of King and Briggs nuclei were transplanted from the lethal hybrid *Rana pipiens* ♀ × *Rana catesbeiana* ♂, back into enucleated *pipiens* eggs, thus providing a test of changes affecting the capacity of the nuclei to continue functioning in the foreign egg cytoplasm. The results of these experiments were clear-cut (Figs. 16 and 17). At the onset of the developmental block and for several hours thereafter the nuclei of the hybrids remained unchanged—when transferred into enucleated *pipiens* eggs they were capable of participating again in the whole course of hybrid development. Only after the hybrids had been arrested for some 10 or

more hours did their nuclei lose the capacity to promote cleavage and development of the recipient eggs. This loss of transplantability was found to coincide with the appearance in the hybrid of cytological abnormalities, such as bridged anaphases, clumped metaphases, and vacuolated interphase nuclei.

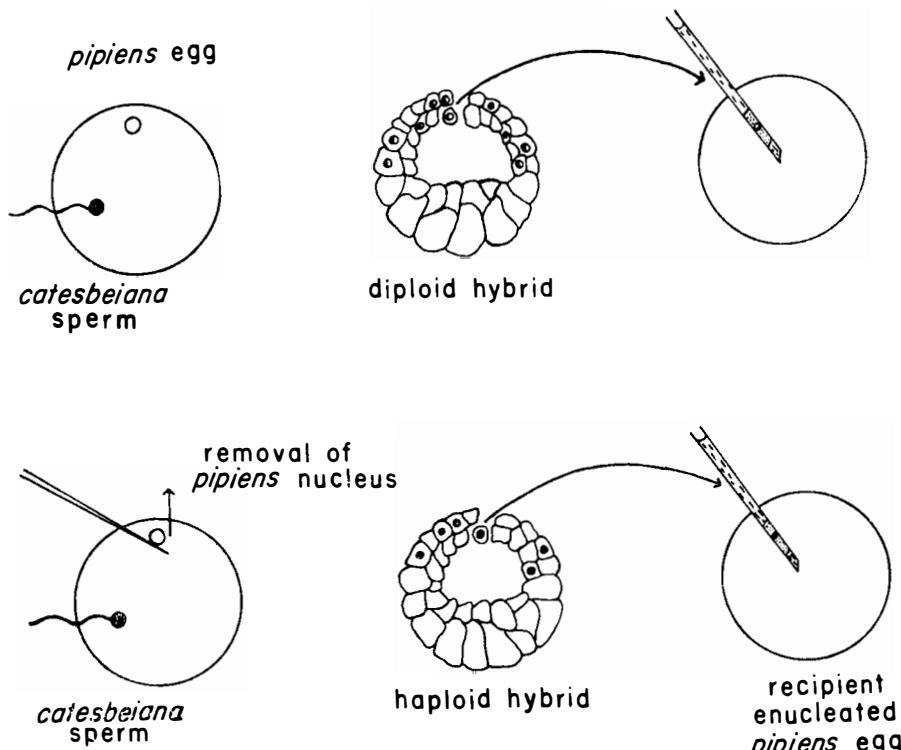


FIG. 16. Diagram illustrating procedure for testing nuclei of hybrids by transplanting them into enucleated eggs. The tests are actually made at considerably later stages of hybrid development than that indicated in the diagram. For details of the nuclear transplantation method see Fig. 23. Results of tests of hybrid nuclei are given in Fig. 17 (King and Briggs, 1953).

The results outlined above show clearly that the arrest of hybrid development at the beginning of gastrulation is *not* due to a "lethal" change in the nuclei. It is possible, however, that the nuclei might be undergoing more subtle changes, affecting their capacity to promote postblastula development, which would not be revealed in tests of the type described above. Recently Moore (1958a) has attempted to detect such nuclear changes in the following way. Working with the lethal combination, *Rana pipiens* and *Rana sylvatica*, Moore first transplanted

diploid nuclei of *pipiens* blastulae into enucleated *sylvatica* eggs. As expected on the basis of previous hybridization studies, the embryos resulting from the nuclear transplantations were arrested in late blastula stage. From such hybrid blastulae the *pipiens* nuclei were now transplanted back into *pipiens* cytoplasm. If the nuclei had remained completely unchanged in the foreign cytoplasm they should, when placed back into their own type of egg cytoplasm, promote complete and normal development. However, in the cases reported the "reconstituted" *pipiens* embryos developed only to early gastrula stages. Moore has emphasized that these results must be regarded as tentative, since they are based on a limited number of cases. If confirmed they would lead to a most important conclusion—namely, that nuclei undergo some sort of genetic transformation while replicating in foreign cytoplasm which limits their capacity to promote differentiation when returned to their own type of cytoplasm.³

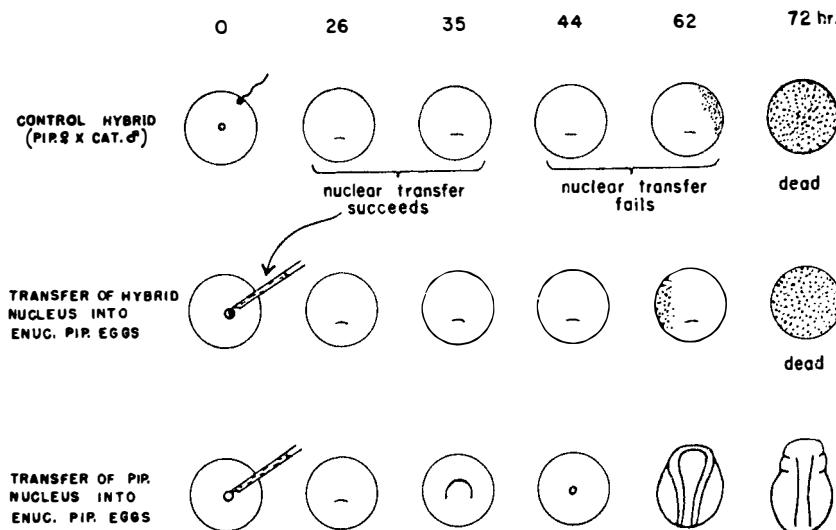


FIG. 17. Results of transplantation tests on nuclei of arrested hybrids (*Rana pipiens* ♀ × *Rana catesbeiana* ♂). At the time of developmental arrest and for several hours thereafter the nuclei retain the capacity to promote typical hybrid development following transfer to enucleated eggs. Later on the nuclei become morphologically abnormal and are no longer able to elicit cleavage in the test eggs. Results similar to those illustrated above for diploid hybrids are obtained also with nuclei of androgenetic haploid hybrids (King and Briggs, 1953).

d. Biochemical studies on lethal hybrids. These have been reviewed recently by Barth and Barth (1954), Moore (1955), and Brachet (1957), so we will give here only a very short account of this work. The results

³ In more recent experiments, Moore (1958b) has obtained a confirmation of the results described above.

of these studies vary somewhat with different hybrids. In the *Rana pipiens* ♀ × *Rana sylvatica* ♂ hybrid the rates of a variety of metabolic activities are lower in the blocked gastrulae than they are in normally developing control gastrulae. (See reviews above for references.) This holds for O₂ consumption, CO₂ production, lactic acid production, glycogenolysis (particularly in the dorsal-lip region), and capacity to maintain ATP (adenosine triphosphate) phosphorylated under anaerobic conditions. Thus there would appear to be a general suppression of intermediary metabolism in this hybrid. This could be due to blocks in the formation or activity of a variety of enzyme systems, both nuclear and cytoplasmic, but as yet there would appear to be little information as to which of these systems are involved. Also, it should be mentioned that lethal hybrids may differ from each other metabolically. For example, in the *Bufo vulgaris* ♀ × *Rana temporaria* ♂ hybrid, oxygen consumption continues to increase for a time at the normal rate after development has become blocked; and the same is true of the *Rana pipiens* ♀ × *Rana clamitans* ♂ cross. Thus, at least with respect to respiration, both of these hybrids differ from the *pipiens* × *sylvatica* hybrid mentioned above.

Nucleic acids in lethal hybrids have been studied by several investigators (see review by Brachet, 1957). With respect to RNA content, it appears that lethal and control embryos do not differ until the beginning of gastrulation, after which there are marked increases in RNA in the controls, but not in the arrested hybrids. The difference between the two types of embryos appears to be mainly or entirely a difference in cytoplasmic RNA. In the controls cytoplasmic RNA increases rapidly during gastrulation and later stages, while in the blocked hybrids there is no perceptible RNA increase in the cytoplasm. Meanwhile, the nuclei of the hybrids show larger than normal amounts of RNA, suggesting that there may be a block in the transfer of RNA from nucleus to cytoplasm. Such a block, if it exists, is reversible, at least in certain hybrids, e.g., the *Rana esculenta* × *Rana temporaria* hybrid investigated by Brachet (1954). The cells of such hybrids begin to show increases in cytoplasmic RNA, and also begin to differentiate following grafting to normal hosts.

With respect to DNA the results of several studies indicate that while it does not increase at the normal rate, it does show a significant increment after development is arrested in lethal hybrids (Gregg and Løvtrup, 1955). According to a recent cytophotometric study of B. C. Moore (1957) the individual nuclei in the blocked *pipiens* × *sylvatica* hybrid build up an amount of DNA greater than that required for continuing mitosis—yet the nuclei fail to divide. Presumably this block is also one that may be overcome, for cells of the *pipiens* × *sylvatica* hybrid are known to be able to resume development on grafting to normal hosts.

As a result of biochemical studies, of which only a few are mentioned

above, we now know that the cessation of development in lethal hybrids is accompanied by pronounced alterations in intermediary and nucleic acid metabolism. However, the problem of relating these alterations to specific biochemical properties of nuclear and cytoplasmic organelles has so far hardly been attacked. For example, Brachet (1957) has suggested that the failure of RNA synthesis in hybrids may be due to inability of the cytoplasm to utilize RNA synthesized in the foreign nucleus. He has also suggested that the reduced respiratory metabolism of certain hybrid cells could be due to a failure of the nucleus to produce coenzymes such as DPN (diphosphopyridine nucleotide). It is well known for certain cell types (e.g., amoebae, mammalian liver cells, etc.) that RNA and DPN are produced in the nucleus and transferred somehow to the cytoplasm (Goldstein and Plaut, 1955; Hogeboom and Schneider, 1952). Thus nucleocytoplasmic interactions of this type may be going on normally in embryos and could be among the most important ones which are blocked in hybrids. There is no experimental proof of this as yet, but methods are now available for tracing the movements of materials between cytoplasm and nucleus. Applied to both hybrids and normal embryos they may eventually reveal those biochemical interactions between cell organelles which are especially important in early morphogenesis.

3. Hybrids Surviving to Postgastrula Stages of Development

Hybrids of this type provide an opportunity for appraising the relative roles of nucleus and cytoplasm in determining specific characteristics of embryonic organs.

a. Developmental rate. Studies on developmental rate in a variety of echinoderm and amphibian hybrids indicate that the nucleus has little influence during cleavage and that it is only at gastrulation that its role in development becomes detectable. The maternal cytoplasm alone determines the rate of cleavage and development until the beginning of gastrulation, after which developmental rate becomes affected by the foreign chromosomes contributed by the sperm nucleus (A. R. Moore, 1933; J. A. Moore, 1941). Although the pregastrula developmental rate of hybrids is thus determined by the maternal cytoplasm, toward the end of cleavage antigens peculiar to the paternal species can be detected serologically (Harding *et al.*, 1954, 1955).

b. Forms of embryonic structures. In addition to the observations on early development, the literature of hybridization, especially echinoderm hybridization, is replete with descriptions of the morphology of later stages (Hertwig, 1936; von Ubisch, 1939; Hörstadius, 1936, 1939). Only a few of these will be referred to here.

i. *Diploid Hybrids.* When eggs of *Strongylocentrotus (Paracentrotus)* are fertilized by sperm of *Sphaerechinus*, the hybrid blastulae that are formed are abnormal (Baltzer, 1910). The few hybrid embryos that reach the pluteus stage resemble the maternal species. Baltzer showed that this could be accounted for on the basis that some of the paternal chromosomes had been eliminated. Chromosomal studies showed that at the time of the first mitosis some of the divided chromosomes failed to separate. Later stages, instead of having a full chromosome complement of 38 (18 from *Strongylocentrotus* and 20 from *Sphaerechinus*), had only about 21; 16 or 17 having been eliminated. That the loss was from the paternal set was shown by the reciprocal cross *Sphaerechinus* ♀ × *Strongylocentrotus* ♂ in which all of the *Strongylocentrotus* chromosomes were able to survive and divide normally, giving a pluteus morphologically intermediate between the plutei of the parental species.

In the interordinal cross of the sea urchin *Strongylocentrotus purpuratus* ♀ and the sand dollar *Dendraster excentricus* ♂, a large number of fully formed plutei are produced which also show evidence of inheritance from both parents (A. R. Moore, 1957). The purely maternal rate of development up to the end of the blastula stage indicates that the cytoplasm dominates development up to that point. When the gastrula stage is reached the situation changes and the nuclear or paternal effects (shape of body and some features of the skeleton) become increasingly apparent, but not dominant. The intermediate character of these embryos is also evidenced in their alkaline phosphatase content (Flickinger, 1957).

ii. *Haploid Hybrids.* Haploid hybrids provide the best test of the relative influence of the nucleus and cytoplasm in the development of species characteristics since the chromosomes are derived entirely from one species and the cytoplasm from another.

(1) Cyttoplasmic control: The androgenetic haploid hybrid *Triton palmatus* (♀) × *Triton cristatus* ♂ normally dies before reaching a stage at which species characteristics become evident; however, as we have seen (page 580) ectodermal portions of such gastrulae grafted to normal hosts (*T. alpestris*) differentiate into normal skin and survive the metamorphosis of the host (Hadorn, 1936). After metamorphosis, the hybrid skin shows the typical protuberances of the *T. palmatus* parent (Fig. 18). Since the *T. palmatus* egg nucleus had been removed and since the *T. cristatus* (paternal) species does not have epidermal protuberances, this is considered a case of *cytoplasmic heredity*. The maternal influence, which in this case appears late in development, is probably "determined" very early in the cytoplasm of the egg before fertilization (Fankhauser, 1952, see p. 187). Although the presence of protuberances

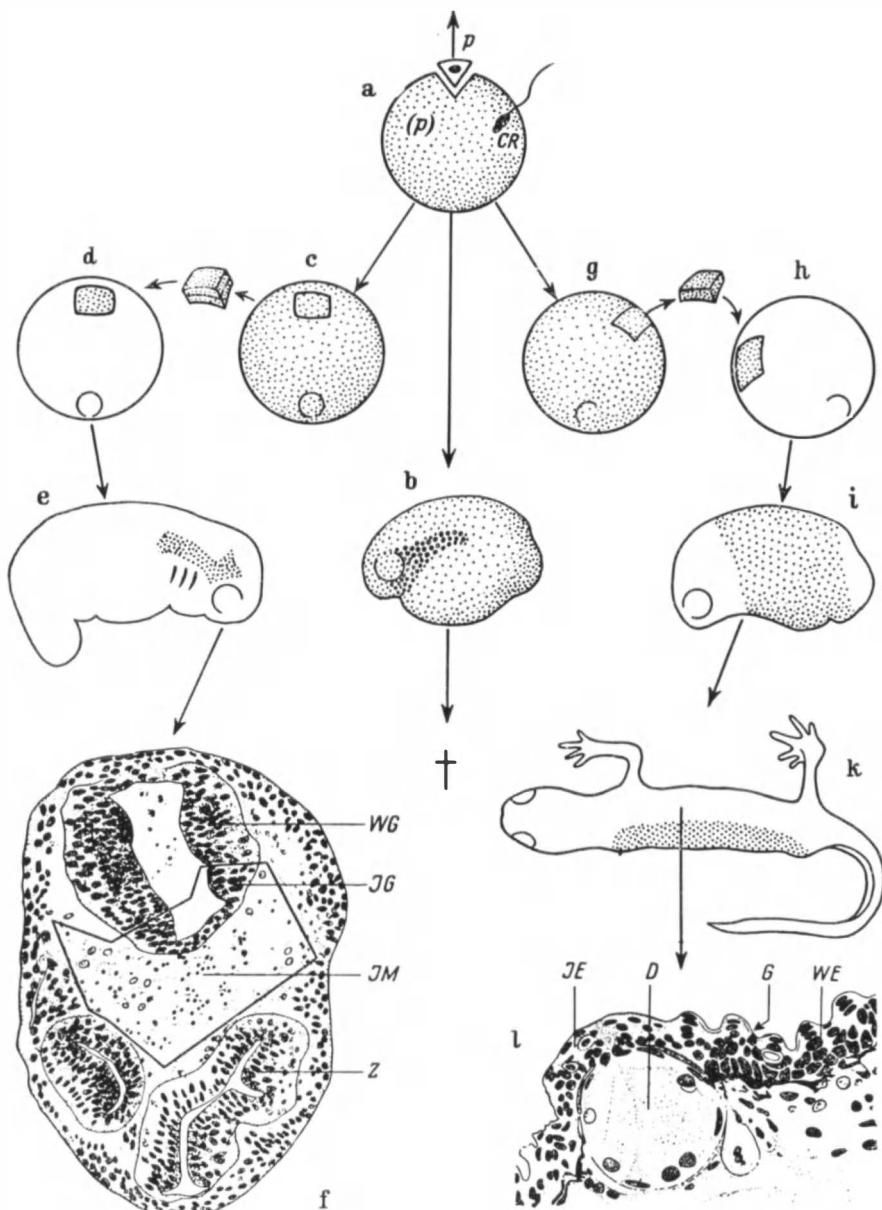


FIG. 18. Diagrammatic summary of some of Hadorn's experiments on the lethal haploid hybrid, *Triton palmatus* (♀) × *Triton cristatus* ♂. a. Method of preparing hybrid by fertilizing enucleated *palmatus* egg with *cristatus* sperm. b. Development of hybrid. Death at the neurula stage, associated with degeneration of head mesoderm. c-f. Transplantation of presumptive brain and archenteron roof

in the epidermis of the *T. alpestris* host makes it impossible to exclude all extraneous morphogenetic influences, the fact remains that "the cytological characters of the skin formed by the fragments of the hybrid merogone have *not* been determined by the nucleus present in it" (Brachet, 1957, see p. 375).

(2) Mainly nuclear control: *Form of sea urchin skeleton.* Among the remarkable experiments of Hörstadius on the mechanics of sea urchin development, one of the nicest is the merogonic combination he made between *Psammechinus* and *Paracentrotus* (*Strongylocentrotus*) (Hörstadius, 1936). When the presumptive ectoderm (animal half + veg₁) of *Paracentrotus* is combined with the micromeres of the heterospermic merogone (*Paracentrotus lividus* cytoplasm + *Psammechinus microtuberculatus* nucleus), the hybrid blastula consists entirely of *Paracentrotus* cytoplasm and nuclei, except for the nuclei of the micromeres (presumptive skeleton-forming cells) from the merogone. Although the *Psammechinus* species was represented only by the nuclei of the skeleton-forming micromeres, these nuclei gave the pluteus the appearance of a *Psammechinus* larva (bent, thickened, irregular club-shaped body rods). In the reciprocal combination (*Psammechinus* cytoplasm and nuclei, except for the *Paracentrotus* nuclei of the skeleton-forming cells) the plutei look like *Paracentrotus* larvae with straight, slender body rods. In this case the rods may show minor irregularities, suggesting the possibility of a minor influence from the *Psammechinus* cytoplasm. However, the main result clearly indicates a predominant control of species characteristics by the nucleus (Fig. 19).

Pigment pattern. Haploid hybrids between two species of California salamanders have been studied by Dalton (1946) in an effort to determine the respective roles of the nucleus and cytoplasm in pigment pattern formation. Since the haploid hybrids do not develop to a stage where the differentiation of the specific pigment pattern of the paternal species becomes evident, it was necessary to transplant pieces of the haploid hybrid merogone to normal embryos of the same age. When the neural crest of *Triturus rivularis* (♀) × *Triturus torosus* ♂ hybrid is grafted to the flank of a diploid *Triturus torosus* neurula, a pigment pattern essentially like that of the nuclear donor species (*T. toro-*

of hybrid to normal host. Contact with normal host tissues improves the differentiation of hybrid brain tissue (JG), but has no beneficial effect on the head mesoderm (JM) which still degenerates. g-l. Transplantation of presumptive epidermis from hybrid to normal host. The hybrid epidermis survives through metamorphosis (k) and displays the surface protuberances characteristic of *palmatus*, the cytoplasmic parent (Hadorn, 1955).

sus) is produced. However, an influence of the cytoplasmic species is not completely lacking, as evidenced by a tendency toward the pattern type of the cytoplasmic-donor species (*rivularis*) in the number of melanophores appearing on the flank and their degree of dispersion there.

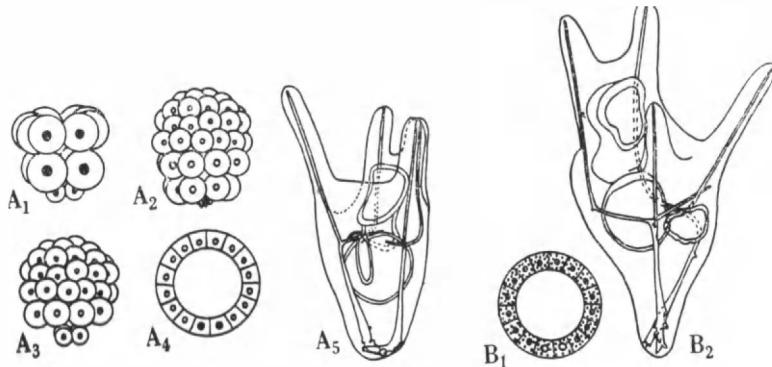


FIG. 19. Nuclear control of skeletal features in hybrid sea urchin larvae. The crosses involve *Paracentrotus lividus*, which normally develops straight body rods; and *Psammechinus microtuberculatus*, which develops thick, bent, club-shaped rods. In the A series illustrated above the animal half of a *Paracentrotus* morula (A₂) is combined with the micromeres of the haploid hybrid consisting of *Paracentrotus* cytoplasm plus *Psammechinus* nuclei (A₁). This gives an embryo (A₃, A₄) consisting of *Paracentrotus* cytoplasm and nuclei, except for the nuclei of the skeleton-forming micromeres, which are *Psammechinus* (shown in black). The body rods, as seen in A₅, develop the clubbed shape characteristic of *Psammechinus*. The results from the reciprocal combination (B₁) are shown in B₂. Here the *Paracentrotus* nuclei (shown in white) control the formation of straight body rods, although the cytoplasm (dotted) and all the other nuclei (black) are derived from *Psammechinus* (Hörstadius, 1939).

4. Summary

The study of hybrids has provided information on nucleocytoplasmic interactions during three phases of development, as follows:

(1) Fertilization and cleavage may occur normally in hybrids composed of widely separated species, e.g., in crosses between *Bufo* and *Rana*, or between *Paracentrotus* (*Strongylocentrotus*) and *Arbacia*. Cleavage rate and development to gastrulation in such crosses appears to be controlled entirely by the egg cytoplasm. Combined with the evidence outlined in the preceding section of this chapter, this result demonstrates that the nuclei make no specific contribution to pregastrula development.

(2) Gastrulation: After proceeding normally through cleavage stages hybrids of the type mentioned above generally are arrested in late blastula and early gastrula stages. The nature of the block to further

development differs in different hybrids. In some the block can be overcome when the hybrid tissues are grafted to normal embryos—suggesting that the deficiency is in the form of some diffusible material which cannot be produced in the hybrid cells, but which can be utilized when it is supplied from without by normal cells. In other cases the block cannot be overcome in this manner—indicating that the incompatibility between nucleus and cytoplasm affects some nondiffusible component of the cells.

Nuclear transplantation work on lethal hybrids shows that the cessation of development is not initially associated with lethal changes in the nuclei. However, more subtle changes in the genetic properties of the nuclei may possibly result from their replications in foreign cytoplasm.

Biochemical studies have shown that the cessation of development in lethal hybrids is accompanied by pronounced alterations in intermediary and nucleic acid metabolism. However, the problem of assigning these biochemical changes to particular alterations in nucleocytoplasmic interactions, and in turn to specific morphogenetic events, remains to be worked out.

(3) Later phases of development: Certain hybrids which develop beyond gastrulation provide an opportunity for determining the relative roles of nucleus and cytoplasm in organogenesis. The general result appears to be that the nucleus begins, during gastrulation, to exert a specific influence on both developmental rate and on the form of the organs that later differentiate. In some cases the nucleus appears to exert the dominant influence in organogenesis, but there are other instances in which a cytoplasmic influence is also apparent.

C. Specific Gene Effects on Development

Hybridization studies of the type described above tell us that the nucleus begins to exert an effect during gastrulation, influencing both the rate of development and the form of certain organs arising in subsequent stages. For a more precise analysis of the role of the nucleus we turn now to a brief consideration of the effects of genes on differentiation. Much work has been done on this subject during the past twenty years or so, which by now leads to the conclusion that normal development, from the earliest stages onward, depends on the presence of a series of genes each affecting specifically some developmental event or combination of events. The evidence for this conclusion comes mainly from studies of the developmental effects of lethal factors in *Drosophila*, the mouse, and the chicken (see reviews by Hadorn, 1948, 1951, 1955, 1956; Poulson, 1945; Gluecksohn-Waelsch, 1953, 1954; Stern, 1954, 1956; Landauer, 1951; Waddington, 1956; and others).

In *Drosophila* a large number of lethal mutants are known, some of which have been extensively studied by Poulson, 1945; Hadorn, 1955, 1956; Ede, 1956; and others. The lethality in different cases is expressed at widely different times in development, ranging from cleavage to adult stages. As an example of mutants affecting very early stages in organogenesis we may mention the series of notch (facet) lethals investigated by Poulson (1945). These involve deficiencies around the facet locus of the X, ranging in size from 45 bands to 0 bands (i.e., no cytologically observable deficiency). Regardless of the size of the deficiency, if it includes the facet locus, the developmental consequences are the same, the outstanding features being: (1) a greatly hypertrophied nervous system; (2) a deficiency in the other ectodermal derivatives; (3) a failure of the mid-gut to form (fore-gut is rudimentary, but hind-gut is nearly normal); (4) a failure of the mesoderm to differentiate. Together these abnormalities constitute what Hadorn (1948) has called a "pattern of damage," attributable specifically to the notch deficiency. Other deficiencies affecting early development produce different patterns of damage. For example, a deficiency at the white locus permits normal ectodermal differentiation, which notch does not, but has a more drastic effect than notch on endodermal and mesodermal differentiation (Poulson, 1945). At later stages of development additional lethal factors come to expression (Hadorn, 1948, 1951, 1955). Thus the mutant, lethal giant, shows no externally visible abnormalities until the beginning of pupation when development ceases with a combination of abnormalities involving the ring gland, gonads, salivary gland, fat body, and imaginal discs. Another mutant, meander lethal, stops development just before pupation with a different combination of abnormalities resulting from a characteristic disproportionate growth of various larval organs. As Hadorn emphasizes, each lethal mutant must be analyzed in detail in order to establish its pattern of damage, but when this is adequately done the pattern is seen to be specific for each locus. In *Drosophila*, therefore, the evidence appears conclusive that each phase of development normally depends upon the activity of specific gene loci.

Of numerous genes affecting development in chickens and mice we will mention here only a few. One group of mutations in chromosome IX of the mouse appears to be of particular embryological interest. The mutants in this group display various abnormalities in the axial structures—for example, duplications in the homozygous kink mutant; and deficiencies in the homozygous short-tail mutant. Apparently the genes in this group are concerned with the development of the primary inducer system; i.e., with the notochord and mesoderm (Gluecksohn-Schoenheimer, 1949a, b; Gluecksohn-Waelsch, 1954). Another very interesting

mutation in the mouse is short tail (*Sd*) (Danforth, 1930). In addition to its effects on the tail this mutant displays a variety of other abnormalities including deficiencies in the development of the kidneys. In normal mice the metanephros develops from two components—the nephrogenic mesenchyme, which gives rise to the secretory tubules and Bowman's capsules; and the ureteric bud, which grows into the mesenchyme and branches extensively to form the collecting tubules. In the *Sd* mutants Gluecksohn-Schoenheimer (1945) could show that the nephrogenic mesenchyme is normal in appearance, but that the ureter varies greatly. In some cases the ureter fails to make contact with the mesenchyme, and kidney differentiation fails completely. In other instances the ureter does extend into the mesenchyme but shows reductions in the number of branching tips it puts out. Secretory tubules develop only from those parts of the mesenchyme in contact with the branches of the ureter, and more or less deficient kidneys result. This evidence suggested that kidney development involves an inductive interaction between the ureter and the nephrogenic mesenchyme (see Grobstein, 1955, for proof of this), and that the effect of the *Sd* mutant is primarily on the growth and branching of the ureteric component of the system (Gluecksohn-Waelsch, 1954).

In the chicken several lethal mutants are known, of which the most thoroughly studied embryologically appear to be the creeper mutant and the wingless and polydactylous mutants (see reviews by Landauer, 1951; Gluecksohn-Waelsch, 1953; Hadorn, 1955). One of the most interesting studies of these mutants, and the only one to be described here, is that of Zwilling on the genetic control of limb development. In the chick, as in other vertebrates, the limb bud consists of a mound of mesoderm covered by ectoderm. The ectoderm at the apex of the bud is thickened to form a ridge. This ridge is essential for limb formation; if it is removed or replaced by other ectoderm, limb outgrowth ceases (Saunders, 1948). The mesoderm of the bud is also essential. When it is replaced by nonlimb mesoderm from the flank, limb development also fails (Zwilling, 1956b; see also recent paper by Saunders *et al.*, 1957, on development of leg mesoderm combined with wing ectoderm). Thus a specific combination of apical ectoderm and bud mesoderm is required for normal limb development; and Zwilling's experiments were designed to determine how these interacting components are affected in the mutants. In the case of the polydactylous mutants it could be definitely shown (Fig. 20, a) that the gene affects primarily the mesoderm; for when mutant mesoderm was combined with normal ectoderm polydactylous limbs were still obtained, while normal mesoderm combined with mutant ectoderm gave normal limbs (Zwilling and Hansborough,

1956). Morphological observations indicated that the mutant mesoderm acts by first inducing in the ectodermal component an enlarged apical ridge. Apparently this is an essential event in the sequence leading to polydactyly, for if some of this ridge material is removed, normal limbs may result despite the continued presence of the mutant mesoderm (Hansborough, quoted in Zwilling, 1956b).

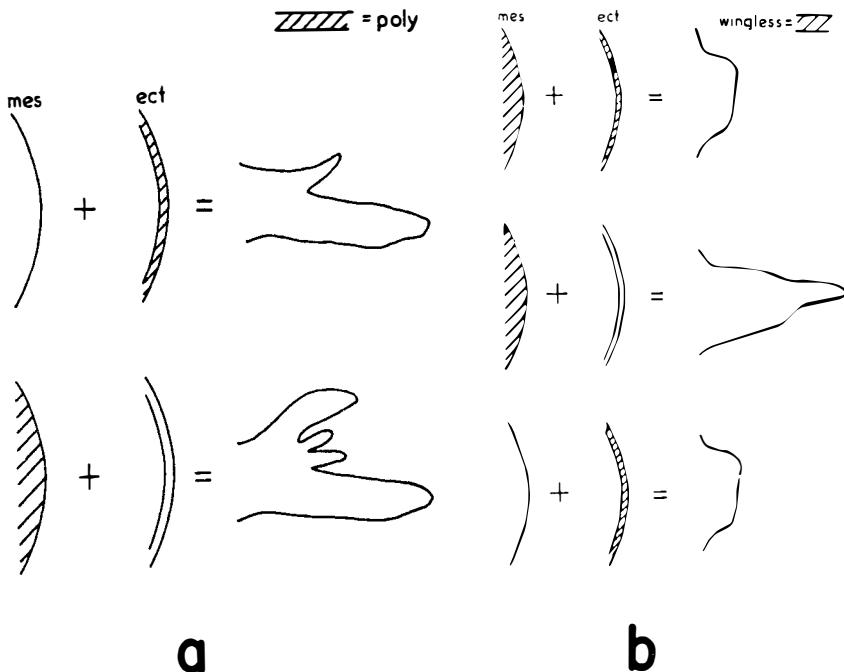


FIG. 20. Roles of wing bud mesoderm and ectoderm in the development of genetically determined polydactyly (a) and winglessness (b) in the chick. See text for description (Zwilling, 1956b).

A comparable analysis of limb development in wingless mutants gave a somewhat different result. In these embryos the wings (and to some extent the legs) fail to develop appreciably beyond the bud stage. Zwilling (1949) first showed that the apical ectodermal ridge of the mutant wing bud begins to develop in normal fashion but soon degenerates. The functional significance of this observation was later proved by combining wingless ectoderm prior to the time of ridge degeneration with normal mesoderm. In this combination the ridge still degenerates and no wing develops (Zwilling, 1956a). The interpretation of this result is somewhat complicated by the fact that the mutant mesoderm, when

combined with normal ectoderm, also shows deficiencies. It produces some outgrowth, but gives at best only a spikelike limb (Fig. 20, b). At first sight it might be supposed that in the wingless limbs both the ectodermal and mesodermal components are affected directly by the mutation, but it is also possible that the primary effect is on only one of the two components, which then produces an irreversible alteration in the second component of the bud. No doubt a continued utilization of recently developed methods for studying inductive tissue interactions (Grobstein, 1953; Zwilling, 1955) will lead to a solution of this type of problem, giving us a much more precise analysis of gene action than we have had heretofore.

Compared with the forms discussed above, the amphibia provide only a small number of identified genes—an unfortunate circumstance in view of all the other advantages these animals would offer for studies of gene action in development. A few genes controlling pigmentation pattern have been identified in anurans (Moore, 1942, 1943; Lantz, 1947; Moriya, 1952; Moriwaki, 1953), and in the urodeles there is the well-known recessive white gene (*d*) in the axolotl (Haecker, 1907). To these we may now add two new genes both affecting early development in the axolotl (Humphrey, 1948, 1952, 1958).

Of the pigmentation genes it appears that the white gene in the axolotl is the only one which has been extensively studied with respect to its mode of action in development. In a series of studies Dalton has shown that the initial effect of this gene is exerted, not on the pigment cells themselves, but rather on the embryonic tissues through which the pigment cells must migrate from their place of origin in the neural crest. Absence of a direct effect on the pigment cells was demonstrated by culturing "white" neural crest *in vitro*. When thus separated from the influence of other "white" tissues the crest material produces normal melanophores in approximately normal numbers (Dalton, 1950a). The controlling influence of the white tissues, particularly epidermis, could be demonstrated in several ways. For example, if part of the epidermis of a white embryo is replaced by that of a normal ("black") donor, the "white" melanoblasts will migrate readily beneath the black graft but not beneath the adjacent white epidermis (Dalton, 1950b). The inhibitory effect of the white tissues can also be demonstrated by grafting black neural crest to a white host. In this combination the "black" melanoblasts are inhibited in their migration, just as are the "white" ones (Dalton, 1950b). Thus, there would seem to be no doubt that the primary effect of the white gene is to alter the tissue environment in some unknown fashion so as to inhibit the migration of the pigment cells.

In later development the pituitary may also be concerned in the

effects of the white gene, but the exact nature of its involvement is so far undetermined (Dalton, 1953; Dalton and Krassner, 1956).

Finally, we would like to mention the two genes discovered during recent years by Humphrey (1948, 1952, 1958). These are particularly important because they effect early development in a form, the axolotl, which offers exceptional advantages for embryological and cytological experimentation. Humphrey's original report (1948) described a lethal condition, appearing in 25% of the offspring from certain matings, and ascribable to what was then thought to be a single recessive gene, *f*, for "fluid imbalance." Embryos homozygous for this gene are first affected

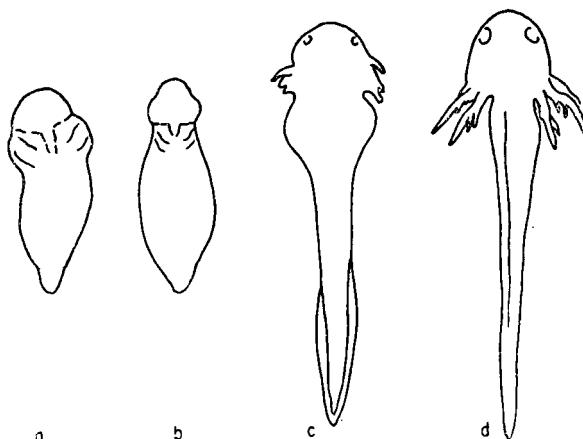


FIG. 21. Gene effects on embryonic development in the axolotl. a. Swelling of the head in tail bud embryos homozygous for the fluid imbalance gene, *f*. c. Appearance of ff embryo at hatching. Swelling is now confined largely to the region of the pronephros. Stunted gills are the result of the presence of an additional recessive gene, *g*, normally, linked with *f*. b. and d. = Normal controls for (a) and (c). (Humphrey, 1948, 1958; diagram from Fankhauser, 1952.)

at the tail bud stage, when a swelling becomes apparent in the head and suprabranchial regions (Fig. 21). This swelling is due mainly to an accumulation of fluid in spaces within the mesoderm of these regions, although excess fluid may distend the archenteron and extend into other spaces as well. Later on the fluid is diminished in amount or sometimes disappears entirely, but now the developing gills become stunted and display subnormal circulation. Embryos with these abnormalities practically always die in prelarval (i.e., prefeeding) stages. However, in rare instances embryos recover from the fluid imbalance, develop normally functional gills, and survive to sexual maturity (Humphrey, 1952). The explanation of these exceptions has recently been found to lie in the

fact that the lethal condition as originally described is caused by two genes (Humphrey, 1958). One of these (*f*) produces the fluid imbalance, and in itself is not invariably lethal. The second gene (*g*) produces as its first obvious effect the stunting of the gills and is 100% lethal. These two genes are normally linked, but in rare cases may be separated by crossing over, thus making possible the occasional *ff* individuals (actually *ffGg* or *ffGG*) that survive to sexual maturity.

In addition to identifying the two genes described above Humphrey (1952) has made a most important observation concerning the mode of action of one of them, *f*. Briefly, this amounts to the fact that the *f* gene can affect embryonic development through an influence previously exerted on the egg cytoplasm during oogenesis. The evidence for this comes from a comparison of offspring of *ff* and *Ff* females showing (1) a precocious and extreme development of fluid imbalance in the *ff* offspring of homozygous mothers, and (2) the appearance of a transient and less extreme imbalance in *Ff* offspring of homozygous mothers—a feature which is *not* seen in *Ff* offspring of heterozygous mothers. These differences in the embryos can only be due to the effects of the different genotypes of the mothers, mediated through the egg cytoplasm.

I. Summary

The fact that the various phases of development require the action of specific genes seems firmly established, particularly in the extensive studies of lethal mutants. The relationship between developmental events and the genes involved in these events is a complex one; but when adequately analyzed each gene is seen to be concerned in a specific way in the development of a particular combination of organs. In a few cases the analysis can be carried still further to show specific gene effects on the component tissues in an inductive combination. Some other aspects of gene action in development will be considered in the section to follow.

D. Genetic Studies on Somatic Cells

Having reviewed some of the evidence for a specific and essential nuclear role in differentiation, we should now consider the central problem of developmental genetics. This has to do with the nature of the interactions between the cytoplasmic localizations and the nuclei, leading to an orderly predictable formation of divergent cell types in development. As Ephrussi (1956) has so clearly pointed out, our approach to this problem has been conditioned by two basic premises which are, (1) that the nuclear genetic complement remains intact and identical in the various somatic cell types, and (2) that the differences

between these cells are nevertheless highly stabilized, if not irreversibly determined. Although much used in the formulation of theories of differentiation, these premises are, of course, only statements of the probable condition of differentiated cells. Far from being firmly established they actually represent the basic problems which for many years could not be subjected to direct experimental attack because of the lack of methods for studying genetic changes in somatic cells. Recently such methods have become available, making it possible now to review the basic problems with some new information at hand.⁴

1. Tissue Culture and Tissue Transplantation Studies

Two kinds of evidence show that the various cell types arising in development possess a high degree of stability in their differentiated forms. First, there is the demonstration from experimental embryology that tissues which have begun one type of differentiation, say through exposure to a particular inductive stimulus, lose their ability to respond to other inducers and to differentiate in other directions. At each such step there is a "restriction of potencies," until eventually a given cell type appears to be restricted to a limited number of possible expressions, in some cases apparently only one. We speak here of a stabilization of differentiated cell types, but it would be more accurate to refer to tissue types since the embryological experiments referred to involve test grafts consisting of large numbers of cells.

In order to determine if differentiation actually does involve stabilized changes in the properties of individual cells, numerous tissue culture studies have been carried out. In some cases cell type transformations occur in culture (Weiss, 1944; Fell and Mellanby, 1953; Fell, 1957; Wilde, 1955); but in general the cultured cells appear to reproduce true to type over extended periods of time (see review by Ephrussi, 1956). There are, however, certain limitations in most tissue culture studies making it difficult or impossible to obtain absolutely decisive information on the character of the differences between individual cell types. These limitations derive from the fact that the cultures are usually kept going by the transfer of large numbers of cells at each subculturing period. Thus, as Puck *et al.* (1957a) point out, more than one cell type could well persist, giving the possibility that the observed character of the cultured cell populations might depend to an unknown degree on the interactions between cells rather than on their intrinsic properties. A second hazard is of course that of selection, which could lead to a

⁴ A very recent paper by Schultz (1958), which we saw in manuscript after this chapter was written, provides a most valuable discussion of genetic mechanisms in somatic cell differentiation.

change in the properties of the cell population that might be mistaken for a transformation of cell type. These particular uncertainties have been eliminated by the development of methods for the cultivation of single cells (Sanford *et al.*, 1948), and the routine establishment of cell clones (Puck and Marcus, 1955; Puck *et al.*, 1956).

Recently Puck *et al.* (1957a, b) have obtained clones from single cells of a variety of human tissues. These tissues give cells which fall into two general classes, being either epithelioid or fibroblastic in culture. The classes have so far been found to differ from each other in morphology, nutritional requirements, sensitivity to X-rays, and sensitivity to tryptic digestion of the bond uniting the cells to glass in the culture dishes. Since these differences are expressed by the individual cells of the respective classes (as determined by the properties of the derived clones) and persist over many cell generations, it may be concluded that they represent true genetic differences between the epithelioid and fibroblastic cell types. Of even greater interest is the fact, mentioned briefly by Puck *et al.* (1957a), that fibroblastic cells from different organs display differences in morphology, nutritional requirements, and susceptibility to viruses.

These experiments on clonal cell cultures (plus some of the nuclear transplantation studies to be described below) provide for the first time the demonstration of a genetic basis for differences among somatic cell types. Do these genetic differences, shown to exist for the cells in culture, exist also *in vivo* prior to culturing? We raise this question because of the possibility that there may be chromosome number changes in cultured cell populations which could confer upon them genetic properties that they do not display *in vivo*. For example, T. C. Hsu (1958) has shown recently that the chromosome number of cultured human cells changes from a predominantly diploid value (46) to irregular near-polyplloid values in the course of five to eight subculturings following the original explantation. A similar phenomenon has also been reported by Bender (1957). Presumably such a shift occurred also in the epithelioid clones mentioned above, for Puck *et al.* (1957b) report that they display chromosome numbers of about 78, compared with a value of about 46 for the fibroblastic clones (see also Levan's (1956) report of hypertriploid numbers in nonclonal cultures of human epithelial cells). Thus, the differences between the two types of clones, while genetically determined, are probably not representative of the homologous cell populations *in vivo*. On the other hand, the differences among the fibroblastic clones, which according to a brief report (Puck *et al.*, 1957b) all display approximately normal chromosome numbers, should more nearly represent the differences among the *in vivo* populations from which

the clones were derived. Thus, the indications are that at least some somatic cells normally differ from each other in a genetic sense, i.e., in the sense that they possess distinctive heritable traits which are passed on unchanged over many cell generations.

2. Nuclear Transplantation Studies

While the clonal cell studies promise to define the genetic properties of somatic cells, they so far provide no information on the nature or intracellular location of the genetic mechanisms involved. For this we turn now to a review of recent nuclear transplantation studies. These investigations are basically extensions of the much older work in which, by constricting or flattening eggs, the cleavage nuclei were forced into different cytoplasmic regions than they would normally occupy. As is well known, in such cases the alteration in the distribution of the nuclei had no effect on the subsequent development, whereas deletions or redistributions of cytoplasmic regions had a profound effect. Thus, it was possible to conclude that it is the distribution of the cytoplasmic materials, and not of different kinds of nuclei, which sets the pattern for differentiation. In reviews of this older literature it is sometimes assumed or implied that the experiments also proved the identity of cleavage nuclei *after* they were distributed to the different cytoplasmic regions, but in fact no such thing was demonstrated. Thus, in one of the most decisive experiments of this type, Wilson (1896) could show that it made no difference which of the cleavage nuclei were distributed to the macromeres and micromeres of the highly mosaic *Nereis* egg. The macromeres still developed into gut, and the micromeres into the various other embryonic structures, according to their mosaic nature. The demonstration of cytoplasmic control of the initial determination of the embryonic regions was unequivocal, but what of the condition of the nuclei *following* their distribution to the cytoplasmic regions? Do they remain identical and totipotent, or do they respond to the special cytoplasmic environments by changing so as to reinforce the differentiation? This could only be tested by transferring nuclei from one specialized cell to another, or to an uncleaved egg, and such experiments were not done.

The same uncertainty concerning the genetic condition of the cleavage nuclei is presented even more obviously in *Ascaris*. We have already described the process of chromosome diminution which occurs in the somatic blastomeres of *Ascaris* embryos, but not in the blastomeres destined to form germ cells. To this should be added a brief account of an experiment of Boveri (1910) demonstrating the cytoplasmic control of the diminution process. Reference to Figure 22 will make the experiment clear. In normal eggs the first cleavage divides the egg into animal

and vegetal blastomeres. At the beginning of the second division the chromosomes of the animal blastomere undergo diminution while those of the vegetal blastomere remain intact. One of the experiments demonstrating the cytoplasmic control of diminution involved centrifuging the uncleaved egg, thus flattening it and forcing the first cleavage to occur at right angles to its normal direction. Thus, in the centrifuged eggs there is no segregation of animal and vegetal cytoplasm such as normally occurs, and in the ensuing prophase neither of the first two nuclei undergoes diminution. From this and other observations it could be concluded that the maintenance of the chromosomes in undiminished

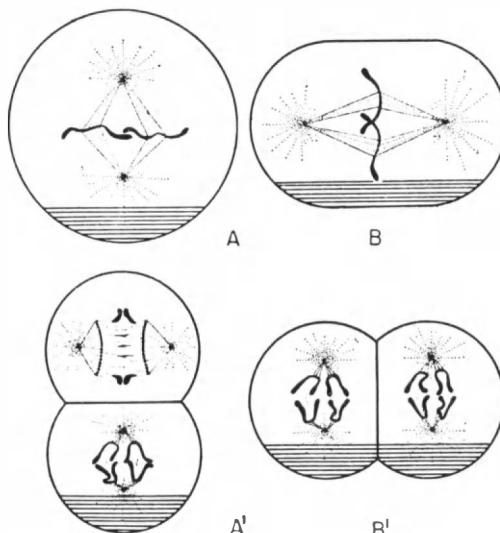


FIG. 22. Cytoplasmic control of chromosome diminution in *Ascaris*. A, A'—normal eggs, B, B'—centrifuged eggs. The shaded area indicates the distribution of a hypothetical cytoplasmic material. In blastomeres containing this material the two large chromosomes remain intact; in blastomeres lacking it the chromosomes undergo diminution (Boveri, 1910; figure from Stern, 1954).

form depends upon some cytoplasmic component originally present in the vegetal portion of the egg. Blastomeres containing this type of cytoplasm retain the two large chromosomes, while in all blastomeres lacking it the chromosomes undergo diminution. Are we to suppose now that the somatic and germ line nuclei are still genetically equivalent despite the striking morphological differences in their chromosomes? Again nuclear transplantation studies would be required to settle the point, and if technically feasible should prove extremely interesting and informative in *Ascaris*.

While mosaic embryos present the problem of cytoplasmically in-

duced nuclear changes in especially clear form, the same problem naturally exists in other types of embryos which show a determination of their organ regions at a later time in development. For technical reasons almost all of the nuclear transplantation work on this problem has been done on one of these embryos, that of the frog, *Rana pipiens*; and has involved transplanting nuclei from various embryonic cells into enucleated eggs. The method used is illustrated in Figure 23. The results are as follows:

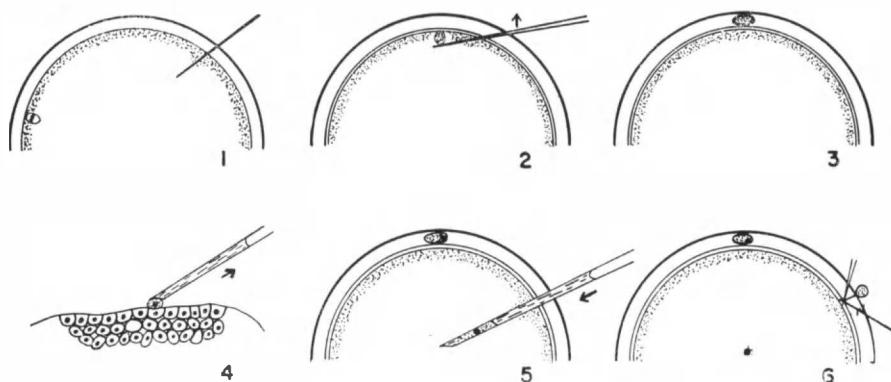


FIG. 23. Diagram illustrating method for transplanting nuclei from embryonic cells into enucleated eggs (*Rana pipiens*).

The upper row of figures shows steps in preparing enucleated eggs. The egg is activated (1), and enucleated (2) with a clean glass needle. The enucleation operation results in the formation of an exovate containing the egg nucleus, shown trapped in the jelly surrounding the egg in (3).

The lower row of figures shows the procedure for transplanting nuclei. The donor cell is isolated on the surface of an intact blastula (which serves as an operating platform) and is then drawn up into a micropipette (4). Since the diameter of the pipette is somewhat smaller than that of the cell, the cell surface is broken. When the broken cell is injected, as shown in 5, the nucleus is liberated undamaged in the egg cytoplasm. As the pipette is withdrawn it tends to draw the surface coat of the egg with it forming a small canal to the exterior which must be severed, as shown in 6, to prevent leakage. (Briggs and King, 1955.)

a. Blastula nuclei. The earlier constriction studies of Spemann (1914) on newt eggs and Seidel (1932) on dragonfly eggs indicated that the nuclei in these forms remain identical for at least the first four to six cleavages, i.e., to about the 128-cell stage. The first nuclear transplantation experiments extended the analysis to nuclei of frog blastulae consisting of 8000 to 16,000 cells (Briggs and King, 1952, 1953). When transferred to enucleated eggs these nuclei elicited completely normal embryonic and larval development. Similar results have since been reported

by Subtelny (1958) and by Moore (1958a). Thus, in the frog it is definite that blastula nuclei are completely undifferentiated and equivalent to the zygote nucleus at the beginning of development. This result is to some extent the expected one. The donor blastula cells are still totipotent, and the transplantation experiments confirm the fact that their nuclei are too.

Some attempts have been made to transplant nuclei of newt blastulae, but the recipient eggs have so far not been observed to develop beyond blastula stages (Waddington and Pantelouris, 1953; H. E. Lehman, 1955, 1957). Lehman's (1955) cytological studies indicate that one of the causes of the arrested development is an irregular distribution of chromosomes, known from Fankhauser's (1934b) work to be lethal. This condition may result from operative damage, to which the newt material may be more susceptible than is that of the frog.

Most recently Fischberg *et al.* (1958) have reported successful transfers of blastula and gastrula nuclei in *Xenopus laevis*. Some of the recipient eggs developed normally, thus confirming the result obtained with frog blastula nuclei. Other test eggs failed to reach the tadpole stage, for reasons as yet unspecified.

b. Early gastrula nuclei. In the early amphibian gastrula we recognize the following principal regions: (1) the presumptive ectoderm and medullary plate material of the animal hemisphere. Cells of this region are known to remain undetermined until exposed to the chorda-mesoderm at a later stage in gastrulation. (2) The marginal zone (including dorsal-lip material), known to be regionally determined to form chordomesoderm. However, it appears that this determination is a property of the region and not of its constituent cells, which still display a wide range of potencies when explanted or transplanted to various embryonic sites (Holtfreter, 1938). (3) Endoderm—according to Holtfreter (1938) this portion of the embryo is already determined at the early gastrula stage.

Transplantation experiments carried out with nuclei from all these regions gave approximately the same result. Regardless of the source of the nuclei, the majority of the test eggs developed into normal embryos (King, Briggs, and Di Berardino, unpublished).⁵ The absence of nuclear differentiation is to be expected in the animal hemisphere and marginal zone in view of the fact that the cells in these regions are not determined. However, the interpretation in the case of the endoderm is less certain. One possibility is that the individual endoderm cells are in fact

⁵ More recent experiments (King, Briggs, and Di Berardino, unpublished) indicate that nuclei of some of the presumptive endoderm cells already may be differentiated in the early gastrula.

determined, and that this involves no stable alteration in the properties of the nuclei. But it is also possible that the endoderm, while determined as a tissue, consists of cells which individually are still labile as to type. The explantation and grafting experiments according to which this tissue was judged to be determined do not exclude this interpretation.

c. *Late gastrula nuclei*. In the late gastrula the main organ areas are laid down and display determination, at least with respect to general tissue type. Tests originally carried out with chorda-mesoderm and medullary plate cells showed that the nuclei of a small proportion of these cells retain the ability to promote normal development of test eggs. The remainder, representing the large majority of nuclei tested, gave either no cleavage or deficient development (King and Briggs, 1954). In view of the possibility that some of the nuclei might have been damaged in these particular tests, the interpretation of the result remained obscure. Later, following some technical improvements which permitted the use of the larger and technically more favorable endoderm cells, an extensive series of experiments was carried out which gave an unequivocal result—showing that the nuclei in these cells do undergo stabilized changes in their properties (King and Briggs, 1955; Briggs and King, 1957). Briefly, these changes amount to restrictions in the capacity of the nuclei to promote differentiation of test eggs. Thus, late gastrula endoderm nuclei (all from the presumptive anterior mid-gut region) fall into the following categories (Table I).

TABLE I
LATER DEVELOPMENT OF EGGS INJECTED WITH
ENDODERM NUCLEI

Stage of Donor	Total No. Normally Cleaved Eggs	Types of Development - % of Total		
		arrested - undifferentiated	abnormal embryos	larvae
(○)	57 (±100%)	10%	13%	77%
(○)	79 (±100%)	27%	53%	20%
(○)	25 (±100%)	72%	24%	4%

(1) About 20% are undifferentiated and promote normal development of the recipient eggs.

(2) Fifty-three per cent promote normal development through gastrulation, but in later stages the test embryos show deficiencies, particularly in the ectodermal derivatives such as nervous systems, sense organs, neural crest, etc. (Fig. 24). Apparently these nuclei have lost the capacity to promote normal differentiation of the ectoderm.

(3) Twenty-seven per cent promote development only to late blastula or early gastrula stages. Thus, nuclei in this group apparently can no longer promote the movements of gastrulation and the formation of mesoderm.

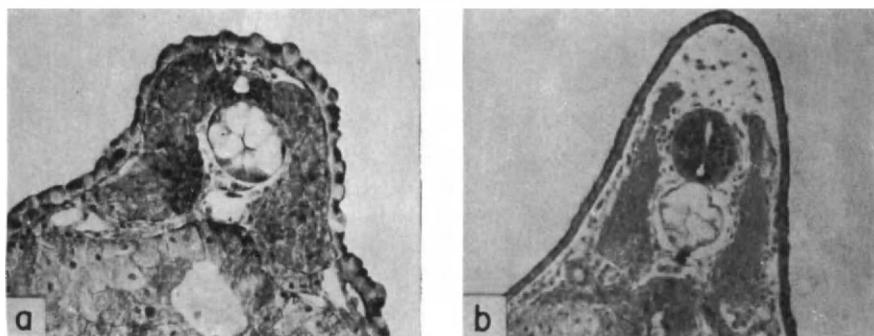


FIG. 24. Section through trunk of (a) "endoderm embryo," and (b) control. The "endoderm embryo" was produced by transplanting a late gastrula endoderm nucleus into an enucleated egg. The embryo gastrulated normally and developed the inductor system (notochord and somites) but displayed rudimentary and degenerating central nervous system and neural crest (Briggs and King, 1957).

At later stages of development (neurula, tail bud) restrictions in the differentiation-promoting ability of endoderm nuclei become more severe. Now most of the test eggs that cleave normally are arrested during gastrulation. Added to this there is a further change which reduces the capacity of the nuclei to elicit even a normal set of cleavages in the recipient eggs (Table II).

These results leave no doubt of the fact that differentiation of endoderm cells is accompanied by definite changes in the nuclei. Several questions now arise concerning the nature of these changes. Of these the most important for an appraisal of their significance in cell differentiation relate to (a) their specificity, and (b) their stability or irreversibility. The stability of the endodermal nuclear changes has been investigated by serial transplantation experiments of the type illustrated in Figures 25 and 26 (King and Briggs, 1956). These experiments involve first a series of nuclear transfers of the usual type from endoderm cells to enucleated eggs. The different eggs, nucleated by descendants

TABLE II
CLEAVAGE OF EGGS INJECTED WITH
ENDODERM NUCLEI

Stage of Donor	Total No. Recipient Eggs	No. of Eggs Showing Cleavage and Blastula Formation	
		No.	%
(○)	101	65	64%
(○)	155	99	64%
(○)	98	32	33%
(○)	130	22	17%

of different endoderm nuclei, display the various types of development already described. One of these original recipients may now be sacrificed in the blastula stage to provide nuclei for a new series of transfers. The enucleated eggs into which these nuclei are placed will all be nucleated by descendants of a single original endoderm nucleus, forming a nuclear clone. In contrast to the varied types of development seen among the recipients of different nuclei, the individuals within a clone develop in a uniform manner. In some clones all individuals are arrested in gastrulation, in others all embryos display the same type of abnormalities in postgastrula development, and so on. Thus, descendants of any given endoderm nucleus express in uniform fashion a particular restriction in differentiation-promoting capacity. The stability of these nuclear changes can be tested by sacrificing clonal blastulae to provide nuclei for two or more clonal generations, as indicated in Figures 25 and 26. The successive generations within a given clone continue to develop in the same uniform fashion, giving no indication of reversal to a more normal type of development. Also, it should be noted that

there is usually no progression of the nuclear change to give more limited differentiation so long as the nuclei are replicating in "undifferentiated" (pregastrula) cytoplasm. However, changes in this direction are seen occasionally (Fig. 26), and the question needs further experimentation.

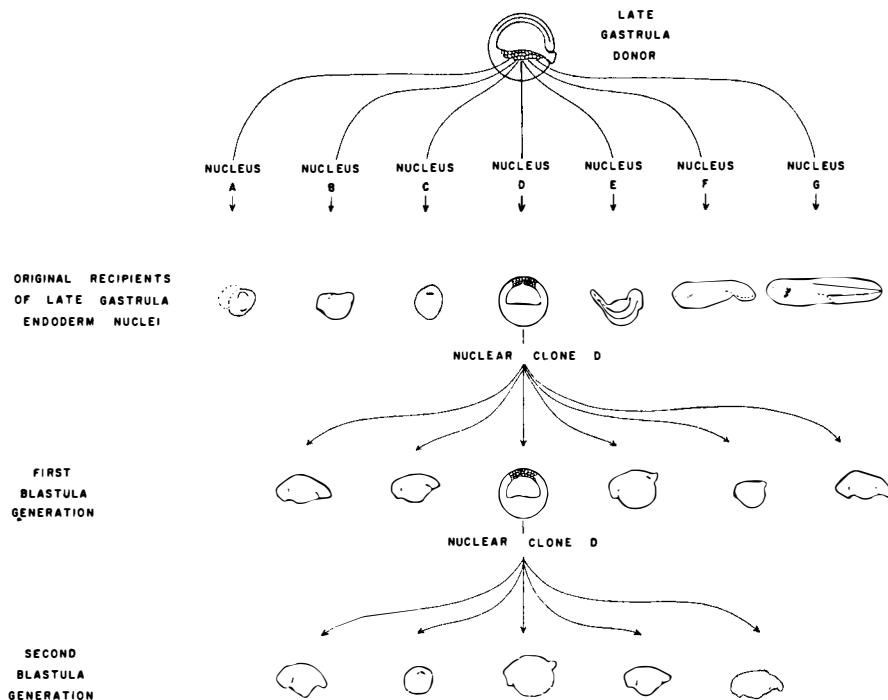


FIG. 25. Diagram illustrating results of an experiment on serial transplantation of endoderm nuclei. See text for description (King and Briggs, 1956).

The clonal experiments show that nuclear changes in differentiating endoderm cells are highly stabilized heritable transformations, at least under the conditions employed in these tests. Are they also specific? Unfortunately, no final answer can yet be supplied to this question. Detailed morphological studies of "endoderm embryos" show that their deficiencies are consistent with the endodermal origin of their nuclei (Briggs and King, 1957). Also, some tests carried out on nuclei of a distinctly different cell type (medullary plate) have given different results than those described above for endoderm nuclei (King and Briggs, unpublished). However, considerably more work will be required before the question of specificity can be regarded as definitely settled.

A few more points should be mentioned briefly before closing this

account of the nuclear transplantation studies. Regardless of the specificity problem, definite alterations in the functional properties of nuclei do occur, and it is of considerable importance to inquire which part of the nuclear complex might be the seat of such changes. Donor cell cytoplasm can be excluded as a controlling factor since control experiments show that it has no effect when injected by itself into either enucleated or normally nucleated eggs (Briggs and King, 1957). This leaves the nucleus proper and the perinuclear structures—notably the

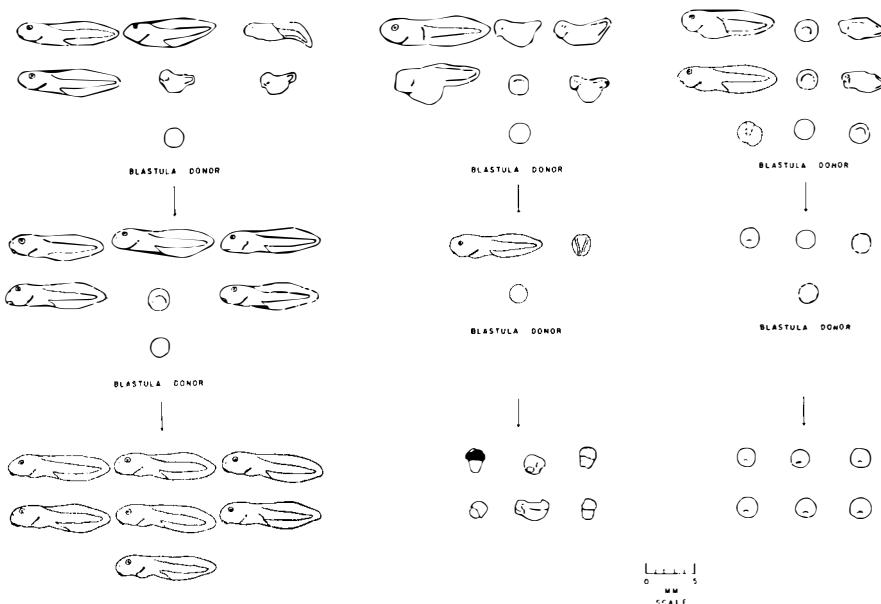


FIG. 26. Results of three serial transplantation experiments with endoderm nuclei. Three late gastrula donors were used (not diagrammed in above figure). Nuclei from these donors were transferred to three groups of enucleated eggs, which developed in the various ways illustrated in the upper part of the figure. The derived clones developed much more uniformly. See text for additional description (King and Briggs, 1956).

centrioles and the Golgi apparatus. Concerning the nucleus proper we can only say that the stabilized changes described above do not involve alterations in chromosome number (King and Briggs, 1956). Of course, changes in the condition of chromosomal loci might occur. Visible chromosomal differentiation can be seen in the puffed loci of giant chromosomes of insects (page 566) and it is possible that comparable changes might be occurring on a submicroscopic scale in the much smaller chromosomes of amphibian cells. If so, the nuclear changes revealed in the transplantation tests could be due to stabilized changes

in the condition of specific gene loci. In view of the evidence for specific gene effects on developmental processes (see preceding section) this is an attractive hypothesis. However, it is strictly conjectural, and in the absence of any direct evidence we would have to consider it equally possible that some nonchromosomal nuclear component, or even the perinuclear organelles, may be responsible for the changes revealed in the transplantation studies.

3. Summary

In the past the analysis of nucleocytoplasmic interactions leading to cell differentiation has been hampered by lack of information on the genetic properties of the end products—the various differentiated cell types. Recent studies, particularly those on cultured cell clones, indicate that in some cases at least somatic cells differ from each other in a genetic sense, i.e., in the sense that they possess distinctive heritable traits which are passed on unchanged over many cell generations. This does not mean that the traits must be regarded as irreversibly determined—it signifies rather that under specific conditions they are highly stabilized and that there is a genetic basis of some sort for this stability.

Efforts to analyze the relative roles of nucleus and cytoplasm in the origin of different cell types dates back to the early embryological work. This showed that the pattern for the formation of the main organ areas is initially determined by the localizations in the egg cytoplasm. The problem whether the nuclei might later undergo stabilized changes in the different cytoplasmic regions remained unexplored until recently when it became possible to test nuclei from different embryonic regions by transplanting them to enucleated eggs. The results of such tests, so far carried out only on amphibian embryos, show that nuclei remain completely undifferentiated through the early gastrula stage, but thereafter undergo highly stabilized restrictions in their ability to promote the complete range of cell differentiations required for normal development. In the one well-analyzed case (endoderm) the restrictions appear to affect preferentially the differentiation of ectodermal and perhaps mesodermal tissues, and are therefore consistent with the endodermal origin of the nuclei. This evidence suggests that the nucleus, or some nucleus-associated organelle, may be undergoing genetic changes of the type required to confer stability on the differentiated cells. But the exact nature and manner of origin of such changes remains to be worked out.

V. GENERAL SUMMARY

In this chapter we have attempted to present an over-all appraisal of the problems of animal development in genetical and cytological

terms. Considered from this point of view development may be divided into three phases, each presenting its own characteristics and its own problems in experimental analysis.

The first phase of development, oogenesis, is already completed at the time of fertilization when the egg cytoplasm is known to possess an array of materials which later determine the pattern of early organogenesis. Concerning the nature of these materials and their arrangements it must be said that we know them mainly by their acts—by the fact that deletions or rearrangements of portions of the egg cytoplasm result in deficiencies or other abnormalities in the ensuing development. Recent genetical, cytochemical, and fine structure studies have increased our general knowledge of the cellular materials that might represent the morphogenetic localizations of eggs; but the application of such techniques to the egg itself is still in its earliest stages.

The manner of origin of the cytoplasmic materials during oogenesis represents one of the most crucial problems of development, and at the same time the most obscure. It goes without saying that the oocyte nucleus plays an essential role in the growth of the egg, but its specific contribution relative to that of the cytoplasm is unknown (except in a few cases, e.g., as in snails, in which nuclear genes are known to impart a specific characteristic on the cytoplasm which can be expressed independently of the genes later in development). However, there is a growing body of evidence that products of the lampbrush chromosomes are passed into the cytoplasm either through the nuclear membrane, or at maturation when the membrane breaks down. Also, fragments of the nuclear membrane itself may be detached and pass into the cytoplasm. To this can be added some evidence pointing to material exchanges between oocytes and follicle or nurse cells, particularly in insect oogenesis. Thus, at least some mechanisms and materials involved in nucleocytoplasmic exchanges are known. What is lacking is an activity test; i.e., a method of assaying known nuclear products, say, for their contribution to the morphogenetic localizations of the egg cytoplasm.

The second phase of development begins at fertilization and includes the events preparatory to overt organogenesis, which in general may be said to begin at gastrulation. Great variations exist in different species of eggs as to detail, but the main events in all would appear to be (a) various cytoplasmic movements or changes in state bringing about the actual localization of morphogenetic regions, and (b) cleavage of the egg, resulting in a rapid reduction of cell size to some value appropriate for cell differentiation later on. The mechanisms involved in the cytoplasmic movements, changes in state, etc., appear to be as mysterious now as they were fifty or more years ago. They occur independently of

the zygote nucleus, and of the details of the cleavage pattern, and thus are inherent in the cytoplasm itself. This cytoplasm always includes the nonchromosomal material of the large germinal vesicle, previously dispersed in it at the time of maturation. What specific contributions this material makes to the cytoplasmic localizations is uncertain.

Cleavage is, of course, a special form of cell division in which the divisions occur very rapidly without detectable cell growth during the short interphases. Cleavage is known to occur without chromosomes, provided an effective "center" is present. The center, or centrosome, appears normally to be derived from the sperm middle piece, but may arise also in association with the egg pronucleus. Apparently the non-chromosomal material of the germinal vesicle must be dispersed in the cytoplasm before asters and spindles will form in association with the centrosome. Whether true centrosomes (centrosomes containing centrioles) ever arise *de novo* in egg cytoplasm is still an unsolved problem, and one of considerable importance.

In the third phase of development the pattern set up by the cytoplasmic localizations is gradually transformed, through continued cell divisions, morphogenetic movements, and inductive interactions, into a coordinated group of organs and their constituent cell types. This phase, beginning with gastrulation, requires specific contributions from the embryonic nuclei. Prior to gastrulation development may proceed with various kinds of abnormal nuclei, or even to some extent with no nuclei. But beginning at this time normal differentiation requires a balanced genome. Even very small chromosome deficiencies or point mutations may have pronounced and specific effects on organogenesis. Thus, quite specific nucleocytoplasmic interactions must be presumed to occur in the course of cell differentiation. While the nature of these interactions is still obscure it appears now that they may not be controlled throughout by the cytoplasmic materials, for in one case at least the nuclei can be shown by transplantation tests to undergo stabilized changes in their properties during cell differentiation.

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CHAPTER 14

The Acquisition of Biological Specificity

By JAMES D. EBERT

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I. BIOLOGICAL SPECIFICITY

It was in 1947, in a far-sighted article entitled "The problem of specificity in growth and development," that Paul Weiss wrote: "The frequency with which such terms as specificity, selectivity, conformity, correspondence, etc., appear in biological literature is ample proof that they denote a universal and fundamental trait, running like a common theme through all manifestations of life. Yet, they are used with so many different shades of meaning and degrees of precision that it is impossible to tell whether the various phenomena to which they are applied bear a purely formal resemblance to each other or whether there is essentially a single principle in back of them all." Now, only a little more than a decade later, we have begun to define precisely, and trace the development of, the characteristics of species, individuals, and tissues that set them apart from their fellows. The fields of immuno-chemistry and transplantation immunity have developed explosively; their impact has made possible the framing of meaningful questions directed toward understanding the development of specificity at the molecular level.

This chapter deals largely with the research of the past decade. Yet most of the problems to be considered are not new, having been formulated long ago, but in different terms. No attempt is made to trace in detail the antecedents of the ideas presented. Being cognizant of

Oppenheimer's (1957) guarded generalization that the ideas of the present are to be understood only in the light of their precursors, the origin of each of the major concepts is stated whenever possible. It is often difficult to assess the extent to which a pioneering effort has contributed to current thought. A case in point is Leo Loeb's (1945) prescient, but frequently obscure, treatise, "The Biological Basis of Individuality." His contributions are rarely quoted, possibly because of the lack of precision of the terms in which the data were presented and the inflexibility of the frame within which the ideas were stated and restated. Nevertheless, as will be apparent in the following, many of Loeb's contributions are incorporated into present-day thinking.

Our discussion of the acquisition of biological specificity is based on the following premises, the soundness of which must be established by an examination of the pertinent evidence: (1) The concept of tissue specificity extends to the molecular level. Although tissue specificity may, in part, reflect differences in associations and numbers of macromolecules which are alike in all tissues, we recognize by immunochemical and physiological techniques, molecular types characteristic of each tissue. (2) The difference between two individuals is based on more than the difference in the sum of their tissue-specific proteins, or other antigenic molecules (or what Loeb called a mosaic basis of individuality); it rests on the existence in each individual (or in all individuals of identical genotype, e.g., identical twins and members of a highly inbred strain) of specific molecules not restricted to certain tissues but common to all or almost all its parts. Loeb spoke of these characteristic molecules as part of the "individuality differential"; we designate them as *isoantigens*. (3) A third, but less secure, assumption states that similarly there are antigens common to all members of a species, the species-specific antigens. Although reference will be made to pertinent findings on species specificity, emphasis will be placed on the tissue-specific antigens and isoantigens. Among the questions to be considered are the following: What are the criteria of isoantigenicity and tissue specificity? By what techniques are tissue specificity and isoantigenicity recognized? Are they properties only of the adult, i.e., do they represent end points of differentiation, or are there tissue-specific molecules and isoantigens characteristic of the embryo, to be modified or replaced during development by related molecules of the adult? Do the isoantigens and tissue-specific molecules develop independently—or are they related in development or function? Do they differ in site and time of formation? Are their synthetic mechanisms based on a single operative principle?

And, finally, when and how does the embryo or immature animal

acquire the ability to recognize that a molecular type or tissue to which it is exposed is foreign, and just how foreign it is? Does every cell of the body have the ability to distinguish like and unlike, or is this ability the property of only a few cell types? The experiments of Moscona (1957) illustrate beautifully that aggregates of intermixed chick and mouse cells of the same type, e.g., chick and mouse chondrogenic cells, reconstruct a chimeric tissue consisting of interspersed cells of the two species. Aggregates of intermixed cells of different types become associated according to type, chick nephrogenic cells in one grouping, mouse chondrogenic cells in another. Thus, at one stage in their history, a type of tissue specificity dominates over species specificity; cells aggregate histotypically regardless of generic origin. Does this pattern change during development? Would adult cells behave similarly *in vitro*? It appears that at least some kinds of adult cells do. What may we learn by a comparison of cell compatibilities and incompatibilities *in vivo* and *in vitro*?

In treating of these questions, we take up first evidence from what is perhaps our most sensitive technique for the demonstration of biological specificity, the study of the fate of transplants of normal tissues.

II. TRANSPLANTATION IMMUNITY

A. Recognition of Individual and Species Differences

Most tissues of the body do not long survive their transplantation between randomly chosen individuals of the same avian or mammalian species. For example, a skin graft made from one mouse to another—a skin homograft—heals in at first and enters a phase of epithelial proliferation; its behavior and gross appearance do not distinguish it from an autograft, a graft made from one part to another of the same animal. Eventually a state of resistance develops in the host resulting in the destruction of the graft, which is sloughed away as a discolored scab. The state of resistance called into being by the transplantation of living tissue between individuals is called transplantation immunity, for, as will be made clear, it is an immune reaction, related to the more familiar defense mechanism in which an animal reacts to infection with heterologous antigens like bacteria (Billingham, 1956).

In a review of the literature of his time, Schöne (1912) repeatedly called attention to the fact that transplants between unrelated individuals rarely succeed, in contrast to the nearly universal success of autoplastic grafts. He cited cases in which skin homografts were terminated earlier when the host had been exposed previously to the transplanted tissue and invoked a secondary immune reaction as one of the

principal mechanisms of homograft destruction. In observing the case of a badly burned young woman, Gibson and Medawar (1943) noted that in contrast to the success of skin autografts to the burned area, homografts from the subject's brother were destroyed during a period of from 15 to 23 days after transplantation. More remarkable, they observed that a second set of homografts from the same donor transplanted on the fifteenth day after the original operations began degenerating immediately, destruction being virtually complete on the eighth postoperative day, paralleling the sloughing of the first set. The course of these reactions clearly suggested that the girl had acquired an immunity to her brother's skin and that the rapid dissolution of the second set of grafts was due to an immune reaction.

Experimenting with rabbits, Medawar (1944, 1945) proved beyond all reasonable doubt that the rejection of a skin homograft leaves its host in a state of heightened resistance, the effect of which is to hasten the rejection of a skin homograft transplanted on a second occasion from the same donor. He showed the survival time of second-set grafts to be inversely proportional to the size of the immunizing dose (first-set grafts) in terms of grams of skin transplanted per rabbit. The median survival time of first-set grafts was 10.4 ± 1.1 to 20.4 ± 8.0 days depending upon the dosage and type of immunizing graft employed, whereas that of second-set grafts was 6.0 ± 0.6 days. Moreover, the reaction was shown to be systemic rather than local, for the heightened resistance applied equally well to second-set grafts made to parts of the body well removed from the site of the first graft. These findings point up all of the main factors which determine the time of onset of a host's reaction against a homograft save one: the genetic relationship between donor and recipient. Work by many investigators has contributed to the latter problem, not always in tangible ways. It would be difficult to trace the complete line of thought, and not necessarily rewarding.

Our present understanding is largely the confluence of two main streams of inquiry: (1) analyses of the fate of transplants of normal tissues with emphasis on the genetic relationship between donor and recipient, and (2) studies of the genetics of tumor transplantation. Over a span of forty years, with numerous collaborators, Loeb carried on an intensive investigation (summarized, 1945) in which he studied the effect on the host-graft reaction of varying the degree of relationship between donor and recipient, employing inbred animals. The choice of tissues for transplantation varied with the experimental design and conditions—among those used were thyroid-parathyroid, cartilage, striated muscle, and bone. The site of transplantation usually was a pocket in the subcutaneous tissue of the back or flank. Autoplastic combinations

served as controls. Among the inbred lines studied most successfully were rats (with H. D. King) and guinea pigs (with Sewall Wright). Loeb's interpretation of the mechanism of host resistance has proved to be inadequate; on the other hand, these pioneering studies showed a definite correlation between the degree of relationship of donor and recipient and the success of transplants. The failure of homografts is due to genetic differences between donor and host. The genes concerned have been given the name "histocompatibility genes" (Snell, 1957a, b), the essential prerequisite for compatibility being that the recipient match the graft genetically. If a graft carries one or more genes (and isoantigens) for which the host has different alleles, it evokes an antagonistic response. Conversely, the absence in the donor of genes present in the host is not significant.

Studies of the genetics of transplantable tumors have been important, not only because they provide a model for studies of transplantation genetics of normal tissues, but also because they focus attention on highly inbred strains of mice as unusually favorable objects of study. Our knowledge of the genetics of tissue transplantation is, in fact, based almost entirely on studies with tumors, as exemplified by studies of Snell, who has shown clearly the importance of single gene differences at histocompatibility loci.

The detailed quantitative analysis of transplantation immunity hinges upon the development of a method of measuring the intensity of the recipient's reaction. The survival end point of a skin homograft may be determined with accuracy only when the graft loses its cuticle and hair, exposing the epithelial surface to direct observation. In the mouse this requires about 10 days (Billingham and Medawar, 1951). More violent reactions which take place in a much shorter time cannot be grouped accurately. Three accurate methods of determining survival time have been developed: (1) determination of the time after transplantation at which the circulation of blood in the graft is halted (Taylor and Lehrfeld, 1953, 1955); (2) transplantation of the homograft back to its original donor or to another member of the donor's inbred strain (Medawar, 1944; Mitchison, 1954); (3) the use of the method of probit transformations to estimate median survival time, employing data derived from scoring the degree of survival of the graft epithelium at the time of its removal (Mitchison, 1954; Medawar, 1944, 1945). The probit method of analysis is similar to that used to measure the LD₅₀ of a toxic drug. Median survival time (MST) is defined as the time after transplantation at which the breakdown of the grafts borne by 50% of their recipients is just complete. Employing the probit method, Billingham *et al.* (1954a, b) estimated the MST of homografts exchanged between

inbred strains of mice. Since repeated reference will be made to these combinations, a summary of the findings is essential. A graft from strain A made into strain CBA survived for 11.0 ± 0.3 days; similarly, CBA into A, 10.2 ± 0.3 ; A into AU, 9.0 ± 0.3 ; AU into A, 9.1 ± 0.4 ; CBA¹ (or F₁) into WG, 9.2 ± 0.2 ; WG (or F₁) into CBA, 8.5 ± 0.2 . Homografts exchanged between members of the same inbred strain could not be distinguished from autografts, but mild incompatibilities were noted when skin grafts were made between members of sublines which were only eight to twelve generations apart. The MST of a second graft of A-line skin, transplanted within 15 days of the first graft to a CBA host is reduced to about 6 days. Immunity decays progressively, but is in force in the combination A → CBA for at least 240 days (Billingham *et al.*, 1956a). As in the earlier, less quantitative studies with rabbits, it was found that differences in age over the interval 6 weeks to one year had no significant effect on the MST, nor did difference of sex. Although antinomies are not uncommon in the field of homotransplantation, there has been general agreement on these points. One exception to the latter statement is found in the report by Eichwald *et al.* (1957) that, in mice of strains A/Jax and C57BL, intrastrain grafts, grafts from pure strains to F₁ hybrids and from one F₁ hybrid to another F₁ hybrid almost always succeed provided donor and recipient are of the same sex, or provided the donor is female and the recipient male. When the donor is male, and the recipient female, however, grafts fail except when donor and recipient are both of the A/Jax strain. In the absence of an adequate test of the possibility of a differential hormonal requirement, the finding only suggests the possibility of histocompatibility genes on the Y chromosome.

There is no body of estimates of survival time of homografts in other species approaching the accuracy of those for inbred mice. As cited earlier, with rabbits chosen at random from members of a heterogeneous stock, Medawar (1944, 1945) found a survival time of 10.4 ± 1.1 days; Sparrow's (1953) similar estimate for the guinea pig was 9.2 days. In rats, Taylor and Lehrfeld (1955) found a mean survival time of 8.1 days, although Billingham *et al.* (1954a, b) believe about 2 days must be added to the estimate to make it equivalent to their own. Anderson *et al.* (1951) found that the majority of skin homografts exchanged between unrelated cattle were destroyed within 9 days; a similar estimate has been made for chickens (Cannon and Longmire, 1952). Although extensive data have not been obtained for homotransplantation in man, the information available is consistent with observations on other species (Rogers, 1957). Accurate mean survival times cannot be given. There have been few detailed studies of homotransplantation reactions in adult

cold-blooded vertebrates. The most striking evidence is that presented by Hildemann (1957), who has demonstrated that homotransplants of scales in goldfish behave similarly to skin grafts in mammals. Second-set homografts regularly break down more rapidly than first-set grafts; in addition, higher water temperature also accelerates destruction. It appears likely that the same fundamental reactions are involved in the destruction of scale transplants in fish as in the sloughing of mammalian skin.

B. Mechanism of Action

Although it is generally accepted that the reaction of the host against a homograft is based on a mechanism of actively acquired immunity, it has long been debated whether the destructive process is caused by cells or by antibodies (cytotoxins) in the serum, or both. Although the question is not fully resolved, it is becoming increasingly clear that in many reactions cells are indispensable. Evidence from two principal lines of investigation may be presented in support of this conclusion: (1) adoptively acquired immunity; and (2) studies using diffusion-chamber methods. Under the latter heading will be considered also the evidence for the role of serum antibody.

1. Adoptively Acquired Immunity

Actively acquired immunity has been defined as the state of heightened resistance to an antigen as a result of a previous exposure to the antigenic stimulus. Passive immunity, of course, ordinarily refers to resistance produced by the transfer of ready-made antibody from an actively immunized donor to a normal subject. In *adoptively acquired immunity*, the subject becomes immune as a result of the transfer not of antibody, but of immunologically activated tissue. As Billingham *et al.* (1954a, b) make clear, it is passive in the sense that it is not the outcome of the subject's own active immunization; but it is active in the sense that it depends on the presence of functional tissue previously exposed to an antigenic stimulus. The idea is not new; the technique of transfer of antibodies by cells and grafts has been confirmed and refined since the turn of the century (Boyd, 1956; Topley, 1930; Harris and Harris, 1951; Hale and Stoner, 1953). Although in many cases the immunological system has been one in which serum antibodies are demonstrable and capable of conferring passive immunity, the principle has been extended to forms of immunity in which serum antibody has not been detected, e.g., tuberculin sensitivity and sensitization produced by simple chemical compounds (Landsteiner and Chase, 1942; Chase, 1951). Mitchison (1954) was the first to show clearly that heightened resistance to trans-

plants can be transferred by living cells but not by serum. Using tumor homografts, he developed the following experimental design: two strains of mice are used, donors and recipients. The recipients are subdivided into primary and secondary hosts. The primary hosts are actively immunized by the transplantation of a tissue from the donor strain. When these grafts have been destroyed, tissues, cells, or fluids are transferred from primary to secondary hosts. The reactivity of the secondary host is now tested by the transplantation of a homograft from a member of the donor strain. The question to be answered: Does the graft to the secondary host behave as an orthodox first-set graft or is its lifetime reduced by the presence of the inoculum from the primary host?

The success of the approach depends on the fulfillment of two prerequisites: (1) the use of a closely inbred strain as the source of the donor animals; and (2) genetic similarity of primary and secondary hosts which insures that a tissue transplanted from the primary to the secondary host will survive without itself provoking an immunological reaction. The test of survival may be transplantation back to the donor strain [employed by Mitchison (1954)] or detailed analysis of MST [employed by Billingham *et al.* (1954a, b)]. In a few experiments, the latter group employed as secondary hosts mice which had become specifically and completely unresponsive to homografts of the donor strain by inoculation with donor cells in fetal life ("tolerant" mice, see page 635). These mice already carried donor homografts of long standing, so that no test grafting was required; moreover, as test mice they had the peculiar advantage of being manifestly unable to react against the graft on their own account.

Billingham *et al.* (1954a, b) extended Mitchison's observations on tumors to normal skin grafts. Normal CBA mice ("secondary hosts") which have been inoculated intraperitoneally with *regional lymph nodes* derived from actively immunized "primary hosts" behave as if they themselves had been actively immunized. To a lesser degree, the spleen of the primary host has the power to transfer immunity. Although the regional nodes are shown to be the principal site of the reaction, other sites must be involved, for the extirpation of the regional nodes after homograft regression does not prevent the host from giving a secondary response, nor does regional lymphadenectomy before grafting reduce the power of the primary response. The following inocula are ineffective in transferring transplantation immunity: killed lymph nodes, nodes transplanted subcutaneously; whole blood, concentrated leucocytes, serum. Thus it is clear that the transfer of transplantation immunity cannot be due to the passive transfer of preformed antibodies; it depends on the incorporation and function of actively immunized cells.

2. Experiments with Porous Membranes

The development of porous membranes that readily permit the passage of large molecules while preventing the passage of cells has provided a valuable tool for the study of immunity to homografts and heterografts. Algire *et al.* (1954, 1956; see also Prehn *et al.*, 1954; Algire, 1957) have made effective use of cellulose ester membrane filters in constructing diffusion chambers in which grafts may be separated from host cells. After establishing first that homografts would survive in diffusion chambers in normal inbred mice, they compared the survival time of homografts of Harderian gland in diffusion chambers in immunized hosts with that of grafts placed directly upon the host tissues. Implants in contact with host tissues were destroyed within 3 days, whereas grafts separated from host cells by a filter survived for the duration of the experiments. In further experiments with filters of greater porosity, permitting the passage of cells, destruction of homografts was observed when cells entered the chambers from immune hosts, but not from normal hosts. In another group of experiments, grafts were combined with washed spleen cells in diffusion chambers, which were placed in mice isologous with the graft. There was extensive destruction of the "target" cells only if the spleen cells came from immunized mice. It was concluded that, under these experimental conditions, the factors destructive to homografts were associated with cells; histologic evidence suggested, but did not prove, that the cells involved were lymphocytes, a finding in general agreement with the results of Mitchison (1954) and Billingham *et al.* (1954a, b).

As the use of membrane filters was accelerated, the question was raised as to the actual porosity of the membranes and the evidence bearing on the passage of antibodies. It had been assumed that cytotoxic antibodies could pass the membrane. It was considered possible that they might not, owing to interaction of the material being filtered and the substance of the membrane. An indirect approach was made to this problem, using animals that had been immunized by heteroplastic grafts. It is well known that tissue culture methods may be used to demonstrate the presence of humoral cytotoxic substances in the body fluids of animals so immunized (Harris, 1943, 1948; see also Ross, 1957; Wissler and Flax, 1957). Algire and his co-workers were able to demonstrate satisfactorily that cytotoxins to heterografts pass into diffusion chambers. When mice were immunized 2 weeks earlier by an injection of human cells (HeLa strain), there was an accelerated destruction of cells implanted in diffusion chambers as compared with nonimmune control animals. Algire argues that, since the cytotoxins elicited by heterografts pass through membrane filters, there is no reason to believe that homograft cytotoxins might behave otherwise.

Algire's principal conclusions are supported by the experiments of Woodruff (1957), who has employed a modification of the diffusion-chamber method for studies of immunity to split-skin grafts in the rat. Woodruff goes one step further, however, and states that his results indicate that homografts are unusually vulnerable to cellular attack when the protection of the membrane is removed. He suggests that prior exposure to humoral antibodies might play a role in rendering the graft more susceptible to attack by cells. The evidence is too fragmentary to warrant further discussion.

In contrast to the failure of Algire and others to demonstrate cytotoxic antibodies to homografts *in vivo*, Gorer (1942) with leukemic cells and Billingham and Sparrow (1954) with skin epithelium have shown that serum antibody from hyperimmune mice can combine with these cells *in vitro* and inhibit their growth on subsequent transfer to susceptible hosts. Gorer and Amos (1956) have demonstrated passive immunity in mice against C57BL leukosis E.L.4., using immune serum. It is fair to state that the evidence for the indispensability of cells in transplantation immunity is more convincing than the evidence for the involvement of serum antibodies. The two possibilities are not mutually exclusive. Inability to demonstrate cytotoxic antibodies to homografts by diffusion-chamber methods may reflect only differences in the choice of tissues, in the dosage, and degree of immunity. There is no doubt of the production of cytotoxins in response to heterotransplants, but the latter represent an extreme degree of tissue incompatibility. The problems arising from the use of cytotoxins as tools in studies of development are discussed further in Section V, D.

C. Immune Reactions in the Transplantation of Tissues Other than Skin

There are grounds for believing that the principle of acquired immunity in homotransplantation applies to tissues other than skin. Although detailed, quantitative evidence is available only for skin transplantation, the bulk of available evidence favors the hypothesis of acquired immunity as an explanation for the rejection of most (if not all) adult tissue homografts (Rogers, 1957; Simonsen, 1955b). Until recently, even a casual review of the literature gave one the impression of a confused state of affairs; within the past decade, however, most of the apparent exceptions have been resolved, as will be illustrated by a brief consideration of the transplantation of cornea, bone, and ovary.

1. Cornea

Corneal transplantation in man is a practical, well-established procedure; the graft usually takes, and remains transparent. It does not differ from normal corneal tissue. Even those grafts which become opaque rarely slough or become necrotic. Thus, it has frequently been

suggested that the cornea either does not elicit an immune reaction or is not affected by the host's response. Recent experiments, however, have shown that corneal tissue is antigenic and that it can be affected by the response of the recipient animal. Billingham and Boswell (1953) have shown that, when homografts of corneal epithelium and stroma are grafted to the chest wall of a recipient, they are destroyed in about 17 days; second-set transplants are destroyed more rapidly—in about 7 days. Moreover, immunization with corneal epithelium or skin will produce an early breakdown of corneal grafts. Conversely, when skin is grafted to a pocket in the center of an avascular cornea in a specifically immunized animal, it is capable of surviving for 20 days, whereas a comparable graft inserted into the chest wall is destroyed in 7 days. Thus, the cornea is not an "immunologically privileged" tissue; it is antigenic and capable of destruction by the immune reactions of the host. In order to understand why corneal grafts are not generally destroyed by the recipient, it is necessary to consider the peculiarities of the corneal site (Maumenee, 1955). Perhaps the critical factor is the avascularity or relative avascularity of the cornea, for it has been shown that vascularization of a homotransplant is essential for its destruction (Medawar, 1948; Billingham and Boswell, 1953). Other important factors include the mechanical structure of the cornea and the amount of engrafted tissue which survives. The graft epithelium and probably the endothelium are replaced by cells of the recipient, only graft stroma remaining; thus, the graft dosage is extremely small compared to that of a skin graft.

The argument that the cornea is a tissue in an "immunologically privileged" position agrees with the observation made repeatedly that homografts succeed when made to the anterior chamber of the eye. Similar results have been reported for grafts made to certain regions of the brain (Medawar, 1948). Successful grafts made to the anterior chamber are not penetrated by blood vessels; successful grafts to the brain may have had an additional advantage in the absence of a regional lymphatic drainage system.

2. Bone

The successful use of relatively small amounts of preserved bone, which has been established beyond question, has led to the establishment of bone banks; the use of massive bone grafts has met with little success, however. Although few well-controlled studies have been made, a review of the evidence leads to the conclusion that fresh and frozen bone homotransplants evoke an immune reaction in the recipient. In the experiments of Bonfiglio *et al.* (1955) which are among the more

revealing, first-set bone homografts in rabbits elicit a maximal reaction at 3 weeks. Second-set grafts provoke a heightened resistance. On the other hand, it has been reported frequently that the ultimate result of autografts and homografts of bone is the same, the only difference being in rate of incorporation, which is slower in the homografts (Bassett, 1955). It appears quite probable that bone homografts elicit an immune response, leading to destruction of the viable cells of the graft. However, the remaining bony matrix then serves as a scaffold for ingrowing undifferentiated mesenchymal cells which are stimulated to osteogenic activity.

3. *Ovary*

There is an impressive body of evidence that homografts of endocrine tissues are far less exacting than skin with regard to their capacity to survive transplantation (Harris and Eakin, 1949; Krohn, 1955; Billingham and Parkes, 1955). According to Halsted's "law," the generalization which is commonly cited to "explain" the success of endocrine grafts, endocrine tissues can be grafted successfully only when there is a deficiency of their secretions in the host. Halsted himself based his proposition on autografts of parathyroid gland, but his generalization was widened by others to cover homografts of other organs, including the adrenal, thyroid, and ovary. The ovary will serve as our example. It is clear that ovarian homografts implanted into ovariectomized recipients are far more successful than skin homografts. The reasons for this difference have not been elucidated. For example, Billingham and Parkes compared the survival of skin homografts in albino rats with that of ovarian homografts made subcutaneously in ovariectomized hosts. They found that a degree of antigenic difference between donor and recipient that will lead to the destruction of skin homografts is only rarely sufficient to affect the continued function of ovarian homografts. On the other hand, if ovarian grafts were made into ovariectomized hosts which had previously reacted vigorously against skin homografts from the same donor, they usually failed to survive, a finding which suggests that, whereas most ovarian homografts are incapable of provoking reactions, they are subject to destruction in immunized animals. Skin and ovary must share some antigens in common. Why are ovarian homografts incapable of eliciting an immune reaction? The most complete discussion of the possible explanations may be found in Billingham and Parkes (1955, pages 559-560). Among the more plausible explanations are the following: (1) Ovarian tissue has fewer genetically defined histocompatibility antigens than skin. (2) The ovarian histocompatibility antigens are capable of eliciting only a weak response. (3) The highly vascular

condition of ovarian grafts—possibly owing to continued pituitary trophic stimulation—prevents an effective concentration of the factors responsible for tissue breakdown. Experiments reported to date are consistent with any of these possibilities, but offer little help in discriminating between them.

D. Nature of the Antigenic Stimulus

From the evidence marshaled in the foregoing sections, we may conclude that antigens issue from a homograft and reach the regional lymph nodes, presumably via the afferent lymphatics. Although under special circumstances whole cells may be transferred, generally the antigenic message must be in the form of cellular fragments or ultra-microscopic particles. Medawar (1957a) has argued effectively that antigens issue constantly from homografts, citing as his most impressive evidence an experiment referred to earlier, in which a successful homograft in a tolerant animal is destroyed by the administration of lymph cells of normal mice. Clearly the experiment does suggest that the homograft is constantly giving forth antigenic matter to which its tolerant host is unable to respond. What is the nature of these antigens? Billingham *et al.* (1956b; see also Brent, 1957; Medawar, 1957a) have reached the tentative conclusion that they are deoxyribonucleic acid (DNA) nucleoproteins. It should be stressed that at this time *the evidence is far from complete*; the findings cannot be evaluated critically. Yet the conclusions are so far reaching that we are compelled to consider the evidence thus far advanced. The work has been done in mice; the experiments consist in the injection of cells and tissue extracts from adult mice of one strain into adult mice of a second strain, which are then challenged with homografts of the donor type, in order to learn whether the inoculation is capable of accelerating destruction of the grafts. In other words, what cells and fractions of cells contain the isoantigen? Whole blood is active, but its immunizing power rests in the leucocytes, being absent from red cells, platelets, and plasma. Moreover, the antigens appear to be represented in all the *nucleated* cells of a single individual or the members of an inbred strain. Nucleated cells need be neither viable nor intact in order to function as antigens. Mouse spleen cells suspended in a variety of media can be disintegrated by ultrasonic irradiation without abolishing their capacity to immunize against skin grafts of the donor strain. From fractionation studies, Medawar and his group conclude that cytoplasmic fractions are totally inactive, whereas nuclei are active. When cells are disintegrated in media in which nucleoproteins are insoluble, then the active fraction is found in the heavy "chromatin" fraction. The active fraction is (1)

destroyed or severely damaged by lyophilization, by repeated freezing and thawing and by heating to 48.5° C. for 20 minutes; (2) insoluble in physiological salt solutions; but (3) soluble in water; and (4) leached out of nuclei by extraction in 2 M NaCl. This and other kindred evidence is in keeping with the idea that the active principle is DNA nucleoprotein. The active fraction is destroyed by incubation with deoxyribonuclease (DNAase), but not by digestion with trypsin. Ribonuclease is ineffective in modifying its antigenicity. Since its vulnerability to DNAase is not as great as might be expected if DNA were the sole active factor, Medawar suggests as a possibility DNA coated with protein and thus partly protected against the enzyme. Medawar is careful to stress the preliminary nature of his findings. That the research is moving rapidly, with attendant changes in conclusions, is illustrated by the fact that in successive publications Medawar (1957a) and Brent (1957) state that (1) although a protein fraction is removed by tryptic digestion, the antigenicity of the nuclear preparation is not destroyed; and (2) the antigens are susceptible to destruction by trypsin under certain conditions. Some uncertainty also exists concerning the involvement of lipoproteins and polysaccharides or mucoproteins (Haskova and Hrubesova, 1958; Medawar, 1958). Only the following comments are warranted: Although the authors appear to have shown that there are transplantation antigens in tissue nuclei, they have not proved that all isoantigens are nuclear. One hesitates before comparing the results of studies with normal tissues with those made on tumors. However, inherent in investigations on transplantable tumors and skin homografts has been the assumption that the types of immunity were the same. As mentioned earlier, Billingham *et al.* (1954a, b) promptly applied to skin homografting the techniques and principles of adoptively acquired immunity established by Mitchison (1954), using tumors. Thus, it is paradoxical that immunity to tumors may be evoked with a variety of nonnuclear agents including cell-free extracts (MacDowell *et al.*, 1941; Kidd, 1946), red cell stromata (Barrett and Hansen, 1953), and microsome fractions (Novikoff, 1957). Although these conflicts are puzzling at the moment, out of such controversies may come clues to basic differences between resistance to tumors and resistance to normal tissues. One can only agree with Medawar that the ideas he has advanced are verifiable, by existing methods or by modifications of those methods.

III. THE ONTOGENY OF TRANSPLANTATION IMMUNITY

It is a commonplace experience that homografts and heterografts are accepted by embryos and maintained throughout most, if not all of the embryonic period. Homoplastic, heteroplastic, and, not infrequently,

xenoplastic combinations are employed widely in experimental embryology; the most popular chimeras are those in which donor and recipient tissues may be distinguished by virtue of clear-cut differences in cell size, chromosome number, or pigmentary features, differences which serve as markers, permitting a conclusion as to relative contributions of the graft and host (Born, 1897; Spemann and Mangold, 1924; Harrison, 1935). Such combinations have been employed widely in an effort to gain insight concerning the distribution and specificity of molecular species active in induction (Holtfreter, 1935; Waddington and Schmidt, 1933). Today their use is exemplified by the studies of Moscona (1957), who has analyzed the fate of mixtures of chick and mouse cells *in vitro*.

In attempting to assess the contribution of these older studies to our current concepts of the development of individual and tissue specificities, the following facts emerge: (1) Since most of the experiments were terminated during embryonic life, incompatibilities were the exception rather than the rule. Moreover, when chimeras were permitted to develop beyond embryonic life, relatively little attention was paid to differences in results with homografts as compared to heterografts. In the latter, the destruction of the graft by the host was commonly encountered in birds and mammals and frequently in the teleosts and amphibians. Perhaps the first clear statement on the development of incompatibility between amphibian hosts and grafts as related to host age is that of Eakin and Harris (1945; see also Harris, 1941), who placed the onset of host antagonism at about the time of metamorphosis. It is important to note that until quite recently the exceptional cases of survival into adult life were accepted almost without question.

Grafts of avian and mammalian tissues succeed in the chick embryo (Sandstrom, 1932, 1934, 1940; Murphy, 1913; Nicholas and Rudnick, 1933). Limb grafts made between bird embryos of different species manifest incompatibility soon after hatching and are destroyed almost without exception by the sixth week post-hatching. Conversely, as a result of the work of Willier and Rawles (1940), Weiss and Andres (1952), and others, we know that neural crest cells transplanted into embryos of the same species may survive beyond hatching and even into adult life. Skin transplants between newly hatched chicks of different breeds result in a number of "takes" (Danforth and Foster, 1929), the incidence of successful grafts decreasing with age (Cannon and Longmire, 1952). Again, the striking difference between the eventual failure of heterografts and the frequent persistence of homografts made to embryos was not emphasized. Throughout the literature, one point is clear, namely that the absence of an incompatibility reaction resulted primarily from a failure on the part of the host to react and not on a lack of antigenic stimulus, for grafts of adult tissue persisted in the embryo almost equally as well as did grafts from embryonic donors.

Although the ontogeny of isoantigens (individual-specific antigens) is by no means understood, it is established that many embryonic tissues are antigenic.

But what of the inference, drawn from reports that *bryophplastic* transplants (grafts of tissue from embryos or newborn animals to adults) succeed, that individual specificity and antigenicity are weak or negligible [summarized by May (1957)] in the embryo? A closer examination of the findings reveals that most successful bryophplastic grafts are made to the anterior chamber of the eye, which is, as we have already seen, an "immunologically privileged" site. Other grafts succeed, at least temporarily, when the host's lymphoid tissues are destroyed by irradiation. In short, most of the evidence does not prove the lack of antigenicity of the graft, but simply that it is not destroyed by the host.

A. *Maturation of the Antibody-forming System*

With the exception of one recent study, that of Green and Lorincz (1957), who attribute the degeneration of Krebs mouse ascites tumor cells in the chick embryo beginning at about the seventeenth day to the formation of a serum protein with the properties of a "natural antibody," there is no well-documented evidence of the ability of an embryo to form circulating antibody. Although the usual reservation must be made concerning the critical value of negative findings, the best available evidence (Freund, 1930; Burnet *et al.*, 1950; Wolfe and Dilks, 1948; Wolfe *et al.*, 1957a) indicates that the ability to produce antibody in response to an antigenic stimulus first develops around the time of birth. Allowing for species variation, and for the fact that only a few of the more common animals have been studied, and with few antigens, the tentative conclusion may be stated that the antibody-forming system reaches a state of functional development within a few days after birth, at which time antibody can be formed in small quantities. The system matures rapidly; in the chick "serological maturity" is reached by about 6 weeks' post-hatching. Although, as we have remarked, there is no obvious direct correlation between the production of circulating antibody and transplantation immunity, there is a striking parallelism in the time course of their development. The ontogeny of the blood serum proteins is considered fully by Schechtman (1955).

IV. ACTIVELY ACQUIRED TOLERANCE

A. *Theory of Acquired Tolerance*

In their thought-provoking monograph, "The Production of Antibodies," Burnet and Fenner (1949) proposed a theory of immunological tolerance. In discussing this theory of far-reaching significance, Owen (1957) has rightly emphasized that its factual cornerstone was the

observation by himself and co-workers that twin cattle showed identical blood types more often than should have been expected from estimates of the occurrence of identical twins. For example, one pair of twins had different sires, yet identical types, which consisted of a mixture of two distinct cell populations. Moreover, similar observations were made on higher multiple births, including a set of quintuplets (Owen *et al.*, 1946). The long-known placental anastomosis of blood vessels in cattle twins (Lillie, 1917) led Owen to propose that the identical blood types resulted from an exchange of hematopoietic cells (hematoblasts) which had been reciprocally "transplanted," an argument that has been upheld effectively upon further analysis. The evidence shows that "the cells were established and retained as intact units, developing autonomously as a function of their own genotypes; there was never any indication that exchanges of determinants at the subcellular level had produced 'transformation' for some of the gene-controlled antigens in which the members of a twin pair differed, and not of others" (Owen, 1957). Parenthetically, it may be noted that comparable findings of spontaneous transfer of hematoblasts have been reported for sheep (Stormont *et al.*, 1953) and man (Dunsford and associates, 1953), and that the phenomenon has been reproduced experimentally in rats (Ripley, cited by Owen, 1957) and embryonic chicks (Hasek, 1956).

Burnet and Fenner predicted that the exposure of an embryo to foreign cells should result in the inability of the animal to respond immunologically against those cells in later life, after its faculty for immunological response had developed. The postulate was tested by Billingham, Medawar, and their co-workers in a series of rewarding experiments. They found that the majority of nonidentical twin calves would accept homografts of each other's skin, whereas grafts exchanged between siblings were rapidly destroyed. The phenomenon was highly specific, for a calf accepted homografts only from its twin and from no other donor (Anderson *et al.*, 1951; Billingham *et al.*, 1952). Shortly thereafter the phenomenon which occurs in twin cattle was reproduced experimentally (Billingham *et al.*, 1953) in mice.

B. The Tolerant State: Conditions for Its Induction

"Actively acquired tolerance" is defined as a specific reduction or suppression of immunological reactivity as the result of exposure of animals to antigenic stimuli before the maturation of the faculty of immunological response. Tolerance of homografts may be brought about by the exposure of embryos or newborn animals to an immunizing dose of foreign homologous cells. The stimulus should be one which in adults would cause them to become immune. What is the optimal time for

the induction of tolerance? Billingham *et al.* (1956a, page 372) state "at some epoch of life, therefore, there must be a transition from one modality of response to another. The transition could hardly be a sudden one . . . the power to confer tolerance falls away to zero as the age of the injected subject increases." As emphasized earlier, the ability to respond to an antigenic stimulus increases with development. Thus, a "null period" or transitional period is common. Although it was thought earlier that mouse embryos younger than 18 days are superior to older embryos in respect of the proportion which become tolerant, Billingham and Brent (1957a) have shown that equally satisfactory results can be obtained using newborn mice. Tolerance of homografts in chickens is obtained by grafting fragments of adult tissues to the chorioallantoic membrane of 10- to 11-day hosts, by intravenous injection at 10 to 11 days, by artificial synchorial parabiosis at 10 days, and by intravenous inoculation within 12 hours after hatching (Billingham *et al.*, 1956a). Simonsen (1956) observed that, when human blood is injected into chick embryos, the depressing effects of the injections on the formation of red blood cell agglutinins are greatest when the embryo is in the seventeenth to nineteenth day of development. The optimal time for the induction of tolerance in other animals is less clear; tolerance can be induced in rats and rabbits, but the data are far less extensive.

Methods for inducing tolerance are described in full by Billingham *et al.* (1956a). In the mouse, for example, a cellular suspension prepared from a variety of tissues of the adult mouse is injected intraembryonically (or intravenously in the newborn animal). Six to eight weeks after its birth, each injected mouse is challenged with a skin homograft from a member of the same inbred strain as the donor of the original inoculum. In no mouse did the test graft fall significantly short of the median survival time (MST) which, as described earlier, is invariant with respect to age and sex. In no case has immunity resulted from the inoculations. However, every degree of tolerance has been observed; tolerance is not an "all-or-none" phenomenon. In some tolerant animals, homografts live a few days beyond the MST; in others, grafts are accepted permanently. A partial inhibition of tolerance may be permanent—for the weakening of the response to a second-set graft in partially tolerant animals is proportional to the weakening of the first.

C. Nature of the Tolerance Reaction

The induction of tolerance is a powerful method of revealing antigenic similarities and differences. Billingham and associates believe that tolerance is not a tissue-specific phenomenon. This statement will be examined during the discussion of the properties and requisites of the

inoculum. It is individual-specific (isoantigenically specific, or specific within a closely inbred strain). An animal injected in fetal life with cells from donor A (or a donor of strain A) is tolerant only of grafts made from donors of the same strain. Tolerance extends to another strain, as might be expected, only if that strain contains no antigens that are not also present in A. Like transplantation immunity, tolerance is systemic and not localized to a single region.

Billingham and colleagues argue that acquired tolerance results from a failure of the basic mechanisms of the immune reaction. It is not caused by a modification of a mechanism at a peripheral level. Their interpretation rests on two chief building stones: (1) Immunological reactivity can be restored to a tolerant animal by inoculating it with cells taken from the regional lymph nodes of actively immunized members of its own strain ("adoptively acquired immunity") or with cells taken from the lymph nodes of normal unimmunized lymph node cells. The tolerant graft is a *constant source* of antigenic stimuli, and remains susceptible to a reaction directed against it. (2) Tolerance is neither caused by nor accompanied by an antigenic adaptation of the grafted cells. Cannon and Longmire (1952; Cannon *et al.*, 1954; Weber *et al.*, 1954) have held that homografts in some way "adapt themselves to foreign soil" (Billingham *et al.*, 1956a). They argue from the following experiments: First, skin homografts to newborn chicks are more often tolerated when the donors were newborn than when the donors were older. Six of 60 grafts were retained when the donors were from 1 to 7 days post-hatching; when the donors were 12 to 13 and 14 to 16 days old, the numbers were 2/62 and 0/82. Second, a supposedly "tolerant" adult chicken, carrying a successful graft, often rejects a second graft from the original donor, although the first homograft survived. The results of the first type of experiment have been verified by Billingham *et al.* (1956a); their interpretation, however, is the opposite of that of Cannon and associates. They emphasize that the stimulus that confers tolerance must be a fully antigenic stimulus, and that the newborn chick is approaching the null period. Since a graft of younger skin "takes" more rapidly, inasmuch as grafts of older skin require longer to establish vascular connections, the younger skin is antigenically more active, being the quicker to act, and acting for a longer time prior to the null period. Insofar as the second line of evidence is concerned, Billingham and co-workers believe that the results indicate a state of incomplete tolerance. In their experience, four of four second-set grafts made to tolerant animals bearing grafts survived. The writer suggests that the argument of Billingham and associates may be too one-sided in view of the indecisive nature of the evidence. Cannon and colleagues have not proved

that the tissue specificity of embryonic or newborn skin is alterable, but their experiments make the goal well worth pursuing. It remains entirely possible that acquired tolerance and graft adaptation can occur simultaneously or independently. Cannon (1957) stresses that not more than one-half of all grafts of day-old skin made to day-old hosts can be returned successfully to the original donor several months later, and that in only one out of twenty-one cases was a repeat homograft between adults successful. The first graft, made to day-old chicks, remains healthy, but the second graft is destroyed.

D. Properties of the Stimulus

Until now the discussion has been confined to acquired tolerance in one well-defined sense: tolerance to homografts (normal tissues, iso-antigens). Tolerance extends to tumors (Koprowski, 1955; Bollag, 1955; Billingham *et al.*, 1956a; see Koprowski, 1957, for a summary and evaluation) and heterografts (Simonsen, 1955a; Billingham *et al.*, 1956a). Of particular note is the observation by Simonsen and Harris (Simonsen, 1955a) that turkeys injected in embryonic life with the blood of chickens become susceptible to infection with the Rous sarcoma virus. In general, the methods employed for homologous normal tissues are only marginally effective with heterologous cells. The question remains whether a high degree of tolerance to heterologous cells might be achieved by earlier inoculation or by other modifications of the procedure. It cannot be emphasized too strongly that the capacity to respond to different antigens may mature at different periods of development.

What are the requirements of the stimulus for the induction of tolerance to homografts? It must be an effectively immunizing dose of foreign homologous cells, i.e., an antigenic stimulus. Among successful inocula may be listed whole blood, concentrated leucocytes, and cellular suspensions of a variety of tissues, including kidney, liver, testis, and spleen. Red blood cells freed from the leucocytes are ineffective. It should be pointed out that it has not been proved that the tolerance induced by suspensions of tissue cells is not dependent on their content of leucocytes. The question of lack of tissue specificity, then, cannot be considered as fully resolved. *Must leucocytes be included?* We return to this question later in our consideration of the graft-versus-host reaction. Drying from the frozen state abolishes the power to induce homograft tolerance. In short, intact (viable?) cells are required; cells which have no power to elicit transplantation immunity are incapable of inducing tolerance of homografts. To complete the parallel, it would only be necessary to add that, like the antigens conferring transplantation immunity, the stimulus for homograft tolerance may be nuclear in origin.

However, insofar as the writer is aware, this link in the chain is yet to be completed. As yet, it has not been demonstrated that transplantation tolerance can be conferred by nuclei in the absence of intact cells. An unexplained observation is the failure of intact avian red blood cells to induce tolerance. The ineffectiveness of mammalian red cells has been attributed to the absence of nuclei, an argument which cannot hold for avian erythrocytes.

E. General Immunological Significance

1. Tolerance to Purified Antigens

The question remains whether tolerance is restricted to transplantation immunity or whether it is a phenomenon of more widespread occurrence and significance. The relation between the depression of agglutinins for injected erythrocytes and the field of tolerance to homografts has been mentioned briefly in the foregoing discussion. The following point should be kept in mind: transplantation immunity differs in several respects from immunity to erythrocytes and soluble antigens in that it appears to resemble delayed hypersensitivity, being cell-borne and cell-mediated. No cause and effect dependence on circulating antibody has been demonstrated. Although it is tempting to attempt a general explanation that might relate the suppression of transplantation immunity to the inhibition of immune responses to other antigenic systems, it appears unwise. One difficulty lies in the fact that most of the studies on the depression of serum antibody response have been done in rabbits, whereas our understanding of acquired tolerance is based chiefly on results in the mouse and chick. Thus, direct comparisons are difficult. Hanan and Oyama (1954) found that the injection of bovine serum albumin (BSA) into pregnant rabbits (and into some of the offspring) three times weekly for three and a half months inhibited the production of antibody to BSA in the treated rabbits at maturity. The inhibition was not completely specific, the response to ovalbumin also being affected. Cinader and Dubert (1955, 1956) described a specific inhibition of response to the injection of human albumin in rabbits that had received from 20 to 1110 mg. of the antigen during the period 9–19 days after birth. These rabbits did not produce antibody during the period covered by the experiment, over 600 days. This report is, in the writer's opinion, the most impressive of the studies with purified antigens: a variety of techniques and adequate controls were employed. One aspect of the work is somewhat less satisfying. Six of the rabbits "tolerant" of human albumin received a modified (diazotized) human albumin (DHA). Of this group, two produced antibody to the DHA, and one of the two lost

its tolerance to human albumin. It is clear that the DHA evoked an antibody capable of cross-reacting with the unmodified antigen, but pending verification further discussion is unwarranted. Dixon and Maurer (1955) injected BSA and human plasma into rabbits six times weekly for over three months; challenged at 10-11 months of age with the homologous antigen, the rabbits failed to respond. It is stated that under these conditions the antigen was no longer detectable. However, the sensitivity of the method employed to determine this is open to question. Although specific depression of immune responses in rabbits has been observed by others, including Smith and Bridges (1956) and Downe (1955), the results of further necessary long-term experiments have not appeared.

Although it would be extremely valuable to have information on the chick or mouse, only two investigations have been reported, both in the chick, and they are conflicting. Cohn (1957) found that the injection of various antigens (diphtheria toxoid, galactosidase, bacteriophage T2, and arsanilic acid-BSA) into chick embryos at 14 days failed to inhibit the ability of 10- to 12-week-old chickens to produce antibody against them. Owen (1957) believes that these negative findings cannot be given their apparent weight in view of Simonsen's report, cited earlier, that the most sensitive period for depression of response was the period from 17 to 19 days of incubation. On the other hand, Owen himself recognizes the likelihood that different antigens might well have different sensitive periods. Cohn cites the work of Green and Lorincz (1957), which suggests that prolonged contact with antigen including living cells is not necessarily an advantage in eliciting tolerance. Chick embryos were injected with mouse ascites cells that proliferate extensively; yet at hatching the chick emerges almost free of the cells. After two months the experimental chicks produced as much antibody against these cells as did uninjected controls. Wolfe *et al.* (1957b) find that the injection of massive doses of BSA into newly hatched chicks results in a significant depression of response 6 weeks later. The depression is transitory, however, for, at 12 weeks, and more clearly at 22 weeks, there is little difference between the experimental and control groups. The antibody response to human gamma globulin was unaffected.

2. Immunological Paralysis

The introduction of the adjective "massive" in the preceding paragraph and, in fact, a consideration of the dosage employed in all the studies of depression of response to soluble antigens raise a serious question, that of the relation of these studies to the phenomenon of immunological paralysis. Felton (*Felton et al.*, 1955) first showed that

massive doses of type-specific pneumococcal polysaccharides caused an impairment of immunity. Moreover, animals already immunized could be "paralyzed" by the addition of sufficient antigen. How are acquired tolerance to homografts, depression of response to soluble antigens, and immunological paralysis related? Are they manifestations of the same basic phenomenon, persistence of antigens in the tissues? In this one respect, acquired tolerance to homografts, resulting from the injection of viable cells, appears not to be the method of choice.

The persistence of purified protein antigens can be approached in several ways, for example, by determining the fate of passively administered homologous antibody. If significant antigen persists, then it would appear that more passive antibody would be bound. Medawar (1957b) believes that the few data available (Dixon and Maurer, 1955; Cinader and Dubert, 1955, 1956) suggest this is not the case, for enfeeblement of response persists after the antigen has disappeared. The writer will point out only how difficult it is to ascertain the length of time an antigen persists in a tissue.

3. Production of Tissue-Specific Antibodies following Induction of Tolerance to Heterologous Tissue Antigens

A limiting factor in studying tissue specificity is the existence in each tissue of antigens common to all tissues or shared with some other tissues. Often these common constituents are strongly antigenic. It is common practice, after production of, say, an antiserum against heart, to absorb the antiserum repeatedly with blood and other tissue extracts to remove antibodies versus the common antigens. The method is not only tedious, but often unsatisfactory, because of the high concentration of non-specific, relative to specific, antibody present. Feldman and Yaffe (1957) point out the possibility that the production of specific antibodies might be augmented in animals which had been made tolerant of heterologous tissue antigens. For example, if the objective is an antiheart serum, the animals destined to produce the antibody might be injected in the fetal or neonatal period with brain or liver, or with a mixture of antigens. Feldman and Yaffe do not distinguish between homograft tolerance and tolerance to specific proteins, nor do they concern themselves with the relation of their idea to immunological paralysis; at the outset, at least, they have been concerned with the purely practical question of the applicability of tolerance as an experimental tool. Their first report is encouraging: the introduction into neonatal rabbits of brain or heart antigens inhibits the later development of the capacity to produce cross-reacting antibodies. Specific antibody response to selected types of antigenic components within the complexes of tissue antigens seems entirely possible.

4. The "Enhancing" Factor: Survival of Tumor Homografts in Mice Pretreated with Killed Donor Tissues

Flexner and Jobling (1907) discovered that a second graft of a transplantable sarcoma grew successfully in many rats in which the first graft of the tumor had failed. Casey and associates (Casey, 1932; Casey *et al.*, 1948) demonstrated that the Brown-Pearce tumor grew more rapidly in rabbits pretreated with dead homologous tumor tissues. The effect was specific, being produced only by factors in tissues derived from the challenging tumor (Casey, 1933). The phenomenon was extended by Snell and co-workers to include a number of tumors and strains of mice (Snell *et al.*, 1946, 1948), and somewhat later Kaliss and associates (Kaliss and Snell, 1951; Day *et al.*, 1954) showed that grafts of tumors would survive in mice pretreated with mouse spleen, kidney, or liver. It has been found that the effect which is species-specific, although not tissue-specific, persists for long periods, often permanently. Several lines of evidence lead to the hypothesis that the effect of pre-treatment by tissue extracts is mediated by the production of antibodies by the host animal. Graft survival is enhanced by the injection not only of tissue extracts but also of isoantisera to such extracts. If antisera are injected one week before inoculating with tumor, most of the grafts survive. As the interval between injection of the antiserum and tumor inoculation increases, the enhancing effect diminishes. Enhancement may be produced, but with less success, by injections of antiserum after administration of the tumor. Effectiveness drops off rapidly, the treatment being unsuccessful when made more than 9 days after grafting. Rabbit antisera have some effect, but for best results isoantisera are required.

When the host's ability to form antibodies is reduced, for example by treatment with cortisone, the enhancing effect of tissue extracts is drastically curtailed, leading Kaliss to believe that a necessary part of the effect is the production of antibodies by the host. What is the role of the antiserum? Kaliss (1957) has discussed several alternative hypotheses and has arrived at the following tentative conclusion: the antiserum acts directly on the graft, altering its responses to the host, so that it can now survive in what would otherwise be a hostile environment. He cites some support for this view in the reports by Barrett and Deringer (1950, 1952) indicating that a tumor homograft that survives in "foreign" mice loses some of its specificity, a finding loosely described as an adaptation of the tumor. An alternative explanation, considered unlikely by Kaliss, holds that the antibodies in some way inactivate the iso-antigens of the graft. Billingham (1957) tends to favor the latter view. He and Sparrow (1955) find that the life of skin homografts in rabbits

may be prolonged at least threefold by the intravenous injection of the recipient with living epidermal cells or whole blood taken from the future donor. Billingham infers that the antibodies responsible for enhancement of tumor survival and the survival of skin homografts are hemagglutinins, antibodies directed against relatively stable blood cell antigens. How these antibodies might cross-react with the isoantigens to neutralize the antigenic stimulus of the graft is not made clear.

As might be expected, attempts have been made to concentrate the enhancing factor and determine its identity. The analysis is not far advanced, and any conclusions would be premature. Kandutsch (1957) has summarized the limited data which suggest that the active principle may be a mucoprotein. This finding agrees with Medawar's recent statement that the "active" transplantation antigen in nuclei may be a polysaccharide (Medawar, 1958).

It appears that the enhancement phenomenon is not directly related to acquired tolerance. *Enhancement* depends upon an *active antibody response* by the host; *tolerance* requires the *suppression* of the animal's capacity to respond to specific antigens.

V. THE DEVELOPMENT OF TISSUE SPECIFICITY

A. Application of the Ideas and Tools of Immunochemistry

The induction of transplantation immunity and tolerance is presumably not tissue-specific: injections of leucocytes or various tissue cells may confer tolerance or immunity to subsequent homografts of skin, depending on the age of the recipient. Skin contains no isoantigens that are not present in leucocytes, or kidney, or liver, or spleen. The evidence available points to the nucleus as the site of at least some of the isoantigens. From this fact, the inference has been drawn that the nuclei of various body tissues are not differentiated as far as their isoantigens are concerned, a statement that is not inconsistent with modern embryological thought. Although Briggs and King (1957) have presented evidence to the effect that endoderm nuclei undergo irreversible changes during differentiation, which restrict their ability to promote the formation of ectodermal and mesenchymal structures, the level at which these changes occur is unknown.

The lack of tissue specificity in transplantation immunity leads us to examine next the concept of tissue specificity. Does tissue specificity extend to the molecular level? Are there, in fact, tissue-specific molecules, or is the concept of tissue specificity based on differences in numbers and associations of molecular types which are in themselves alike in all tissues? To the extent that they contain specialized products of

differentiation, cells and tissues clearly are tissue-specific; the reader may wish to supply his own illustrations drawing from his field of interest; among the better-known examples are insulin of the pancreatic beta cell, actin, myosin, and tropomyosin of the muscle fiber, α -crystallin of the lens. As one reflects on these examples, however, he realizes at once that their tissue specificity is apparent from their functional specificity; it is unnecessary to consider their antigenic specificity unless the use of the methods of immunochemistry affords an uncommon advantage for the study of localization or synthesis. As a general rule, these tissue-specific molecules exhibit species specificity to some degree, making analysis by immunochemical techniques possible; the principal advantage of these methods is their exquisite sensitivity, which makes possible the analysis of the rate of synthesis and accumulation and site of localization of proteins or other macromolecules present in embryos in trace amounts. The principal difficulty, one which is often not appreciated, is that antigenic specificity depends upon relatively small determinant groups rather than on the complete structure of the molecule, and that the molecule may contain more than one kind of determinant group. Little is known of the kind, number, and size of determinant groups of natural proteins. The antigenically active groups and physiologically active groups of a molecule may not be identical. Similarity in immunochemical properties of natural molecules of diverse origin may result from (1) identity of one or more antigenic determinant groups, (2) a degree of structural similarity, or (3) the presence of one as an undetected trace contaminant in the other, for, inasmuch as traces of antigen may evoke large amounts of antibody, such contaminants tend to be revealed.

It must be emphasized that molecules that behave alike immunochemically are not necessarily identical in chemical constitution. The reader of the literature in the field of immunoembryology must be aware also of the frequent incorrect usage of "antigen." An antigen is defined properly on the basis of its ability to elicit antibody production; not infrequently reference is made to the development of specific antigens, when in fact the data demonstrate only the development of specific determinant (combining) groups. It was thought at one time that the proteins of eggs and embryos lacked antigenicity; although this generalization has been disproved, critical studies of the antigenicity of embryonic proteins are few, many investigations having been concerned with the ability of embryonic molecules to interact with antibodies produced to molecules from the adult. I do not wish to imply that the use of antibodies directed against adult antigens has not contributed to studies of the synthesis and localization of proteins during ontogeny. In fact, a comparison of the immunochemical reactivity of a protein at

successive stages in development, employing quantitative techniques and reciprocal absorptions with antibodies directed against the corresponding embryonic and adult antigen should yield information bearing on the important question as to whether there are progressive transformations with age within a single molecular species. At this time there is no evidence of a critical nature that demonstrates whether, once formed, a molecule changes with increasing age. In fact, the data available suggest that, at least in the better-known cases, e.g., the hemoglobins, the differences in the fetal and adult molecules reflect the sequential synthesis of populations of closely related molecules. Whether the latter reflect, in turn, the changing sequence in sites of hemoglobin synthesis is not known (Ebert, 1958a). The success of immunochemical techniques depends on a number of factors: the nature of the antigens injected, the nature of the substances used in testing the reactivity of the antibodies produced, the very nature of the animal used for antibody production. Often several interpretations are possible; for example, an experiment in which the precipitate recovered in an antigen-antibody reaction is larger at one stage than at the preceding stage might suggest that the antigen has increased in quantity, in availability to react with the antibody, or in avidity (combining power).

The major shortcoming in the use of antibodies directed against specific, well-characterized adult proteins lies in the preoccupation with the end products of development without elucidation of the processes by which they were formed. Nace (1955) stated the problem cogently, when he said this approach "carries our knowledge from the adult level back to the embryonic level, which important though this is, does not suggest the morphogenetic significance of protein systems limited to the embryonic period, or of only minor importance to the adult, or which, though functional in the adult, have not yet been studied." The basic assumption behind much of the research in immunoembryology has been that unknown but vital components exist, which may be revealed by immunochemical analysis. To what extent has this proved to be true? The problem confronting us has been put aptly by Coons (1957), who wrote: "An inescapable condition for the employment of immunological methods is the recognition and acceptance of the fundamentally circular character of the situation. The injection of a mixture of substances of unknown composition or variety results in the synthesis of antibodies specifically reactive with some of them. It is this circular dependence on induced biosynthesis that invariably amuses students first exposed to the fundamental definitions of immunology, and that accounts for the long lag that intervened between the discovery of the basic immunological reactions and the isolation and characterization of

the antigenic materials responsible for them. Even in the case of bacterial antigens this process is only now well under way. The exquisite specificity of the immune mechanism favors the discovery and differentiation of new substances, but the discovery is essentially a blind one. This logical posture attains its full awkwardness in the study of naturally occurring components of tissues."

In seeking paths out of this circle, recently developed methods have been employed in the enumeration and identification of embryonic molecules. In the sections to follow we shall be concerned less with the historical development of the field than with the findings which have resulted in the formulation of new concepts and the modification of older views. A complete enumeration of the investigations in the field is not intended; much of the work has been reviewed (see Ebert, 1955, 1958a; Nace, 1955; Tyler, 1955, 1957). Insofar as possible, examples have been chosen which permit a comparison of the results obtained with several techniques and demonstrate the advantages and pitfalls in their use.

B. Development of the Lens

The subject has been considered recently by van Doorenmaalen (1957), Langman (1956; also see Langman *et al.*, 1956) and Ebert (1958a). In only one study have antibodies against embryonic lens antigens been employed, by Burke *et al.* (1944), who made a comparison of the ability of embryonic lens proteins to react in complement fixation and precipitin tests with antibodies prepared against both 160-hour embryonic lens antigens and adult lens proteins. There has been a tendency to disregard the findings of Burke and associates since several groups, e.g., ten Cate and van Doorenmaalen (1950), Flickinger *et al.* (1955), and Langman *et al.* (1956), using several methods including precipitin techniques and studies of the toxic effects of antibodies, described the occurrence of substances in the chick embryo capable of combining with adult lens antibodies as early as 35–50 hours of incubation. The question of the nature of these combining groups, and their relation to the embryonic lens antigens described by Burke *et al.*, becomes more significant in view of the results of van Doorenmaalen's (1957) analysis using fluorescein-labeled antiadult lens serum (Ebert, 1958a). Although the antibodies employed by van Doorenmaalen reacted in precipitin tests with one or more components in the embryo at 50–60 hours, and with extracts of iris, a point of some interest in view of the role of the iris in providing materials for lens regeneration [reviewed by Nicholas (1955)], clear-cut specific localization of the labeled antiserum was not obtained until the fifth to sixth day of

development, at which time the marginal zone of the lens epithelium showed a brilliant fluorescence of the cytoplasm. Van Doorenmaalen describes the appearance of the nucleus as an oval, unstained space, surrounded by an intensely-stained zone of cytoplasm. The staining fades out in the direction of the epithelium of the anterior wall of the lens. The lens fibers are best stained on the retinal side, where they are seen as green, compact bands.

This disparity in results achieved by one investigator employing the same antisera in two different techniques does more than point up the need for more frequent comparisons of this type. It raises the fundamental question as to whether the combining groups required for reaction with antibody in the test tube differ from those required for a reaction with the antigen *in situ*. Or is it simply that there are more reactive groups available (exposed) in the test tube than in the tissue (Ebert, 1958a)? Langman *et al.* (1956) reported that the toxic effect of antilens serum in tissue cultures is a more sensitive tool than the precipitin technique; it should be interesting to test van Doorenmaalen's antibodies for their ability to prevent lens formation.

Langman and associates also observed that the localization of the lens proteins involves a spatial restriction, the groups capable of combination with antiadult lens sera being distributed more widely in the head ectoderm in younger than in older embryos. As lens induction proceeds, the reactive groups are restricted. In a discussion of comparisons of precipitin and tissue localization techniques, another experiment may be suggested. It might be rewarding to repeat the experiment described by Woerdeman (1955), who reported the synthesis of substances capable of reacting with antisera against adult lens following the admixture of extracts of optic vesicle and ectoderm *in vitro*, using a labeled antiserum, in order to determine whether the substances synthesized exhibit a fibrous pattern of organization.

C. Myogenesis

1. Cardiac

In the chick, as in all vertebrates, the heart arises by the fusion of two primordia which lie on either side of the mid-line. The process of fusion proceeds progressively from anterior to posterior as the result of a series of integrated morphogenetic movements. The dual origin of the heart has been shown by simple experiments in which the fusion of the heart-forming regions is prevented by a mechanical obstruction [reviewed by Copenhaver (1955)], resulting in the formation of two beating hearts. Recently DeHaan (1958) has succeeded in producing

cardia bifida by altering the chemical environment. In early blastoderms cultured endoderm surface up (New, 1955), the cardiac primordia can be prevented from fusing by treatment with acetylcholine. Since acetate also is an effective agent, and since it is known to form coordination complexes with heavy metals, DeHaan has advanced the hypothesis that acetylcholine acts by displacing calcium in producing double hearts. Other agents which sequester calcium and divalent ions have been shown to act in a similar manner, lending support to this view. Compare figures 1 and 2.

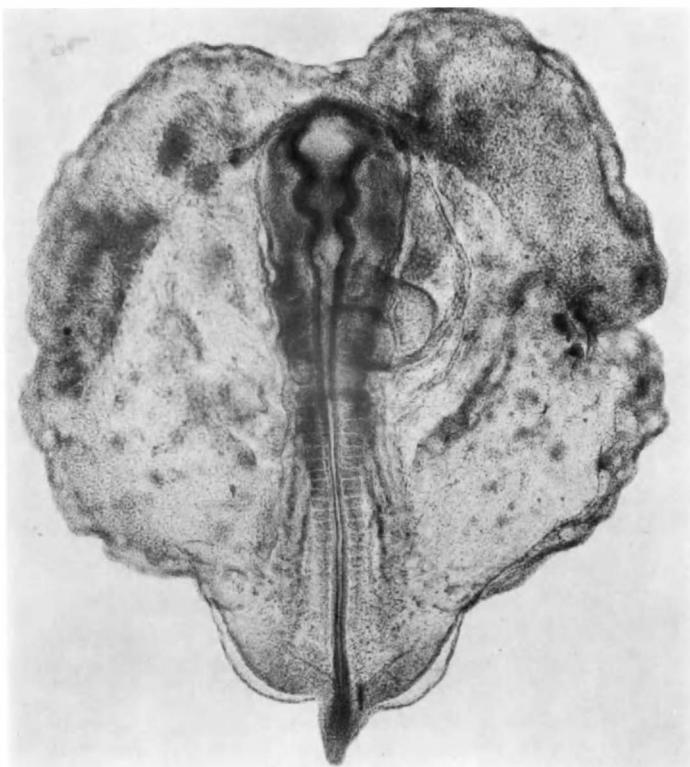


FIG. 1. Chick embryo cultivated *in vitro* for 24 hours following explantation at 4-somite stage. (Duffey and Ebert, 1958).

Inextricably interwoven with the structural development of the heart is the onset of contractility. As the heart is formed, so it begins to beat (Ebert, 1953). The first beats of the heart can be made out early in the second day of development. The first twitching is along the right

margin of the developing ventricle, the beat being at first slow but rhythmical and gradually involving the whole wall of the ventricle, spreading from the posterior to the anterior end. These fibrillations are followed in succession by a concerted movement of the right side of the ventricle and then by similar involvement of the left side, so that soon the entire muscle mass of the ventricle undergoes synchronized

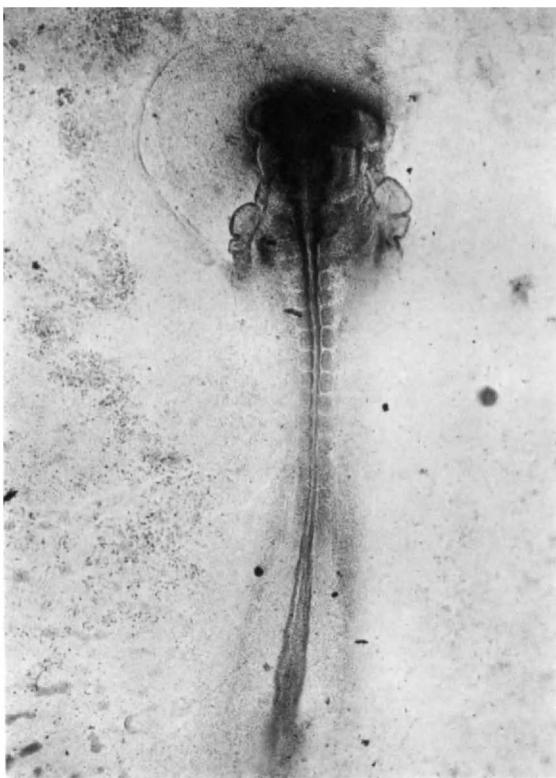


FIG. 2. Cardia bifida in the chick embryo produced by placing a crystal of acetyl-choline on the endoderm of the area pellucida 24 hours earlier. (Courtesy Dr. R. L. DeHaan).

contractions which have been described as rhythmic but intermittent, that is, rhythmic contractions interrupted by rest periods. Subsequently the atrium and then the pacemaker begin to contract at a rate higher than that of the ventricle, thus stepping up the rate of the entire structure, for the part of the tube with the highest rate sets the pace for the heart as a whole. These events occur before any of the nerve fibers which grow out from the central nervous system approach the heart and before the specialized system for conducting impulses in the heart is estab-

lished. However, the absence of organized neural structures does not rule out the possible activity of chemical regulators of contraction.

In the chick embryo, the formation and first pulsations of the heart, which occur early in the second day of development, are preceded by the localization of cardiogenic cells to well-defined regions of the embryo as early as the head-process stage. It has been known for nearly thirty years that pulsating cardiac muscle will develop in cultures of tissue taken from peripheral regions of unincubated eggs but not in explants made from the central square millimeter of the embryo. The capacity of the embryo to form heart is first progressively localized to its posterior half and then to large regions on either side of the anterior end of the primitive streak (reviewed by Ebert *et al.*, 1955). Rawles (1943) established the extent of the heart-forming regions at the head-process stage. In the center of each of the lateral regions, the capacity for the formation of cardiac muscle is highest; toward the periphery the capacity diminishes. It appears that under experimental conditions a much larger population of cells of the embryo has the capacity to form heart than actively participates in cardiogenesis during normal development. In the course of her study, Rawles also proved that even in the first day of development the left side of the embryo is superior to the right in its ability to form cardiac muscle. There is no striking difference in shape or extent of the right and left heart-forming areas, but there is a clear-cut difference in the frequency with which heart tissue is produced. In interpreting these experiments, two points have to be emphasized. First, each of the transplanted pieces of the embryo contains three layers, and, although the heart muscle arises from the mesoderm, we cannot state whether it develops independently or whether interactions between the tissue layers are required. The significance of this point may be realized if it is pointed out that, in the amphibian embryo, evidence has been presented which demonstrates that an interaction between mesoderm and endoderm is a requisite for normal development of the heart (Bacon, 1945). The situation in the chick is not altogether clear; it has been reported that, although pulsating masses can develop from pieces of the chick embryo from which the endoderm has been removed, the differentiation of the heart is much poorer (Rudnick, 1938). Second, the tissue fragments which are transplanted contain a relatively large population of cells. Thus, by this technique, one tests the potentialities of a tissue mass and not of a restricted population of cells or of a specific line or strain of cells.

We inquire next to what extent such differences in heart-forming capacity reflect detectable differences in chemical composition or metabolism, differences which might suggest suitable targets for further

experiments. How do the heart-forming areas differ chemically from adjacent regions of the embryo? Ebert (1950) showed that if an anti-serum elicited in rabbits by extracts of adult heart (and subsequently absorbed with extracts of other organs to remove antibodies against antigens held in common by several tissues) is mixed with an extract of the chick embryo at the primitive-streak stage, a time well before morphogenesis of the heart, a clear-cut precipitin reaction is observed. This finding suggests that the early embryo must contain determinant groups identical with or closely related to those of the adult heart, a conclusion supported by the additional finding that when early embryos are cultured in media containing antiheart serum the development of the heart is differentially suppressed. Embryos develop in which the heart is absent or retarded in its development.

Whereas these findings are of considerable interest in themselves, their greatest value lies in the further questions which they raise. Since the antiheart sera employed contain more than one antibody owing to the complex nature of the antigenic mixture which elicited them, one is led to ask which of the embryonic combining groups is so vital to the metabolism of the embryo that combination with its specific antibody results in a suppression of differentiation (Nace, 1955). This question may have more meaning if I re-emphasize that the combination of an antigen with its specific antibody is not necessarily accompanied by the loss of the physiological activity of the antigen or, in the case of combination *in vivo*, by the impairment or destruction of the tissue antigen. As shown by Owen and Markert (1955) and independently in our own laboratory, following the injection of an enzyme, e.g., tyrosinase, into rabbits, some animals produce antibodies which combine with the enzyme but do not affect its catalytic activity, whereas other rabbits produce antibodies which combine with the enzyme and completely block its action. Other antibodies are produced which fall in between these two extremes. Similarly, studies by Mellors, Pressman, and others have shown that when antibodies, for example, antibodies produced in rabbits against rat kidney are labeled (with radioactive isotopes or the fluorescent dye, fluorescein isocyanate) and injected into living rats, they tend to localize in the homologous organ, the kidney. In some cases the presence of the antibody results in damage to the tissue at the site of localization; in others no toxic effects are observed [summarized by Edds (1958)]. The observation that an antiserum is capable of producing a specific defect in the embryo suggests that the isolation and identification of the specific antigen that evoked the antibody might yield information concerning cellular components important to development, and points to the feasibility of investigating the synthesis of one

or more specific proteins and determining the effect of various experimental conditions on the process. Thus, it is possible to ask meaningful questions concerning the synthesis of the contractile proteins, actin and myosin, in the development of the heart. A complete discussion of the chemistry of the contractile proteins and the fine structure of muscle is beyond the scope of this chapter. Three proteins, myosin, actin, and tropomyosin make up about 75–80% of the total protein of muscle. We shall be concerned only with myosin and actin, for little is known of the developmental history of tropomyosin and the proteins of the poorly defined residual fraction. It is entirely possible that from the standpoint of the development of the muscle fiber, elements of the "unknown" fraction may prove to be more interesting than myosin or actin. One is intrigued by the observation that not only can myosin be eluted from its position in the muscle fiber, the anisotropic (A) band, but it also can be replaced, the contractility of the fiber being restored in the process. What are the properties of the "residual" fiber which enable the specific recombination with myosin to occur? The basic premise at the outset of the research on the formation of the cardiac proteins (Ebert, 1953) was the oversimplified, textbook statement that the final contractile substance of muscle is actomyosin, a statement based principally on the discoveries that the "structural protein," myosin, has the enzymatic capacity to catalyze the transformation of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and that filaments made from a mixture of actin and myosin will contract upon the addition of ATP. Although it was realized that new findings, for example, the demonstration that myosin can be split into discrete subunits, the meromyosins, might require some modification in thinking, the work assumed only that the contractile system involves the interaction of actin, myosin, and ATP. The development of cardiac myosin was examined first. In the course of this work, several unexpected and puzzling observations were made (Ebert, 1953, 1955, 1957; Ebert *et al.*, 1955).

Myosin extracted from hearts of adult chickens contains one major and two minor components, as determined by electrophoresis. Injection of this material into rabbits elicits antisera, which, after absorption with myosin extracted from breast and limb muscle, react specifically with cardiac myosin. This observation is critical, for it permits us to say that antibodies can be used to distinguish determinant groups of cardiac myosin from those of skeletal myosin. When extracts of the early chick embryo are mixed with anticardiac myosin sera absorbed in this manner, clear evidence of the combination of antigen and antibody was obtained. The youngest embryos from which extracts are capable of reacting with the specific antisera are those at the intermediate streak stage, during

the movement of mesoderm through the patent primitive streak. In further experiments, it was found that at this stage the reactive groups of cardiac myosin can be detected throughout the epiblast; they are absent from the hypoblast. A few hours later the reactive groups are confined to the regions described by Rawles as the heart-forming regions of the embryo. It is surprising to find that prior to the formation of the heart the capacity to synthesize myosin is found throughout the epiblast. It might have been expected that the synthetic abilities of the cells in the heart-forming areas would be established only after they had reached their definitive position in the embryo.

In contrast to the development of myosin, actin can be detected first by immunological methods similar to those employed for myosin at the time at which myosin is being restricted to the heart-forming areas. Moreover, when actin is first detected, it is confined to the heart-forming regions. Again, it should be made clear that in our experience (Ebert *et al.*, 1955) cardiac actin consists of at least three antigenic components of which at least one is found also in actin derived from limb muscle. The sequence of development of the cardiac contractile proteins in the chick embryo has been summarized as follows: The synthesis of cardiac myosin is detected during the formation of the mesoderm. At the definitive primitive-streak stage, cardiac myosin is distributed throughout the epiblast. It has not been detected in the hypoblast. Within 2-3 hours, the protein is restricted to the heart-forming regions of the embryo. Cardiac actin is detected first in the heart-forming areas. It cannot be stressed too strongly that the methods employed detect only determinant groups of actin and myosin. They do not prove that the proteins of the adult and the embryo are identical. Evidence from other sources to be discussed below does indicate that the myosins detected by immunochemical and physiological techniques are identical or closely related, and perhaps laboring the point does a disservice to those employing immunochemical tools. The sequence of events related to the localization of myosin is particularly intriguing. At least two possible explanations have been advanced. It was held possible that the segregation of the protein results from the movements of cells, that is, cells containing myosin aggregate in the heart-forming regions. Another view held that the disappearance of myosin from the regions peripheral to the heart-forming areas results from the failure or inhibition of one or more steps in the pathway of myosin synthesis. It is important to remember that Rawles' transplantation studies show that within the cardiogenic areas heart-forming ability falls off from the center toward the periphery. Experiments designed to determine which of these hypotheses is more correct have failed to do so.

As illustrated in the accompanying diagram (Fig. 3, Ebert, 1957), primitive-streak embryos were cut into three fragments, and each piece was grown *in vitro* before being analyzed for its content of myosin with the thought that it might show whether the loss of ability to synthesize myosin is independent of the relationship of a given mass of cells to adjacent tissues.

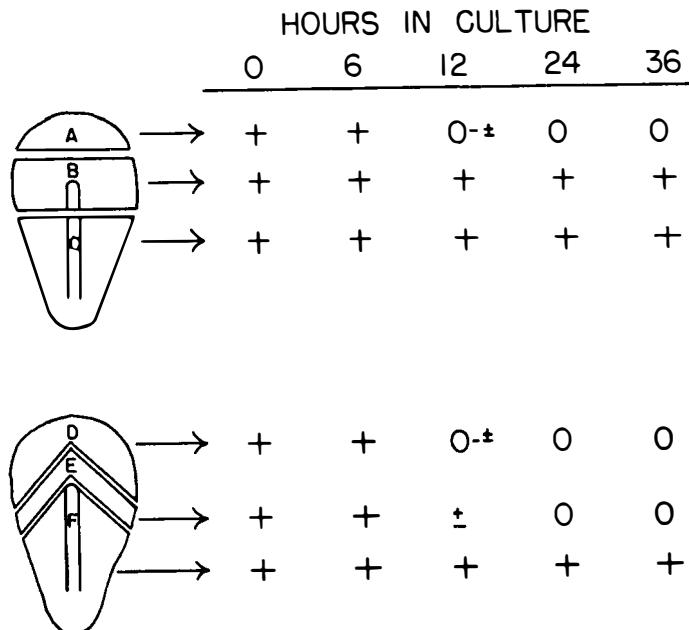


FIG. 3. Serological determination of cardiac myosin in pieces of the early chick embryo after isolation and cultivation *in vitro* for varying periods. (Ebert, 1957).

In the whole embryo the anterior piece (*A*) exhibits the same behavior as the posterior piece (*C*), namely it loses its capacity to synthesize myosin. The mid-section (*B*) retains its synthetic power. In isolation, however, *A* behaves quite differently from *C*. The posterior piece, *C*, retains its capacity to develop a pulsating heart and even after 36 hours *in vitro* retains the immunologically reactive groups of cardiac myosin. The anterior piece, *A*, does not form pulsating masses and loses its myosin, although not immediately; the protein can be detected for at least 6, and occasionally for as long as 12, hours. If the embryo is cut somewhat differently, following an earlier experiment by Rudnick, then only the posterior piece, *F*, retains myosin for more than 12 hours. In the absence of precise quantitative evidence, it cannot be said with

certainty whether sections *D* and *E* differ in the time at which they lose the ability to form cardiac tissue. It can be stated, however, that there is no striking difference.

Johnson and Leone (1955) have confirmed the major aspects of this work. Antisera prepared in rabbits against several proteins including actomyosin, myosin, and "myogen" extracted from adult chicken hearts were used as reagents to detect the time of origin of combining groups specific to cardiac muscle proteins. Unfortunately, although some preliminary absorption studies were carried out, the antisera employed as diagnostic tools were not absorbed routinely. Precipitin tests indicate the following developmental sequence: "myogen" is detected first at 20 hours, followed by actomyosin at 40 hours of incubation. However, primitive-streak stage embryos cultivated in antiactomyosin sera are abnormal in that many lack hearts or have malformed hearts, leading the authors to conclude that specific combining groups of actomyosin are present in the definitive primitive-streak embryo. Johnson and Leone propose that the determinant groups are part of an "incomplete" antigen, since they cannot be detected by precipitin tests until somewhat later. The chief drawback in the work lies in the failure to use absorbed antisera, resulting in many nonspecific effects on the embryo.

Since much of our information on the structure of adult muscle has been obtained with the electron microscope [see Porter and Palade (1957) for references], it is logical to ask whether this tool has been brought to bear on the problems of heart formation. According to Hibbs (1956), the muscle of the early chick heart consists of a network of loosely arranged cells. Between 24 and 30 hours of incubation, these cells assume a more compact arrangement, and sometime prior to 30 hours slender filaments are formed within the cytoplasm. Some of the myofilaments are oriented to form loose bundles. About 6 hours later, the first indications of banding appear as a dense line which appears to be continuous across the width of the fiber (the Z line). Although contractions are noted at about 30 hours, the dense substance of the A band, the site of localization of myosin, is laid down gradually beginning at between 48 and 60 hours of incubation. A gradual increase in density of the A band is noted. Thus, we see an apparent contradiction. On the one hand, there is no doubt that the heart begins to fibrillate early in the second day of development; moreover, immunological tests indicate the presence of a substance able to react with antimyosin sera even before that time. On the other hand, by electron microscopy the A band does not make its appearance until late in the second or early in the third day of incubation. It is quite possible that this apparent conflict of views involves little more than differences in resolving power of the several techniques employed. It is possible that in earlier stages the A substance may be present although evenly distributed. There seems

little doubt that the contractile system can exist in primitive form less highly organized than the discrete muscle fiber.

What other lines of evidence have been presented in support of the contention that the heart-forming regions are differentiated chemically well in advance of the appearance of structural characteristics of the muscle? Clues have been obtained from studies of the metabolic properties of the early embryo. The points of contact between this study and the work just described involve chiefly the question of the sources of energy for cardiac metabolism and development. Several years ago, Spratt (1950), arguing on the basis of results derived from a comparison of the effects of several metabolic inhibitors on the early embryo, suggested that the metabolic pathways operating during the development of the embryonic heart and brain differed markedly. Support of this argument comes from studies completed recently by Duffey and Ebert (1957). By cultivating early chick embryos in a medium containing traces of the metabolic inhibitor, antimycin A, it was shown that the metabolic pathways of mesodermal derivatives, including the heart, but not the blood-forming tissues, differ characteristically from brain and spinal cord, for concentrations of this inhibitor as low as one-tenth of a microgram per embryo inhibit the development of organized mesoderm almost completely, while leaving the developing brain and spinal cord intact. The use of slightly lower concentrations of antimycin A shows that the heart may be somewhat more sensitive than other mesodermal derivatives. Spratt had reported earlier that low concentrations of sodium fluoride also inhibited development of the heart, whereas higher concentrations produced destruction of the entire embryo. As Duffey first observed, however, under the latter conditions the disintegration of the embryo follows a clear-cut, reproducible pattern in that the initial sites of inhibition are the regions of the heart-forming cells. At any given stage of development beginning with the primitive-streak stage through the establishment of the heart, the locations of the cells destroyed by fluoride reflect the sites of the highest ability to form a pulsatile heart and the area of greatest capacity for the synthesis of actin and myosin. It seems unlikely that all of the cells in the heart-forming areas are specific cardiogenic cells. It should be particularly interesting to combine the chemical or immunochemical techniques with the procedures for tissue disaggregation to determine whether it would be possible by chemoselection or immunoselection to single out a cardiogenic strain.

2. *Skeletal*

The somites are laid down progressively from anterior to posterior; yet it appears that in a developmental sense the more posterior somites are only slightly behind the older anterior ones in their stages of differ-

entiation. Measurements by Herrmann (1952) show that the more posterior somites are larger at the time of segmentation than are the anterior ones and that their volume increases at a greater rate so as to quickly overtake the anterior ones. A similar inference may be drawn from the work of Holtzer *et al.* (1957), who found that myosin-synthesizing capacity spreads posteriorly more rapidly than segmentation. Herrmann and co-workers (Herrmann, 1953; Rothfels, 1954) have found, by culturing early chick embryos *in vitro* in the presence of amino acid analogs, that in contrast to analogs of methionine and phenylalanine, which have a general inhibitory effect on the embryo, analogs of leucine, isoleucine, and valine have a specific effect upon segmentation. The somites fail to split off, but instead the entire segmental plate or some part of it may undergo normal histological differentiation as a unit. No explanation for such specificity has been proposed.

It is not possible to state whether the somite undergoes independent differentiation with respect to myogenesis or whether such differentiation is induced by interaction with other tissues, e.g., notochord or neural tube. However, recent work by Muchmore (1957a, b) indicates that the development of muscle depends upon specific interactions. As neural tube, gut, and/or notochord are removed, the extent and normality of muscle differentiation decrease in proportion to the degree to which the normal relations of the somite are disrupted, but the most striking results are obtained by the addition or removal of neural tissues (Muchmore, 1958). Holtzer and Detwiler (1954) specify the dependence of somitic muscle differentiation on interactions with the neural axis. Although Muchmore's data do not rule out a possible specific influence of the neural axis, his working hypothesis may be taken as the best available at this time. It is interesting to note that, apart from Muchmore's work, most of the literature on cartilage and muscle formation in explants or grafts of somite is aimed at showing the activity of specific inductors. "Simple" explanations for the influence of other tissues, for example, that they prevent dispersal of somite cells, have been considered only infrequently. It would be interesting to know whether isolation of a somite plus its normal neighbors, ectoderm and endoderm, would result in muscle formation.

Similarly, there is controversy concerning the extent to which the somites contribute to the trunk musculature. Carbon-marking data by Straus and Rawles (1953) in the chick embryo show clearly that the somites produce only the dorsal third of the axial musculature, with the ventral half arising from the lateral plate and the remaining sixth being a joint effort of the two. In *Ambystoma*, however, Detwiler's (1955) results appear equally conclusive that all axial musculature is formed

from the somite. Attempts to explain the difference in findings on the basis of class differences fail in the light of a small number of extirpation experiments by Straus and Rawles, using *Amblystoma*, which lead them to the same conclusions they reached for the chick. Hence the question remains unanswered.

In the foregoing discussion, mention was made of the localization of myosin to the A band of striated muscle; initially this conclusion was reached on the basis of extraction techniques for the differential removal of myosin from the muscle fiber. Hasselbach (1953) and Hanson and Huxley (1953) extracted isolated fibers with solutions known to remove myosin and found that, concomitant with the extraction, the anisotropic (A) band disappeared. However, work by Szent-Györgyi *et al.* (1955) and de Villafranca (1956) suggests that these techniques remove additional soluble proteins. In an important technical advance, Finck *et al.* (1956) applied the fluorescent antibody technique (Coons and Kaplan, 1950; Marshall, 1951) to the localization of myosin, and found that fluorescent antibodies against myosin of chicken skeletal muscle could be used to detect myosin in the A band of glycerinated mature and embryonic muscle fibers. These labeled antibodies were used as staining reagents to analyze the development of trunk myoblasts in the chick embryo (Holtzer *et al.*, 1957). The following account is drawn from the authors' thought-provoking exposition. Myoblasts from the brachial myotomes of chick embryos (Hamburger and Hamilton stages 12 to 25) were studied by (1) conventional histological methods, (2) phase contrast microscopy of myoblasts, both living and glycerol-extracted, and (3) observation by fluorescence and phase contrast microscopy of embryo trunks stained with fluorescent antimyosin. The antibodies were prepared in rabbits against skeletal myosin from adult chickens and were unabsorbed. In the series studied by phase contrast microscopy alone, striated myofibrils were detected at stages 22 to 23; in preparations stained with iron hematoxylin, at stages 18 to 19. From the studies with fluorescent antimyosin the following picture emerges: "The brachial myotomes of stage 13 to 14 embryos do not bind the antibody. In the more anterior brachial myotomes of the stage 15 embryo, there are seen under the fluorescence microscope several dozen slender fluorescent filaments or fibrils. The earliest fibrils appear uniformly fluorescent without evidence of cross striation. These are the first definitely stained elements to appear in the developing myoblasts in the brachial myotomes." At stage 16, although several hundred delicate, uniformly fluorescent fibrils can be seen in a myotome, no cross striations are visible. By stage 17 or 18, however, cross-striated fibrils clearly are present. By stage 20, a combination of fluorescence and phase contrast microscopy reveals that

the labeled antibody is confined to an area that has the dimensions of a mature A band. Z bands were not observed at this stage. Thus, although myosin is clearly found in the A bands after stage 16 to 17, it is difficult to assess the significance of the finding that delicate filaments without cross striations are also stained for a brief period before the detection of cross banding. The writers raise the possibility that, although the definitive sarcomere pattern is achieved very early, it may be preceded by a less organized stage. Holtzer and associates emphasize also their opinion that the early myofibril grows laterally as a thin sheet associated with the sarcolemma, and that growth in length occurs in the growth tips of the elongating myoblast.

The authors' critical comments on fluorescence microscopy and antibody staining (Holtzer *et al.*, 1957, pages 716-718) warrant summarization: (1) the limiting sensitivity of the technique is not known, for it varies with the nature of the specimen and with a number of technical details. As with any cytological technique, the limitation is not the total quantity of the protein present, but its concentration within a restricted area. If myosin were present evenly throughout the cell, a larger amount might be present and yet remain undetected. Thus, the findings do not prove that myosin is synthesized on the myofibril.

(2) A limiting factor is the immunochemical specificity of the reaction. Although the myosin employed was highly purified, it contained one major and one, possibly two, minor components, as determined by serum-agar diffusion studies. This observation is in agreement with that of Ebert (1953), whose preparations of cardiac myosin were shown by electrophoresis to have a like number of components. Holtzer and co-workers report that the antibodies produced against chicken skeletal myosin are neither tissue-specific nor species-specific, cross-reacting on the one hand with chicken cardiac myosin and on the other with skeletal myosins from several species, including the salamander. In spite of the presence of trace components and the extensive cross reactivity, no absorptions were done. The authors rely on the consistency of localization of the antibody concentration as indicating that trace components do not contribute significantly to the staining effectiveness of the antibody. In view of the demonstration by Ebert (1953) of distinct immunochemical differences in cardiac and skeletal myosins and by DeHaan (1956) of species differences in actomyosins, absorption studies would appear to be justified. Additional evidence cited by Holtzer and colleagues relates to the uniform ability of antimyosin preparations to inhibit contraction of the glycerinated myofibril affected by ATP. Again in contrast, Ebert (1958c) finds that only about one-third of the antibodies to cardiac myosin blocked contraction of actomyosin filaments. It must be borne in mind that combination with antibody may occur

only with certain reactive groups on the myosin molecule. Thus, myosins from different sources may differ in immunochemical reactivity. Two points—the lack of species and tissue specificity of the antibodies and the consistent inhibition of contractility—suggest to the writer that the antibodies employed by Holtzer and associates are directed against physiologically active groupings of the myosin molecule, possibly enzymatically active groups (ATPase) or sites of interaction with other proteins. Such a conclusion is entirely consistent with evidence from studies on the species and tissue specificities of purified enzymes.

(3) The fluorescence method permits cytological precision, and in this respect it is more effective than refined precipitin techniques. Another interesting contrast is found in the work of Hibbs (1956) and Holtzer *et al.* (1957). In the myotome, the latter find a localization of myosin to the A band shortly after the appearance of the myofibril; the Z band was not observed at this stage. In the heart, Hibbs describes the Z band at about 36 hours, the A band not until about a day later. Is an explanation to be sought in differences in developmental patterns in cardiac skeletal muscle or in differences in the limitations and resolving power of the techniques employed? Holtzer and colleagues caution that the latter may be the case, in view of the difficulties in fixation of relaxed muscle.

Two recent investigations lend support to the tentative view of Holtzer and associates that unstriated filaments contain myosin reactive groups. DeHaan (1956) demonstrated that proteins immunologically indistinguishable from those of fully formed muscle are present in the regenerating limb of *Ambystoma mexicanum* at least as early as the appearance of the first unsegmented myofibrils and before striae occur. In a study of the regeneration of the forelimb of the newt, *Triturus*, Laufer (1958) made the following observations: (1) The agar-diffusion technique (reviewed by Grabar, 1957) revealed immunological reactivity to specific muscle antibodies before cross striations are visible in the regenerate. The reactions at palette and early digital stages are weak, and the antigens cannot be identified conclusively as actomyosin or myosin. The appearance of actomyosin and myosin was preceded by an increase in an unidentified nucleoprotein, which later decreased to a level characteristic of adult muscles. (2) Autoradiograms of limbs infused with I^{131} -labeled antiactomyosin demonstrated the disposition of the antibodies within the regenerate. There was evidence of localization of the antibodies in muscle cells. (3) Infusion of antimuscle serum into the regenerate produced a cytotoxic reaction, resulting in a temporary block to regeneration. Sarcolysis attributable to the specific effects of the antisera was described. The agar-diffusion technique is illustrated in Figure 4.

We have centered our attention on the initial synthesis of the contractile proteins and their fibrillar organization. Other questions emerge from studies of the accumulation of the muscle proteins during embryonic and neonatal life. For example, it has been suggested by Kasavina and Torchinsky (1956) that in animals like chicks and guinea pigs which are independent at birth the muscle proteins reach physiological maturity during embryonic life, whereas in "dependent" animals like mice and rats the muscle proteins mature more slowly after birth. Kasavina and Torchinsky and Ivanov *et al.* (1956), employing electro-

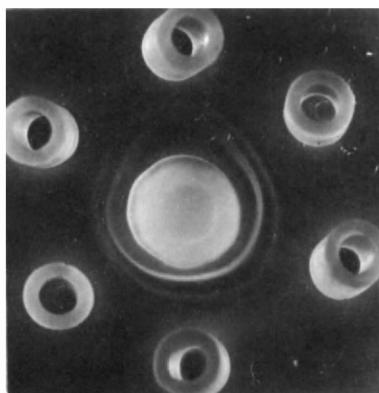


FIG. 4. Reaction of a 0.6 M KCl extract of newt muscle with anti-actomyosin sera (serum-agar diffusion method of Ouchterlony). The center well contains muscle extract, the peripheral reservoirs 6 different anti-actomyosin sera. Note confluence of two distinct bands marking reaction of the antisera with actomyosin and myosin. Some sera distinguish a third component, believed to be nucleoprotein. (Courtesy Dr. Hans Laufer).

phoretic techniques, suggest that the gradual increase in actomyosin during development is correlated with a decrease in a so-called "pro-actomyosin complex," including "myoalbumin." Evidence of a causal relation between the two fractions has not been presented. Analyses of the chemical and physiological maturation of the contractile proteins will be found in the work of Herrmann and associates (Herrmann, 1952, 1955, 1958).

D. Embryonic Antigens

The distinction has been made between the occurrence in embryos of combining groups identical with those of adult antigens and the occurrence of embryonic antigens. The antigenicity of embryonic tissues has been demonstrated in several species, for example, in amphibians (Cooper, 1948, 1950; Flickinger and Nace, 1952; Clayton, 1953) and in the sea urchin (Perlmann, 1954; Perlmann and Perlmann, 1957). Until

recently, however, critical evidence for the antigenicity of substances in the early chick embryo was lacking. After complete absorption with adult laying hen serum, antisera against the serum of the 10-day chick embryo showed positive precipitating activity with the homologous antigens (Schechtman *et al.*, 1954). Similar experiments led Levi and Schechtman (1954) to conclude that the 12-day chick embryo contained distinct embryonic red blood cell antigens. Nettleship (1953) injected 1-, 2-, and 6-day chick embryos, homogenized in normal saline, into hamsters; the hamster antichick sera were dropped or injected into or near the "embryo site" of unincubated eggs. Precipitin titers of the antisera were not determined; in fact, it was not reported that antisera were tested against extracts of embryos of the same or different stages. Nettleship reported that antiserum "placed in proximity to the preincubated chick embryo stops the development of these embryos at a time which corresponds to the time the embryo antigen was obtained." Tyler's (1957) comment on this report is particularly appropriate, "For an unabsorbed antiserum of this type to produce a highly specific effect does seem surprising, and one wonders whether or not this might be due to fortuitous variation in the antibody content of the various antisera."

Mun (1956) has demonstrated convincingly that the 72-hour chick embryo (Hamburger and Hamilton stages 16 to 18) contains specific antigens. In the course of this work, Mun was able not only to remove by absorption methods Forssman antigens and other cross-reacting antigens, but also to inactivate the heat-labile, nonspecific cytotoxic factor of rabbit serum. A full discussion of the evidence is beyond the scope of this chapter (see Ebert, 1958a). Mun's work points up the need for answers to fundamental questions concerning the effects of antibodies on living cells. Can specific toxic antibodies be produced? How can one best differentiate nonspecific toxins from antibodies? In relatively few instances (e.g., Nace and Inoue, 1957) have toxic effects attributed to antibody been associated with a specifically absorbable gamma globulin. Quantitative data are lacking. Although studies of toxicity using labeled antibodies will be helpful, it is already clear that specific localization of an antiserum does not necessarily lead to toxic effects (see the discussion by Mellors and Pressman in Edds, 1958). One sees frequent reference to *cytotoxic* sera, but we have little information on the cellular level. For references to the literature and thoughtful appraisal of cytotoxins, the reader is referred to the papers by Ross (1957) and Wissler and Flax (1957).

Reference has been made to the effects of antibodies against adult antigens on embryonic development (e.g., Ebert, 1950; Ebert *et al.*,

1955; Johnson and Leone, 1955; Langman *et al.*, 1957; Laufer, 1958). Apart from the above-mentioned work, experiments with antibodies against embryonic antigens have given indication of stage-specific inhibitory action (Flickinger and Nace, 1952; Clayton, 1953). Perhaps the most striking recent evidence of the effect of a specific antiserum in a developmental system has been provided by Tyler and Brookbank (1956; see also Tyler, 1957). In early experiments, the authors found that antibody produced against extracts of sea urchin eggs blocked cleavage; at first the effect was believed to be stage-specific, but it was learned that the time of the cleavage block was dependent on the strength of the serum employed. Antisera against extracts from early stages would block at any later stage, and anti-late stage antibodies would block early if the concentrations were appropriate. Subsequently, it was shown that the inhibition of cell division can be obtained with an antiserum produced against the chemically defined constituent of the gelatinous coat of the egg, fertilizin. The reaction is not species-specific. The antisera also block further development of hatched blastulae and gastrulae. Tyler (1957) infers that the antigens affected are surface antigens and further suggests that surface antigens of the uncleaved egg persist until at least the gastrula stage. The relation of this finding to reports of the synthesis of new antigens in the sea urchin at gastrulation (Perlmann and Gustafson, 1948; Perlmann, 1953; Harding *et al.*, 1954) is not clear.

Perlmann and Perlmann (1957) have reported that specific surface antigens can be recognized in the egg of *Paracentrotus lividus*. Their recent experiments stem from an earlier finding that antibodies against egg components produced multiple effects on development, including precipitation of the jelly layer, parthenogenetic activation, damage to the egg cortex, and a depression of the rate of fertilization. It is now reported that these effects are in fact attributable to the presence of at least four different antibodies, directed against at least four specific surface antigens: *J-ag* (induces an antibody which precipitates the jelly); *A-ag* (evokes the "egg-activating" antibody); *C-ag* (cortex-damaging antibody); and *F-ag* (fertilization-depressing antibody). *J-* and *A-* are thought to be polysaccharides, *C-* a protein. These findings, as well as Tyler's observations also have interest to students of fertilization. Discussion of these relationships is reserved for another chapter (see Volume III, Chapter 14).

Among the more tantalizing recent experiments are those reported recently by Inoue and Ishikawa. I say tantalizing because, despite their potential significance, data have not been presented in a form sufficiently instructive to enable a full evaluation. The research is described

because of its potential value, in full realization of the fact that the experiments have not been verified in other laboratories. The following summary was prepared by Edds (1958).

"Antisera were prepared in the rabbit to various regions of amphibian neurulae (*Triturus* and *Hynobius*), including the mesoderm (dorsal, intermediate and lateral), the notochord and the ectoderm. The differentiation of cells from these various regions when cultured either in saline solution or in 1:5 dilutions of normal serum was then compared with that of similar cells cultured in the various antisera. The expected normal differentiation according to cell type occurred in the control cultures. Antiserum to notochord prevented the differentiation of notochord cells in explants of the notochord rudiment. Comparable results were obtained using homologous sera on other rudiments. Heterologous sera permitted the normal differentiation of cells in some cases but also induced the appearance of quite atypical cell types. For example, in cultures of dorsal mesoderm treated with anti-ectoderm sera or with anti-ectoderm and anti-ventral mesoderm serum, smooth-muscle, mesenchyme, heart and pronephros were all encountered. These results are, of course, reminiscent of those obtained by Yamada in the occurrence of what he called ventralization of the explanted cells.

"Inoue and Ishikawa suggested that inactivating a particular antigen by coupling it with antibody permits a rechanneling of the development of the affected cells. The inference, of course, is that antigens are of critical importance for differentiation and that one may select among them favoring one, inhibiting another and thus modifying development. It is also conceivable, however, that particular cell types rather than antigens were selectively inactivated by the antibodies. In this regard, it would be important to establish whether selective cytolysis of certain cell types occurred in the cultures. Further, since ventralization but no dorsalization was encountered by these workers, it could be argued that the effect was non-specific. The importance of cautious interpretation in an investigation of this type is indicated by Tyler's observation that antisera prepared in the rabbit against 16-cell sea urchin eggs, inhibit cleavage at the 16-cell stage but only if proper concentrations of the antisera are employed. Otherwise, the stage at which cleavage is blocked is not specific."

Nace and Inoue (1957) believe that they have been able to distinguish two classes of antibodies in antisera prepared in rabbits against complex antigenic mixtures of entire *Rana pipiens* neurulae. They base their idea on the following evidence: (1) development *in vitro* of dorsal trunk tissues from stage 15 neurulae in the gamma globulin fraction of normal serum was essentially normal (cell types included neuroblasts,

striated fibroblasts, chorda, and others). (2) Tissues treated with unabsorbed antineurula gamma globulin absorbed with neurula pseudoglobulin survived, but showed only epidermal and mesenchymal differentiation. They suggest that the cytolytic action of antineurula sera can be referred to antigenic groups represented on pseudoglobulin and that other antigens may be more closely related to differentiation.

E. Immunological Models of Cell Surface Interactions

According to the hypotheses of Tyler (1942, 1947, 1955, 1957) and Weiss (1947, 1949, 1950, 1955), contiguous cell surfaces are normally held together, at least in part, by forces like those between antigens and their specific antibodies. The idea of mutually complementary surface molecules has been applied to the problems of selective reaggregation of dissociated cells (the means by which cells recognize each other and their appropriate environments, as Weiss aptly puts it) and specific adhesion. Striking examples of the specific interaction of cells are found in the work of Weiss and Andres (1952), who showed that melanoblasts of dissociated embryonic chick cell suspensions when injected into the early embryo intravascularly expressed their capacity for melanin synthesis only in regions favorable for melanogenesis, and Moscona (1957), who showed that dissociated cells from various organ rudiments of chick and mouse embryos when intermixed aggregate and combine to form composite, chimeric tissues. It is a commonplace experience that such reconstituted tissues differentiate histotypically; there can be no doubt that in the course of tissue reorganization following disaggregation many dissociated embryonic cells become grouped preferentially not according to generic origin, but according to tissue type.

Experiments to test the hypothesis of mutual complementarity of configurations of large molecules at cell surfaces have been few. One of the first attempts was that of Spiegel (1954a, b, 1955), who tried to gain insight into the forces that bind adjacent cells, using two species of sponge, *Microciona* sp. and *Cliona* sp. A companion study on embryonic amphibian cells appears less critical and will not be discussed. Antisera were made in rabbits to cell suspensions of each species and to a mixture of cells. Reaggregation of dissociated sponge cells (Wilson, 1910, 1932) was inhibited reversibly in homologous antiserum. In an antiserum against a mixture of cells, aggregates were observed which consisted of cells of both species. Spiegel's discussion (1954a, pages 143-146) should be read for a complete analysis of his argument concerning the extent to which his findings fit his working hypothesis. His experimental findings are convincing; it is clear that specific antibodies were engendered by the injection of sponge cells and that these antibodies have a selective

action. His inference that the responsible antigens are surface antigens is difficult to contradict, although direct evidence is lacking. However, one cannot conclude that the antigenic structures that combine with the antibodies are the same as those concerned in aggregation. This reservation applies also to the recent experiments of Gregg (1956), who has shown that in the slime mold *Dictyostelium* new antigens, presumably surface antigens, are detected at the time of aggregation of the ameboid stage to form a cell mass.

At least two major steps are required for a more convincing demonstration: (1) the use of a labeling technique to prove the surface nature of the antigens; and (2) extraction and characterization of the substances. Steinberg (1958; see also Curtis, 1957) has offered an alternative explanation for type-specific association; he proposes that the surface of tissue cells is ordered in the tangential direction, an order reflected in a latticework of ionized acidic groups which bind calcium or magnesium. Steinberg proposes that "the lattice spacings are peculiar to specific cell types at specific stages in their differentiation and wide enough to preclude the formation of slightly soluble calcium or magnesium salts involving two acidic groups on the same cell surface." He believes also that cell-to-cell binding occurs by the formation of calcium or magnesium salts between adjacent cell surfaces, the binding strength being dependent on the relative dimensions of the lattices. The intriguing possibilities in this scheme, supporting evidence, and a discussion of experiments which might test the hypothesis will be found in Steinberg's article.

F. Interactions of Embryonic Inductive Agents

A logical outcome of interest in immunoembryology is the use of immunological techniques in clarifying the nature of substances concerned in embryonic induction. Attempts have been made in two directions; neither approach has been especially fruitful. Vainio (1957) thought he might be able to demonstrate the necessity of "complementary interactions" of inductive and reacting tissue by interposing an antibody against the inductor, thus blocking it. In experiments with *Triturus gastrulae*, the inductive effect of guinea pig liver, kidney, and bone marrow tissue treated with ethanol was studied after pretreatment with normal rabbit sera or antisera against the homologous tissue. The reader will recall that, according to Toivonen (1938, 1950, 1953, 1954) and others, bone marrow and liver are primarily "mesodermal" and "neuralizing" agents, respectively, kidney being a composite of the two types. The inductive action of bone marrow was reduced drastically by treatment with homologous antiserum. The inductive capacity of liver

was modified by homologous antiserum (and to a lesser extent by normal serum); after exposure to the serum, it lost its "neutralizing" ability and showed instead "mesodermal" inductive ability. Kidney was not affected profoundly in either direction. The systems employed are so complex that they render interpretation of the findings difficult, to say the least. Vainio (1956) has reported also that antisera prepared against the blastopore of *Triturus* cross-react significantly with extracts of guinea pig bone marrow, a fact deemed important by those who believe in the chemical identity or similarity of "inductors" from different species.

Waddington (1957) and co-workers attempted to detect, by immunological methods, the passage of substances from an inductor into the reacting tissue. Grafts of *Triton alpestris* tissues were made into axolotl hosts, as were chick grafts made into duck hosts. Sections were treated with labeled antisera made against the donor species and absorbed with the host species. In neither case was it possible to detect the passage of donor combining groups into host cells. Employing a similar technique in combinations of *Triturus* and *Rana* tissues, Rounds and Flickinger (cited by Flickinger, 1957) obtained inconclusive evidence of a "passage of macromolecules" from mesoderm to ectoderm during induction. In neither of these cases has evidence been presented in full.

VI. OTHER RESPONSES OF DEVELOPING SYSTEMS TO IMPLANTS OF ADULT TISSUE

A. *Reactions Mediated by Immune Processes*

It is a fundamental premise of immunology that in order for a substance to be antigenic it must be foreign to the test animal. As we have seen, Burnet and Fenner (1949) believe that each cell acquires during its development a "self-marker" component, which prevents the organism from making an immune response to constituents of its own cells. Recent intensive research has clarified and extended our understanding of the "exceptions to the rule." If autologous or homologous tissues are modified slightly, they may stimulate an immune response. Moreover, although the alteration has been sufficiently great to make the inoculum antigenic, the antigens are still closely related to the proteins of the host, so closely related that the immune reaction which is elicited cannot distinguish the altered from the normal form. As a consequence, the normal host tissue is destroyed. For example, Morgan (1947) has shown that the injection of normal monkey nervous tissue combined with adjuvant into monkeys results in encephalomyelitis. In a thorough chapter entitled "Autoimmunization and disease," Boyd (1956) comments on our growing awareness that under some circumstances antigens of an individual may

call forth in this same individual the production of antibodies, or elicit a cellular response without artificial intervention. The process is called autoimmunization, and it is thought that a number of serious diseases may be caused in this way (e.g., acquired hemolytic anemia, rheumatic fever, rheumatoid arthritis, glomerulonephritis, sympathetic ophthalmia). Almost without exception the process which enhances the antigenicity of the patient's own tissues is obscure.

1. Induced Aspermatogenesis

Autoimmunization is an important basic problem, but our immediate concern is with the modification of development by the immunization of an animal with homologous tissues. Into this category fall the experiments of Freund *et al.* (1953) who were able to interfere with normal spermatogenesis in guinea pigs by injecting an emulsion containing guinea pig testis and adjuvants. Katsh (1958; see also Ebert, 1957; Katsh and Bishop, 1958) has shown that the sera of injected animals contain sperm-immobilizing, sperm-agglutinating, and complement-fixing antibodies. Yet there are reasons for believing that these circulating antibodies may not be instrumental in inducing aspermatogenesis. For example, it has not been possible to demonstrate a correlation between antibody content and aspermatogenic effectiveness. In addition, although the rabbit is highly efficient in producing circulating antibodies against sperm and testis, aspermatogenesis can be induced only with difficulty. Since it has been possible to show that the ileum of the sensitized guinea pig injected with homologous sperm or testis responds *in vitro* to a challenging dose of guinea pig sperm by contracting, Katsh has suggested that aspermatogenesis may be mediated by an allergic reaction of the anaphylactoid type. He has not yet performed what appears to the writer to be an essential experiment, that of attempting to demonstrate "adoptively acquired" aspermatogenesis (transfer by lymph cells). Among other findings of importance are the following: The antigens are not species-specific, and are only partially organ-specific, being shared by brain and testis. Within the testis, the effective substances are confined to the spermatogenic elements, as established by the failure of homogenates of testes deprived of spermatogenic tissue to elicit a reaction. Katsh suggests, but has not proved, that the antigenic stimulus is provided by a mixture of bacterial and testicular lipopolysaccharides.

2. Maternal Immunization and Embryonic Differentiation

Gluecksohn-Waelsch (1957) has studied the effect on differentiation of injecting emulsions of adult tissues into female mice prior to conception. When an extract of adult mouse brain, emulsified with adjuvant,

was injected into females of the DBA/1 strain, 8-9% of the offspring showed abnormalities of the nervous system. Microcephaly and abnormalities of closure of the neural folds were among the modifications observed. As controls, female sibs of brain-injected mothers were given heart-emulsions; no abnormalities of the nervous system were observed. It is also of interest to note that of 214 embryos in the latter group, only one had an abnormal heart. Although the evidence does not exclude the possibility that constituents of the brain extracts might be transmitted across the maternal-fetal barriers to exert their effect on differentiation directly, Gluecksohn-Waelsch has taken as her working hypothesis an alternative explanation. She believes it more likely that the effects on the nervous system result from the action of maternally produced antibodies on the developing nervous system. She cites preliminary results which indicate the presence in the serum of brain-injected mothers of precipitating antibodies. Again, the possibility that the reaction is cell-mediated has not been considered seriously. Gluecksohn-Waelsch points out the similarity in placentation between the mouse and the rabbit and stresses the convincing experiments of Brambell (1954) in support of her contention that antibodies may be transmitted to the embryo. Progress in the field of macromolecular transfer has been reviewed by Brambell (1954), Schechtman (1956), and Ebert (1958b).

B. Regulation of Organ Growth

The administration to embryos of constituents of adult tissues (in the form of grafts, cell suspensions, crude homogenates, or particulate fractions) has served as a useful tool in examining the concept (Weiss, 1947, 1949, 1950, 1955; Weiss and Kavanau, 1957) that the coordination and regulation of developmental processes presupposes the generation in each cell strain of paired compounds of complementary configuration and that differentiation and growth of a cell strain are governed by a balance of cell type-specific molecules. The writer has pointed out that, although several lines of evidence have established the working concept of selective chemical coordination among cells of identical type by direct exchange of type-specific compounds, research has only begun on the nature of the mechanisms involved (Ebert, 1956).

We have seen that the transplantation of adult cells to an adult organism of different genetic make-up commonly results in an immune reaction, leading to the death of the implant. When adult cells are transplanted to an embryo, however, the transplant survives throughout most or all of embryonic life and may persist after birth. The exposure of embryos in the latter part of the developmental period, and newborn animals, to adult cells frequently results in a state of tolerance, for,

when these animals are challenged with the homologous isoantigens in later life, they are incapable of producing an immune reaction. When adult cells are transplanted to the early embryo, well in advance of the critical period for the induction of tolerance, another consequence of fundamental importance is observed, namely a stimulation in the growth of the homologous tissue (Weiss, 1947), as exemplified by the stimulation of growth of the embryonic chick spleen following transplantation of a fragment of adult chicken spleen to the chorioallantoic membrane of the 9-day host embryo. Ebert, whose observations stemmed from the pioneering observations of Murphy (1916), Danchakoff (1918), Willier (1924), and others, has shown the reaction to be quantitatively organ-specific, i.e., of eleven donor tissues studied, only three are capable of evoking the response; these are, in order of decreasing effectiveness: spleen > thymus > liver. The reaction is class-specific but not species-specific—adult mammalian spleen being ineffective, whereas fragments of spleen of other avian species do stimulate the growth of the chick spleen although never to the same extent as does the homologous tissue. Earlier observations showed also that the age of the donor animal is a critical consideration. The effective factor or factors are absent from the embryonic spleen during the first two-thirds of the developmental period, making their appearance first at about 14 days of incubation. Hence grafts of embryonic spleen from donors younger than 14 days are ineffective. The effective factors are accumulated slowly, reaching the "adult" level by 6 weeks' post-hatching. In immunochemical studies, although Ebert was able to demonstrate the appearance of at least three specific splenic antigens at about the fourteenth day of incubation, he did not succeed in establishing a causal relationship between their appearance and the onset of specific stimulatory capacity (Ebert, 1951, 1952). (See Figure 5.) Of particular interest is the polysaccharide nature of several of the splenic antigens (Ebert, 1952) and the accumulation of polysaccharide in stimulated spleens (Ebert *et al.*, 1959).

The next major step in the analysis was based on the use of radioactively labeled transplants; these studies showed that when the proteins of the donor tissue, e.g., kidney or spleen, were radioactively tagged by injecting into the intact animal S^{35} -methionine in quantities sufficient to insure maximal labeling of the tissue proteins without radiation damage labeled materials from the graft were localized predominantly in the homologous organ of the host. The tentative hypothesis was advanced that macromolecular components released by the graft were localized selectively in the homologous organ; it was held likely that, although these macromolecules were not incorporated directly into the host proteins, the amino acids and peptides into which they were fragmented

before incorporation never entered the general amino acid pool. The results of this phase of the investigation, which included cytologic and autoradiographic studies and analyses of deoxyribonucleic acid in stimulated tissues, demonstrated that the stimulation did not result from a massive transfer of intact cells (Ebert, 1954). On the other hand, they did not rule out the transfer of a seed population of viable cells, a possibility made attractive by the rapid development of other studies demonstrating the successful transplantation of bone marrow and other sources of hematoblasts (Ebert, 1955). Several considerations argue against the importance of the transfer of viable cells in mediating the

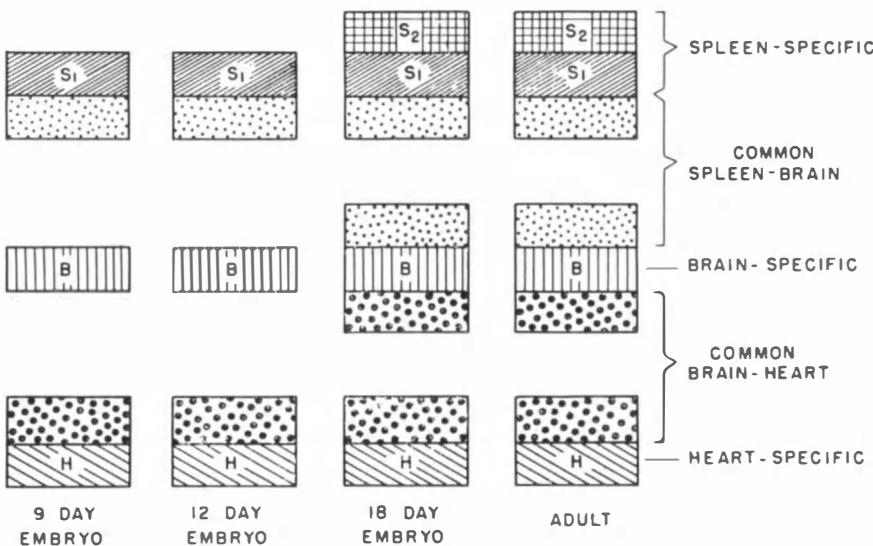


FIG. 5. Saline-soluble organ antigens of the chick embryo. Each of the spleen-specific groups, S₁ and S₂, was shown subsequently to consist of at least 3 antigens. Two antigens of the S₁ group are believed to be carbohydrates. (Ebert, 1951).

growth stimulation, for example: the observed difference in effectiveness between embryonic and adult grafts, whereas such a difference has not been observed in hematoblastic seeding (in fact, it has been said, the more potential hematoblasts, the better); the absence of plasma cells, characteristic of the adult, in the affected host tissues; and the experiments of others, e.g., Andres (1955), which indicate the stimulatory effectiveness of frozen, thawed triturated tissues. In fact, devitalization often has enhanced specificity and stimulatory effectiveness. Nevertheless, it cannot be stated that the requirement for intact cells was ruled out completely. Recent findings (Ebert, 1957; Simonsen, 1957) require a re-evaluation of the earlier conclusions.

1. Cytologic Analysis of the Development of the Normal and Stimulated Spleen

In the chick the primordium of the spleen is observed first at stage 23 of the Hamburger-Hamilton series (about 3½ days) as a dome of mesenchyme immediately dorsal to the region of the stomach and duodenum. Although splenic sinuses and the beginnings of the splenic vein are visible on the fourth day of incubation, little cellular differentiation is observed. This dome of generalized mesenchyme persists until stage 25 (4½ days), when differential growth and other morphogenetic processes result in a shifting of the spleen to the mesentery as an isolated mass. The first distinct cellular differentiation is observed at approximately stage 35 (8–9 days), at which time one can identify granuloblasts, recognizable by their thin basophilic cytoplasm, large clear nuclei, and large, acidophilic nucleoli. Granulopoiesis continues as the primary activity through stage 37 (the eleventh day), when the arterial supply is observed. All during this time mitotic activity continues unabated. The first signs of tissue organization are noted at stage 40 (14 days), and by the seventeenth day (stage 43) the follicles with central arteries and the granulocytic interfollicular pulp are relatively distinct, this condition becoming more and more pronounced in the hatching and post-hatching periods (DeLaney and Ebert, 1959; Ebert, 1957).

Is this sequence of events altered when the growth of the spleen is stimulated by a homologous graft to the chorioallantoic membrane? It is not altered significantly during the period from the tenth through the seventeenth days. The growth of the spleen results from a small increase in cell size and a pronounced increase in cell number. The pattern of cellular differentiation and morphogenesis in the experimental spleens during this period shows no more variation than does the series of normal spleens. The investigators find no evidence of massive cell transfer; moreover, if seed populations of cells are localized in the spleen, they must be incorporated into the normal architecture of the spleen; of course, one might expect, from studies by Moscona and others of dissociation and reassociation of embryonic tissues, that such could be the case. However, cell types characteristic of the adult, e.g., plasma cells, have not been observed. In a small, but important, proportion of rapidly growing stimulated spleens, a distinct, characteristic change is noted beginning on or after the eighteenth day of incubation. This significant departure from the normal has been observed most frequently in cases in which the transplanted spleen was implanted somewhat earlier than usual, i.e., on the seventh rather than the ninth day. The cellular nature of this phenomenon and the conditions resulting in its

expression lead DeLanney and Ebert to postulate that shortly before hatching the transplanted adult spleen produces an immune response directed against its host. This hypothesis will be treated more fully later.

2. Growth Stimulation in the Decapitated Chick

In a recent discussion of phases in embryonic development, Willier (1954) emphasized that one may characterize the main features peculiar to the different periods of development; he described the period in the chick between the tenth and thirteenth days of development as the time

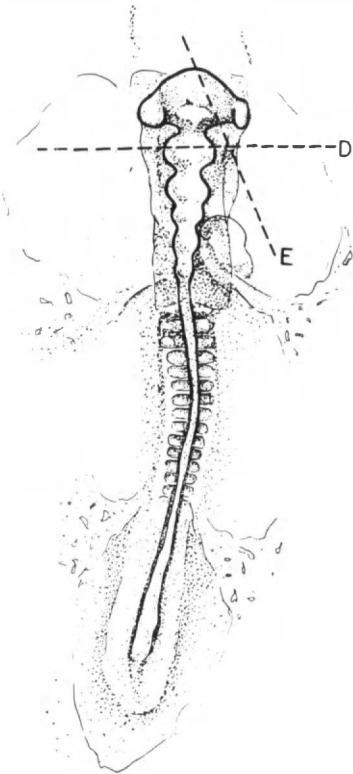


FIG. 6. Chick embryo at stage 11⁺ (38-40 hours), showing levels at which cuts are made for partial decapitation (D) and removal of an eye cup (E). (Ebert, 1957).

of functional integration, the period in which the humoral connections between organs via the blood-vascular system seem to make their appearance. It is during this period that the embryonic pituitary gland begins to function and exert influence on the so-called target organs; the evidence supporting this argument need not be reviewed here. It will

suffice to point out, first, that the ability of the embryonic spleen to act in promoting the growth of the homologous organ when transplanted arises first just at the close of this period of functional integration; and, second, that Vogel (1956) has demonstrated that when the embryo is hypophysectomized the synthesis of cholesterol and growth of the embryonic spleen are modified beginning at 12 to 14 days. It seemed of interest to determine whether the hypophysis was, indeed, an intermediary in the stimulation of splenic growth by splenic grafts.



FIG. 7. Partially decapitated and normal chick embryos at 17 days. (Ebert, 1957).

As a result of experiments carried out by Ebert and Coffman (Ebert *et al.*, 1959; Ebert, 1957), it can be stated that the reaction proceeds without modification in the absence of the hypophysis. The technique and results are illustrated in Figures 6 and 7. During the second day of development, the embryo is partially decapitated by severing the anterior brain region, including the hypophysis. Although the mortality resulting from this operation, originally described by Fugo (1940) and modified by Vogel and others, is high, many embryos survive within the shell, even beyond the expected time of hatching. The experimental cases are compared with several types of controls, including animals in which an eye cup is removed. The decapitated embryos were

permitted to develop until the ninth day, at which time a graft of adult spleen or another adult tissue was made to the chorioallantoic membrane. The embryos were reincubated and allowed to develop for from 5 to 8 additional days, when they were recovered for study. The results confirm fully the finding of Vogel that the spleen grows somewhat more rapidly in the decapitated chick than in the normal, and show clearly, moreover, that the spleen of the decapitated embryo responds equally as well as does the spleen of the normal embryo to a homologous transplant. These results are of interest in another connection: the enhancement reaction, in which the administration to mice of extracts of certain tumors results in increased tumor survival, appears to be mediated by antibodies, the production of which can be reduced by modifying the pituitary-adrenal axis. Although it is generally stated that the embryo is incapable of forming antibodies, it is known from the demonstration of acquired tolerance that the antibody-forming system can be altered during development; hence the possibility that the splenic growth phenomenon might be mediated by an immune mechanism, of a type hitherto undescribed, cannot be discounted. Although the experiments just reported do not provide convincing evidence of the dispensability of such an immune mechanism on the part of the embryo, they do provide additional indirect evidence that this may be the case.

3. Localization of Injected Subcellular Fractions

As cited above, it has been suggested on several occasions that not only are devitalized tissues effective in promoting homologous organ growth, but also that in some instances such devitalized tissues show an augmented specificity and stimulatory effect. For several years, Ebert has been engaged in extending his own study in an effort to define more precisely the nature of the substances transported from graft to host tissue and the manner in which they act. The approach has been a straightforward attempt, first, to determine whether radioactively labeled nonviable homogenates of organs injected into the embryo via the vascular system tend to localize differentially in the homologous organs. Having shown such to be the case, he proceeded to the second objective, that of determining whether all of the constituents of the homogenate behaved in the same manner or whether only certain fractions showed a predominant localization in the homologous organ. The experiments can be summarized briefly: adult tissues were labeled, as in earlier experiments, by the injection of methionine labeled with S^{35} . These organs were stored in the frozen state, after which they were homogenized, and a small amount of the homogenate was injected directly into the chorioallantoic vessels of the 9-day embryo. It should be noted

parenthetically that it was learned quite early that predominant localization does not result from injection by way of the yolk sac; hence it is necessary to inject intravascularly. Moreover, by the latter route the embryo would tolerate an injection of no more than 0.1 ml. of the homogenate; several factors, including the absence of a clotting mechanism, result in a relatively low frequency of successful operations. A recent modification of the procedure (Terasaki and Cannon, 1957) may lead to substantial improvement. The results of these experiments, in which labeled kidney and spleen homogenates were injected on either the ninth or tenth day of incubation with recovery after 24 hours show that the exposure of the embryo to a very small quantity of tissue homogenate for only 24 hours results in a differential incorporation of the label in the homologous organ. The differential is not of the same order as that obtained with living transplants, acting over several days, but it is consistent and reproducible. The next step was the fractionation of the homogenate following standard procedures for the separation of nuclear, mitochondrial, microsomal, and supernatant fractions. The findings show clearly a selective localization only of the microsomal and supernatant fractions, and not of the nuclear and mitochondrial fractions. The properties of the microsomal and supernatant fractions which determine the specificity or which are essential to the localization are non-dialyzable and are destroyed by heating at 80° C. for 5 minutes, and by ashing. It does not seem wise to speculate broadly on the significance of the findings. The possible implications are apparent to all those who are familiar with the importance of the interaction of the enzymes and ribonucleic acid of the supernatant fraction and the ribonucleoprotein of the microsomes in the incorporation of amino acids and synthesis of protein. Yet, it must be emphasized, the experiments have at least one major shortcoming. They deal only with one aspect of the problem, namely *predominant localization*. They do not necessarily contribute to an understanding of why the homologous organ is stimulated. The small quantities of subcellular fractions tolerated by the embryo *do not result* in a *significant growth stimulation*. It appears that a continuing supply of injected materials might be necessary in order to bridge the gap between the transplantation and injection experiments. This objective has not yet been achieved.

4. *Serial Transfer of Growth-promoting Activity*

The experiments to be described arose from an observation made in an attempt to determine the effect of grafts of adult chicken spleen made to the coelom of the host chick embryo at 3 days of incubation, a time at which the host spleen has not yet appeared. The developing

spleen of the host was stimulated greatly by the tenth day, and the question was raised as to whether such a stimulated 10-day embryonic spleen might now in itself be an effective donor. It will be recalled that normally the 10-day spleen is ineffective. This idea has been tested (Ebert, 1957; Ebert *et al.*, 1959). The experiment may be outlined briefly: A number of grafts of spleen from embryonic, juvenile, and adult donors were made to the coelom of the 4-day chick embryo, following a method described by Dossel (1954). The findings confirmed Ebert's earlier finding with respect to the importance of the age of the donor tissue. Utilizing the data obtained as a base line, grafts were made to a number of 4-day embryos, which were permitted to develop until the tenth day, when the host spleens were harvested and used as the donor tissue for a second set of grafts, and so on. The experiment was terminated after the fourth set of grafts was recovered. The growth-promoting activity is not diluted by serial passage, but is maintained relatively constant, considering the qualitative nature of the experimental approach. A simple explanation of these observations is the colonization of the host spleen by whole cells from the donor, cells capable of reproduction. As in the earlier studies employing the chorioallantoic technique, histological analysis of the host spleen denies the transfer of large populations of adult cells; in fact, the evidence is more clear, in view of the primitive architecture of the host spleen in the period covered by the experiment. Again, however, the transfer of a seed population of graft cells is not ruled out.

One must inquire: Why should adult cells, which do not proliferate in the graft, proliferate extensively in the splenic primordium, whereas embryonic spleen cells proliferate when grafted but do not stimulate the host spleen? Colonization does not offer a fully satisfactory explanation. The following possibilities must be considered: (1) a predominant localization in the homologous host tissue of specific particulate or macromolecular constituents of the host; the evidence shows that such a predominant localization is possible under certain experimental conditions, but does not prove that the mechanism applies in transplantation. But what of the serial transfer of growth-promoting activity? The most economical explanation is a transfer of viable cells, but the transfer of subcellular particles is not ruled out. Moreover, the evidence does not permit the exclusion of a combination of colonization and cellular transfer of materials. (2) The growth of the homologous organ is the result of a colonization by living adult cells which, as a result of constituents transferred from the *host*, now acquire new properties which enable them to grow rapidly. (3) Circulating constituents of the host stimulate the graft *in situ* to activities directed toward the host. Thus, we are led

to consider further the idea of continuous and reciprocal exchange between the tissues of the organism, both in the embryo and adult, and between the organism and an implanted tissue. This idea is not new; it is inherent in the theoretical discussions by Weiss (1947; Weiss and Kavanau, 1957) and Rose (1955) and is stated more explicitly in a stimulating paper by Medawar (1957a), stressing the evidence, cited earlier, of the destruction of a homograft in a tolerant mouse by an adaptive immune reaction. Further, striking evidence of the impact on the graft of host "antigens" is seen in the following section.

C. An Immune Reaction: "Graft-against-the-Host"

In studies of the destruction of kidney homografts, Dempster (1951, 1953) and Simonsen (1953) posed the question whether a graft containing cellular elements normally active in immune processes might react against its host, a possibility overlooked by others who were focusing attention on the host versus graft reactions (e.g., Ebert, 1951). The "graft-against-the-host reaction" may become an important link in our understanding of graft-host relationships as a result of a series of recent independent findings in which cells and tissues involved in immune processes have been implanted into hosts which are incapable of an immune reaction.

1. In Bone Marrow Transplantation to Irradiated Animals

The pioneering observations of Jacobson (reviewed, 1952), who showed that the lethal effects of whole body irradiation could be prevented by shielding the animal's spleen, led to a series of striking discoveries. Jacobson proposed several possible explanations for the spleen-shielding experiments, including: (1) humoral stimulation by a subcellular agent; (2) colonization of damaged areas by cells of the shielded tissue. Following the demonstration that several species of animals subjected to a lethal dose of irradiation are kept alive by intravenous injection of a normal bone marrow cell suspension after exposure (Lorenz *et al.*, 1951; reviewed by Congdon, 1957), it was established that blood-forming tissues are specific in causing recovery; hematopoietic cells are required. It has been shown convincingly that, when the hematopoietic tissues of the host have been destroyed, "reseeding" by blood-forming cells is a strict requirement for recovery. It is a question of some controversy whether recovery can be obtained with cell-free extracts of hematopoietic tissues (cf. Ellinger, 1957). Although the evidence strongly favors the involvement of cells, the possibility that cell-free extracts may be effective when the host's blood-forming tissues are not completely destroyed cannot be discounted altogether. The proof that

bone marrow and peripheral blood cells in the animals receiving bone marrow injections are derived from donor cells was obtained by a variety of techniques, applying the basic principle that homologous or heterologous donor cells can be identified by virtue of differences between them and the corresponding host cells. The immunochemical, cytochemical, and cytological methods which have been employed are summarized by Congdon (1957); perhaps the most elegant demonstration is that of Ford *et al.* (1956, 1957), who showed that dividing cells in bone marrow and lymph nodes of surviving host mice had chromosomes cytologically resembling the homologous donor cells which carried a chromosome marker.

However, when homologous or heterologous animals are employed as donors (rather than isologous animals, animals of the same inbred strain), recovery and survival for 30 days is followed by the death of many of the hosts (Congdon and Urso, 1957). The response is characteristic; the lymph nodes and to some extent the spleen go through a granulomatous reaction, resulting in fibrosis and fibrinoid necrosis. This delayed reaction is interpreted by many investigators as an immune reaction, in which the host tissues are affected by immune reactions of the donor cells. Makinodan (1957) believes this explanation to be an oversimplification, and, in fact, favors the view that death is due to a recovery of the radiosensitive antibody-producing cells of the host, which now react against the proliferating foreign bone marrow. Recognition is made also of the possibility that both reactions may occur. His conclusions are based on the following evidence: (1) In irradiated mice injected with rat bone marrow, rat serum proteins could not be detected; if present, the concentration was lower than 12 μg . of protein per milliliter of serum. Makinodan suggests this indicates that the graft is not producing antibodies against the host. His argument holds only for circulating antibody, but ordinarily in heterologous combinations circulating antibodies can be detected. (2) When normal isologous nucleated bone marrow cells were injected into irradiated mice along with rat red blood cells, circulating agglutinins against the rat cells were not detected, indicating inability of the bone marrow cells to produce antibody. (3) The time of the delayed bone marrow reaction and the severity of the reaction are dependent on the dosage of irradiation (faster recovery of immune reactions following lower dosage, leading to a more severe reaction). (4) If antibody-producing cells were derived from the transplant, then one should find an increase in death with an increase in bone marrow dose. The data refute this explanation. Makinodan has dealt largely with heterologous combinations in which the host reaction appears to predominate. In homologous combinations, the graft reaction

appears to take precedence (Loutit, 1957), suggesting that the ability of antibody-producing cells to recognize "foreignness" has quantitative implications (Makinodan, 1957).

2. In Newborn Mice

Billingham and Brent (1957b) have shown that acquired tolerance may be brought about by the intravenous injection of newborn mice with cell suspensions, the success of the operations being dependent to a considerable extent on the strains and combinations of mice employed. A number of mice of strain A made tolerant by injecting adult CBA spleen cells died within 2-3 months after birth. Characteristically, the lymphoid tissues of these animals are grossly aberrant: the lymph nodes are lacking, the spleens fibrotic and deficient in Malpighian corpuscles; even Peyer's patches are massing. Billingham and Brent interpret the findings as a graft-against-the-host reaction.

3. In the Chick Embryo and Larval Salamander

As described earlier, chorioallantoic grafts of adult spleen result in the growth of the homologous organ; when grafts are allowed to continue beyond the seventeenth day, however, a small but consistent fraction of the host spleens undergoes a striking degenerative change characterized by the destruction or modification of the vascular bed, which leads to a stasis in flow of granulocytes, their accumulation in cystic masses in the spleen, and eventual death (DeLaney and Ebert, 1959; Ebert, 1957). The cystic condition of the spleen occurs with a frequency approximating that of the death of chicks at or shortly after hatching. It is believed that the graft produces an immune reaction directed against the host's vascular bed, an argument supported by the findings of others (see Edds, 1958), who have found that in many instances anti-organ sera labeled with radioisotopes or fluorescent dyes are localized principally in the vascular bed of the target organ. Recently it has been observed that when intracoelomic grafts of adult chicken spleen are permitted to remain in position for at least 8-9 days, approximately 30% of them exert a profound effect on the vascular system, leading to death (Ebert *et al.*, 1959). The method and results are shown in Figures 8 and 9.

Simonsen (1957) has demonstrated that a splenic enlargement typically follows the intravenous injection of leucocytes or spleen cells of juvenile or adult chickens into the 18-day chick embryo. His conclusion that the splenic enlargement in this case is due to colonization by donor cells is almost inescapable. Simonsen has been able to propagate the cells through nine consecutive passages without significant

loss of activity. These two findings seem to be of a conclusive nature. Simonsen, however, holds that in theory there is no essential difference between the reaction of the spleen of the 9-day chick embryo to a graft of homologous spleen and the reaction of the spleen of the 18-day embryo to an intravenous injection of homologous cells. He states that the recipients have to be of an age in which tolerance of homologous cells can be induced and that donors must be old enough to form antibodies. Several striking differences in the results of the two approaches should be noted. He remarks (page 498), "Preliminary evidence in support of this hypothesis can be seen in experiments whereby 11-day or 18-day embryos of White Leghorn stock were inoculated intravenously

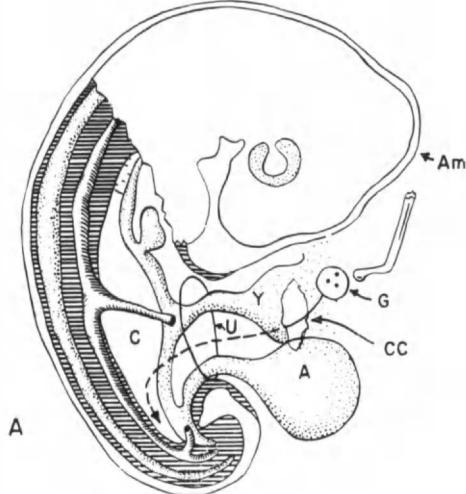


FIG. 8. Diagram of 4-day chick embryo showing insertion of intracelomic graft. (Ebert, 1957, after Dossel, 1954).

with spleen cells of 18-day Brown Leghorn embryos. The group injected on the eleventh embryonic day comprised 5 animals. They were all killed at 3 days after hatching and showed no anatomical changes. . . . The group injected on the eighteenth embryonic day totalled 31 animals, 14 of which were likewise killed at 3 or 4 days after hatching and found then perfectly normal." And (page 499), "Transplantation of embryonic chicken spleen cells give rise to no splenic enlargement in embryonic chicken hosts. . . ." These findings are not consistent with previous reports that implantation of splenic fragments from embryos 14 days and older produced a significant weight increase by the eighteenth embryonic day. Another inconsistency is found in Simonsen's observation that leucocytes of other avian species, pigeon and turkey, did not cause splenic enlarge-

ment in the chick. It is difficult to reconcile this observation with the graft-against-the-host hypothesis.

Finally, DeLanney's observations on the salamander may be described briefly. Grafts of adult salamander spleen have been made either into the coelom or into a pocket in the dorsal fin of the larval salamander. Although there are differences in the behavior of the transplanted tissues in the two sites, grafts in both sites lead not to growth stimulation but



FIG. 9. Hemorrhagic destruction of chick embryos at 12 and 14 days of incubation as a result of intracoelomic transplantation of adult chicken spleen. (Ebert, 1958).

to suppression of the host spleen. The analysis is not yet sufficiently advanced to permit further discussion, but it may be said that DeLanney has taken as his working hypothesis a "graft-against-the-host" immune mechanism.

4. Significance in Embryology and Immunology

The importance of these observations is beyond question. They afford a further demonstration of graft-host interaction. They clearly suggest the passage of host substances capable of an *antigenic* stimulus, thus lending further support to the idea of a continuous circulation of specific products in the embryo.

In this one respect, lymphoid tissues provide a highly sensitive technique for the detection of possible organ-specific molecules or particles.

However, in view of the ultimate destruction of the host tissues, the question must be raised whether the use of organs like spleen for further tests of organ-specific regulation of differentiation and growth is advisable. Although organ-specific agents other than those involved in the immune response may be involved, the overwhelming power of the immune mechanism may render the description of other systems difficult. In fact, Simonsen believes the "graft-against-the-host" reaction to be the single mechanism involved. The evidence does not permit such a conclusion. However, the experiments which should permit a definitive answer are clearly indicated. Well-designed experiments of any of the following types should prove illuminating: (1) a repetition of the experiments of Ebert and Simonsen using a highly inbred line of chickens, in which incompatibility should not be manifested; (2) positive evidence of a growth-stimulating effect of a noncellular organ preparation. It is clear that such organ preparations exhibit the properties enabling them to localize predominantly in the homologous tissue (Ebert, 1957; Walter *et al.*, 1956; Tumanishivili *et al.*, 1956). Evidence may be cited of the efficacy of frozen, thawed, and other nonviable preparations (Andres, 1955; Teir *et al.*, 1957); yet most of it falls short of being wholly convincing. Some danger attaches to criticisms of the hypothesis as a whole stemming from criticisms of the use of lymphoid tissues. Preferably, a tissue like heart or other organs lacking in cells of the antibody series are the experimental materials of choice. Improvements in the technique of intravascular injection into chick embryos (Terasaki and Cannon, 1957) make experiments in significant numbers more feasible. (3) Experiments employing filters which preclude cellular transfer from graft to host.

The graft-versus-host reaction has forced abandonment of plans for attempting to introduce lymphatic tissues into agammaglobulinemic patients in efforts to restore immunological capacity. In fact, Good (1957) has described in such patients effects similar, at least in his first approximation, to those observed in experimental animals.

VII. CONCLUDING REMARKS: PERSPECTIVES

Like its immediate predecessor, "immunogenetics," the compound, "immunoembryology," has been incorporated into the literature of the day, including these pages. It is a convenient term when used solely to designate the application of immunological tools to the solution of embryological problems. Indeed, there is only one embryology, upon which a combination of techniques from many scientific disciplines must be brought to bear. The word "immunoembryology" has one unfortunate connotation. It has nurtured the overemphasis on embryology at the level

of chemical description, a trend characterized earlier by studies enumerating enzymes in ontogeny (Brachet, 1957). For better or worse, the embryology of the past decade has been, at least in large part, descriptive rather than analytical. One of the more fruitful applications of immunological tools to descriptive problems may be found in following the synthesis of specific molecules under conditions in which the findings can be correlated with the results of experiments employing the methods of experimental morphology and information obtained from studying embryonic metabolism.

Nearly ten years ago, in a penetrating article entitled "Immunological studies in embryology and genetics," Irwin (1949) looked ahead to a combination of these disciplines with biochemistry in fashioning a concerted attack on problems of development. One of the major contributions of this chapter has been the demonstration that Irwin's prediction has not yet been realized. Although progress has been made in the genetics of transplantable tumors, few laboratories have had the facilities, know-how, and, it would appear, the desire to engage in the kind of long-term study that is urgently demanded if we are to understand the relationships of genes, isoantigens, and immune processes in the transplantation of normal tissues. Although the information available is fragmentary, the inauguration of research directed at the chemical nature of transplantation antigens is encouraging, for, if Medawar's arguments prove correct, even in part, biochemistry, genetics, and immunology converge at a common meeting ground, DNA-protein.

The gap between the immunochemical studies of tissue-specific proteins and the other disciplines is far greater. Ideas from those studying the biochemistry of cell particulates, microbial genetics, and the use of clonal lines of tissue cells have yet to make their full impact on investigators working with immunochemical tools. To a considerable degree the converse is also true. For example, specific antibodies might be of unusual value in the study of nuclear, even chromosomal, differentiation. They should furnish a precise, sensitive technique for following the synthesis of a specific protein *in vitro* by a nuclear or microsomal preparation. Are the enzymes or the structural constituents of the mitochondria (if, indeed one can distinguish *between* enzymatic and structural proteins) tissue-specific? Are there clear antigenic differences in mitochondria and microsomes? It does not tax the imagination too greatly to propose that with the use of specific antibodies the embryologist may be able to perform "defect" experiments at the intracellular level. The direct immunochemical attack on the nucleus has been delayed not only by the poor antigenic properties of the nucleic acids and nucleoproteins, but also by difficulties in measuring their interactions with antibodies,

using conventional methods. Newer methods of preparing nucleic acids, and technical modifications, including the use of labeled antibodies, promise to overcome these obstacles (Blix *et al.*, 1954; Phillips, *et al.*, 1957). In dealing with microsomes and mitochondria, their complexity is the paramount problem. It may be necessary to isolate some of the components of these particulates before specific antibodies can be obtained (Albright, 1956; Weiler, 1956). However, even complex anti-sera may be valuable tools. In a study of unusual interest, Weiler prepared antibodies against microsomes and mitochondria of normal rat liver. Although the antisera were powerful, complete cross reactivity was the rule. Hepatomata induced by feeding dimethylaminoazobenzol failed to react with antibodies against the normal particulates. The fluorescent antibody technique was employed with much success: normal liver reacted with the labeled antibodies; hepatoma failed to react indicating a loss of specific reactive groups. More striking, however, was the observation that, when experimental animals were sacrificed at intervals shorter than necessary to produce the tumor, small groups of non-reactive cells were observed consistently among the normal cells, a clear instance of a change in immunological reactivity before overt malignancy.

Weiler's provocative experiment, like most of the experiments discussed in the foregoing pages, must serve only as a stimulus to further analysis. This chapter, then, should be regarded not as a summary but as a preface to more intensive research.

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CHAPTER 15

Effects of Radiations on Cells*

By MAURICE ERRERA

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INTRODUCTION

The harmful effects of radiations on cells and organisms were discovered almost at the same time as X-rays and radioactivity, at the end of last century: many of the early investigators died or suffered as victims of this early work. Despite the great amount of effort and a reasonably good understanding of the fundamental physical processes involved in the absorption of radiant energy, we do not yet understand how radiation affects cells: we have quite a good descriptive picture of various effects of radiation, but the fundamental mechanisms are not known. The reason is that most radiations affect in a random manner the intricate mechanisms of the cells, which therefore need to be understood primarily.

We will attempt to give, in this section, an over-all view of the chemical and biological events as we at present know them.

II. ACTION OF RADIATIONS AT THE MOLECULAR LEVEL

A. *Physical Events*

Radiations have a certain energy which may be absorbed by the various cellular constituents and alter their chemical properties; some of these alterations are presumed to initiate a chain of biochemical reactions which will result in cellular damage. Detailed considerations of the

physical events are to be found in papers by Fano (1954), Franck and Platzman (1954), and in the books by Lea (1946) and Bacq and Alexander (1955).

1. Ionization

If the energy is great enough, as in the case of *ionizing radiations*, it will be sufficient to expel an external electron (e^-) from the atom (A) which happens to absorb the energy, the atom thus becoming positively charged: $A \xrightarrow{h\nu} A^+ + e^-$. This electron may then collide with a new atom $A + e^- = A^-$, and thus an *ion pair* is formed. Usually as in the case of X- or γ -rays, the expelled electron (β -ray) has sufficient energy and can induce further ionization in the atoms it hits; many ionizations may then occur as a result of the absorption of one quantum of X- or γ -“light.” The “hole” left by the expelled electron can become occupied by an adjacent electron of the molecule, therefore the ionized atom is not necessarily the one where the process was initiated. The *corpuscular type of radiation*, such as α -rays (helium nuclei) or protons (hydrogen nuclei) or other charged particles, expels electrons from atoms the particles collide with; the uncharged neutron penetrates the electron cloud around the atom and transmutes its nucleus with the emission of an ionizing proton. The feature which distinguishes these different types of ionizing radiations is their *linear energy transfer* (LET): this is the amount of energy dissipated in a given distance. Helium nuclei (α -particles) are not penetrating and are completely absorbed after traveling a few microns in water or organic material: many ionizations occur in a small distance. On the contrary, if the radiation is highly penetrating, like γ -rays or fast electrons, the dissipation of energy takes place over great distances. In terms of ionization, α -particles give a very high ion density and γ -rays give in contrast a very low density. Neutrons and X-rays come in between (the neutron producing the highest ion density of the two), and the β -particles emitted by most radioactive elements (P^{32} , C^{14} , Sr^{90}) are not penetrating and have a relatively high LET, but when accelerated in an electric field, the energy of electrons may be enormous and the LET will become low.

As the cellular constituents are not distributed at random but are organized into more or less important cellular structures, the relative biological effectiveness (RBE = efficiency of radiation/efficiency of γ -rays) of each type of radiation depends on its LET. It has been observed that the more densely ionizing radiations are more efficient, per ion pair formed, for many biological effects. Many references can be found in a paper by Zirkle (1954). One can hope, by comparing the effectiveness of radiations giving different ion densities, to learn some-

thing about the size of the cellular structures in which energy must be absorbed in order to produce a specific effect.

2. *Excitation*

The energy of the radiation may not be sufficient to remove an electron from an atom or molecule, but it may still be sufficient to excite it, that is, to change its usual gravitational position around the atom and thus render the molecule it belongs to more reactive during this short period of excitation. If the absorbed energy exceeds that of a chemical bond of the molecule, it can break it open; if no reaction occurs, the energy is lost as heat or light.

Excitation is the usual mode of energy transfer in the case of *ultra-violet* (UV) or *visible radiation*; it may also occur in the case of ionizing radiation, but to what extent it plays a role in radiobiology is not well known. A detailed consideration on the processes of excitation can be found in the paper by Franck and Platzman (1954).

Infrared light (IR) can also be absorbed by cells, but in this case all the energy is dissipated as heat, and its effects are very unspecific. The absorption of the low-energy radiations (UV, visible, IR) takes place only in special atoms of the molecules constituting the chromophore groups, which determine the absorption spectrum of the compound.

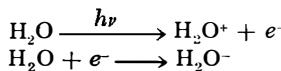
The absorption spectrum of the various cellular constituents will determine whether or not a cell is susceptible to a given wavelength of radiation: many cells are almost transparent to visible light, which will therefore not affect them much (we are of course excluding all the photosynthesizing organisms); the use of low-energy radiations, especially UV, is important because these are absorbed much more specifically than the ionizing radiations. The establishment of an *action spectrum* (relating the efficiency of a radiation to its wavelength) may give indicative leads as to which are the cellular constituents *absorbing* the energy; these may not necessarily be the ones that are altered, because it is established that energy may be transferred through a molecule several atoms away from its "point" of absorption and break a chemical bond after having traveled over several more resistant bonds.

B. *Chemical Events*

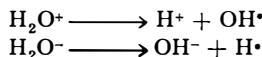
1. *Action of Ionizing Radiations on Water Solutions: Indirect Effects*

a. *Water.* [See for a full discussion the papers by Lefort (1955), by Dale (1955), or by Dainton (1956).]

The loss of an electron by a water molecule, as a result of ionization, will lead to the following reactions:



As neither of these water ions are stable, they dissociate almost immediately:



The OH^\bullet (hydroxyl radical) and H^\bullet (hydrogen atom), having an incomplete electronic equipment, have a high chemical reactivity, and they can either recombine to form a new water molecule or react with whatever molecule they happen to encounter. The life of these radicals may be as short as 10^{-6} seconds in dilute solution.

As the ion density will vary with the type of radiation used, the concentration of OH^\bullet radicals or H^\bullet atoms along the radiation track may vary considerably: in the case of α -rays, the concentration of OH^\bullet can reach molarity, whereas in the case of fast electrons (60 kilo-electron volts) it may be only 10^4 times less. Hydrogen peroxide H_2O_2 is formed with higher density radiation as a result of the reaction of two OH^\bullet , even if the solution has been completely rid of oxygen.

When the water is in equilibrium with air, the H^\bullet atom is thought to react with oxygen (O_2) and give rise to $\text{O}_2\text{H}^\bullet$ (perhydroxyl radical).

b. The solutes present in the water can react with H^\bullet , OH^\bullet , and $\text{O}_2\text{H}^\bullet$ and become chemically altered. As most reactions appear to be oxidative (Bacq and Alexander, 1955, see p. 131; Dainton, 1956), the existence of an independent H^\bullet atom has been questioned by some; a substance only becomes reduced when it has a very high redox potential (greater than 0.9–1.0 for effects of X-rays in the absence of oxygen (Lefort, 1955, see p. 162)).

Many types of oxidative reactions have been described (Lefort, 1955: (1) $\text{Fe}^{++} \longrightarrow \text{Fe}^{+++}$, the electron being transferred to OH^\bullet ; (2) An H^\bullet atom can be removed as in the oxidation of acetic to succinic acid, $2\text{CH}_3 - \text{COOH} \longrightarrow 2\text{C}^\bullet\text{H}_2 - \text{COOH} \longrightarrow \text{COOH} - \text{CH}_2 - \text{CH}_2 - \text{COOH}$, and longer chains still have been observed as reaction products (Stein and Weiss, 1951). (3) An H^\bullet atom can be substituted for an OH^\bullet as in the oxidation of benzene to phenol.

In a similar fashion, small organic molecules like acids, aldehydes, or alcohols can be oxidized (Lefort, 1955; Dale, 1955; Bacq and Alexander, 1955, see p. 46); decarboxylation or oxidative deamination (Lefort, 1955) may take place, and sulfhydryl groups may become linked in S–S bonds (Barron and Johnson, 1956). Much rarer are the *reducing reactions* observed: however, a certain number of redox indicators have been reversibly bleached in the absence of oxygen, and coenzyme I

(diphosphopyridine nucleotide) can be reduced probably to an unnatural dimer, but only in the presence of an H donor like ethanol, which becomes oxidized to acetaldehyde (Swallow, 1956). Oxidized hemoglobin (Fe^{+++}) also can be reduced to the Fe^{++} form by X-rays. In the case of complex molecules such as enzymes and other proteins, nucleic acids, lipids, etc., much less is known about the specific reactions taking place, but in many instances the loss of typical biological or physical properties has been observed: loss of asymmetry of fibrous proteins (Bacq and Alexander, 1955, see p. 171), hyaluronic acid (Schoenberd *et al.*, 1949), or deoxyribonucleic acid (DNA) (Errera, 1957, see p. 107; Davison *et al.*, 1954). However, it has been demonstrated that the most sensitive chemical group of enzymes is the *SH group*, which can be reversibly oxidized (after small doses) to $\text{S}-\text{S}$, either when adjacent groups of the same molecule or groups from different molecules (cross-linking) react together (Barron, 1954). Other types of *cross-linking* (formation of C to C linkage) has been observed in irradiated polyvinyl alcohol (Alexander and Charlesby, 1957). There is reasonably good evidence that such cross-linking also occurs in the case of protein (Alexander *et al.*, 1956) and of DNA (Kaplan, 1955).

2. Direct Effects of Ionizing Radiations

Direct effects occur when ionization *takes place in the molecule or cellular structure* under study: it is probable that the energy absorbed in one part of the molecule will be transferred over the whole structure and ionization or excitation phenomena will not necessarily occur at the point of first interaction (Franck and Platzman, 1954). The existence of unpaired electrons has been demonstrated in irradiated proteins in paramagnetic resonance experiments: in the absence of water, free radicals can still be found after weeks or even months; in water solution and probably in living cells, the life of the radicals is much shorter (probably not more than a few minutes (Gordy, 1955; Fairbanks, Jr., 1957). In the dry state, irradiation can also be the cause of cross-linking reactions or breakages of chemical bonds, and excitation processes are believed to exist (Charlesby, 1953; Alexander *et al.*, 1956).

3. Effects of Ultraviolet and Visible Light

It is not our purpose to go into many details on photochemistry, because the detailed reactions taking place in the cells are not known (except to some extent in the case of photosynthesis, which requires a special treatment). In this case, the energy is capable only of exciting the atoms of the chemical groups which absorb it (the chromophore groups like purines and pyrimidines for $253.7 \text{ m}\mu$ UV). The energy

absorbed may, however, be transferred a certain distance from the chromophore group to other chemical groups of the same molecule or even of adjacent molecules: this is the basis for explaining *photosensitized reactions*; for instance, tobacco mosaic virus can be inactivated by visible light in the presence of acriflavine, with which it combines (Oster and McLaren, 1950). [Many details and references can be found in papers on photochemistry by McLaren (1949), Errera (1953), and Livingston (1955) or in the book by Blum (1941).]

In general, it can be stated that free radicals can be formed by UV; however, these have chiefly been identified during experiments in the gaseous phase. Cellular effects of UV could be due to the breakage of single bonds like hydrogen bonds, peptide and amide bonds, disulfide bonds; the organic radicals presumably formed may reunite to reconstitute the initial molecule, but new combinations and cross-linking could very well occur. When proteins are irradiated, for instance, one does not find the increase in amino or carboxyl groups to be expected if peptide bonds were only broken; and disulfide bonds have been shown to form upon irradiation of a sulphydryl compound.

4. Effects of Oxygen

It has been observed that oxidative reactions due to ionizing radiations occur much faster in the presence of oxygen, and furthermore some reactions appear to be possible only in its presence, as in the case of the oxidative deamination of alanine to pyruvate (Johnson *et al.*, 1951; Lefort, 1955). Even with X- and γ -rays, H_2O_2 can be detected in appreciable amounts and the radical O_2H^\bullet (perhydroxyl) is also believed to be produced as a result of the reduction of molecular O_2 with the H^\bullet atom (Lefort, 1955). Other explanations are also possible. Oxygen could react with the irradiated molecule and make it more sensitive to irradiation. In oxygen, many effects, including biological effects, are often increased by a factor of 3 to 5. In the case of the more densely ionizing radiations, which produce this type of radical even in anoxic conditions, no appreciable oxygen effect is found (Gray, 1954). The study of an oxygen effect in macromolecules has revealed many interesting points:

(a) The inactivation of SH enzymes is very dependent on oxygen tension, but the non-SH enzymes investigated are not inactivated appreciably faster in oxygen (Bacq and Alexander, 1955, see p. 178).

(b) Bacteriophage (Alper, 1954) or DNA are inactivated by X-rays independently of the O_2 tension (Ephrussi-Taylor and Latarjet, 1955; Davison *et al.*, 1954; Scholes *et al.*, 1956).

(c) Even when irradiated in the dry state, with X- and γ -rays, the inactivation of trypsin is oxygen-dependent, and this could perhaps be

explained by postulating a direct attack of the protein by O_2^- ions (Alexander, 1957a).

During UV or *visible irradiation* the presence of oxygen does not appear to be necessary when the absorbing molecule is itself denatured—but oxygen is definitely necessary in photosensitized reactions (see Blum, 1941 and Errera, 1953).

5. Aftereffects

It has often been observed that the molecules under study continue to undergo alteration *after* the exposure to irradiation has ceased. This is the case for the oxidation of tyrosine (Loiseleur and Sauvage, 1953) or the inactivation of some proteins (Anderson, 1954), nucleic acids (Davison *et al.*, 1954), bacteriophage (Alper, 1954), other nucleoproteins (Errera, 1947), or hyaluronic acid (Schoenberd *et al.*, 1950). Pneumococcus DNA when tested for transforming activity does not appear to show any aftereffect after irradiation in 1% yeast extract (Ephrussi-Taylor and Latarjet, 1955).

The aftereffect seems to be the result of a primary process taking place chiefly in the presence of dissolved oxygen, but which may not be sufficient in itself to inactivate the molecule. It could, although not necessarily, be due to the H_2O_2 (Alper, 1955) or to the organic hydroperoxides formed in the solution (Latarjet, 1956).

The case of deoxyribonucleic acid has been the most studied: many mechanisms—such as the oxidative formation of labile phosphate links with the sugar rings of the macromolecular chain or the slow unwinding of the double helical structure of deoxyribonucleic acid—have been postulated (Scholes and Weiss, 1956; Conway, 1954). Although H_2O_2 formed in the solution does not appear to be necessary in the case of deoxyribonucleic acid (Conway, 1954), it may have a very pronounced effect on bacteriophage S_{13} which becomes more sensitive to this agent after irradiation; S_{13} also becomes more sensitive to some reducing agents, e.g., ascorbic acid (Alper, 1955). The aftereffect does not appear to exist after irradiation in the dry state: this is the case for DNA (Alexander, 1957b). It does appear to be the consequence of an indirect effect of irradiation: one will not be able to estimate its contribution in irradiated organisms before one knows more about direct and indirect actions *in vivo*.

In the case of UV the possibility of the occurrence of aftereffects has been envisaged (Errera, 1953), but very little is actually known

6. Protection

a. *Water solutions.* In a radiation-induced reaction *taking place in water*, the fact that the major part of the effect is of indirect origin has

fundamental as well as important practical consequences. Any other solute reacting with the free radicals formed in water will render them less available to the substance under study and protect it, possibly by a competitive mechanism (Dale, 1942, 1955). Many organic or inorganic compounds are efficient *in vitro*—amongst these: thiourea, aniline, phenol, cysteamine and its oxidation derivative cystamine (Bacq and Alexander, 1955), and S-aminoethylisothiuronium·Br·HBr (AET) (Hollaender and Stapleton, 1956). Substances capable of reacting with essential groups of enzymes may, when present during irradiation, protect the group; removal of the agent after irradiation uncovers an unaltered group: this has been shown to be the mechanism in the case of SH enzymes protected *in vitro* by some SH reagents (Barron *et al.*, 1949; Barron, 1954). Many enzymes are also protected by their substrate (Doherty, 1952), their coenzyme, or by competitive inhibitors (Dale, 1940, 1942; Gray, 1946), probably also because the biologically active sites of the enzyme molecules are masked by the protector. It has been suggested, furthermore, that the SH group of cysteamine can protect SH groups of enzymes by becoming linked to it reversibly through an S—S bridge. Similar dissociable complexes can be postulated in other instances (Eldjarn and Pihl, 1956; Littman *et al.*, 1957). Prevention of diffusion from one another of organic radicals originating from irradiated molecules and thus favoring their rejoining, is probably also one of the mechanisms of radioprotection; this can be achieved by freezing at low temperatures (see Errera, 1957). Reduction of the oxygen tension will inhibit those effects of radiation, which are increased in oxygen. There are many ways of producing anoxic conditions, including the use of chemicals such as hydrosulfite, cysteine, or cysteamine (Bacq and Alexander, 1955; Gray, 1956) and of more usual respiratory inhibitors. *In vivo* many reducing organic substrates which consume the cellular oxygen by way of the normal respiratory processes probably also produce anoxic conditions (Hollaender and Stapleton, 1953; Hollaender, 1954). It is difficult at present to know the exact contribution of these mechanisms for certain protecting agents like cysteine or cysteamine; it is probable that it varies according to the type of substrate, the presence of other solutes and the concentration of the different substances present.

b. In the dry state. However, it is possible to protect molecules in the dry state. It has been shown that thiourea, aniline, phenol, and cysteamine when incorporated into a synthetic polymethacrylate, protect it during irradiation even when in the dry state (Alexander, 1957b). In the solid state, no water radicals being present, the protective action is probably due to a transfer of energy through the polymer molecules to the radioprotector. This would be the mechanism of protection in

the case of a *direct* action of radiation on the molecule. The ribonucleic acid of tobacco mosaic virus also appears to be protected against direct effects by cysteine (Ginoza and Norman, 1957).

c. *Ultraviolet irradiation.* In the case of UV, the role of protecting agent is not clear, although several examples are known, including the case of protecting aromatic compounds against photooxidation (see references in Errera, 1953).

7. Dose-Effect Relationship: The Target Theory

The concept of a "target" was introduced more than thirty years ago (Blau and Altenburger, 1923; Crowther, 1924) to explain the effects of ionizing radiation. A target is a sensitive cellular structure whose inactivation by one or several ionizations (hits) would initiate the observed biological effect. If ionizations take place exclusively in the sensitive structure (direct effect) or in its immediate vicinity, the probability of a target being hit depends on its size; and, knowing the proportion of cells affected for each dose, one can determine the size of the target responsible for the cellular function under observation.

When the dose-effect relationship is a simple one, such as the exponential curve found when one plots the log of the number of survivors (in this case the observed function is survival) against dose, the treatment is simple and it can be concluded that one primary event inside the target (or in its immediate neighborhood) is sufficient to inactivate it. If, on the contrary, several ionizations are needed, the response becomes manifest only after a certain dose has accumulated and the curve becomes sigmoid. The number of targets can then be calculated if there are only a few of them. When they are numerous as in the case of kappa particles in paramecia, their number can be estimated (Nanney, 1957).

The best present evidence in support of this concept lies in the increasing number of targets one is able to "observe" when multiplying bacteriophages are irradiated in infected bacteria (Luria and Latarjet, 1947; Latarjet, 1953), or when the response curves of haploid or diploid microorganisms are compared (Latarjet and Ephrussi, 1949).

Since the discovery of indirect effects, such a simple treatment has raised much opposition: so that it may be applicable, one must control or suppress completely all indirect mechanisms or take the diffusion of radicals into consideration. Many attempts have been made to distinguish between direct and indirect mechanisms or to determine their relative importance.

8. Distinction between Direct and Indirect Effects

a. *Dilution effect.* It is possible to distinguish between direct and indirect effects in a simple system by increasing the concentration of

the molecules under study. In the case of indirect effects, the yield of altered solute molecules decreases with increasing concentration of the solute (Dale, 1956; Bacq and Alexander, 1955). It has thus been calculated that in a 1% solution of the enzyme carboxypeptidase, more than 90% of the inactivation is indirect; in a 20% solution, only 60% of the effect is indirect (Dale, 1956).

b. Desiccation. One can also obtain information on the relative importance of direct and indirect mechanisms by comparing the yield of a radiation reaction on the same substrate after desiccation, in a completely protected solution and in the absence of any protector, although it is probable that one will not be able to protect exclusively for indirect effects (Lea, 1946). Hutchinson (1956) compared the effect of α -particles, X- and γ -rays on dried or hydrated yeasts and came to the conclusion that 50% of the inactivation of alcoholic dehydrogenase, invertase, and coenzyme A take place as a result of *direct* inactivation. Small viruses have been studied in highly protected media quite successfully and, in a certain number of instances, their size has been calculated from the exponential inactivation curves and the values obtained are in good agreement with those given by other methods, showing that under well-defined conditions the concept of target can lead to very useful applications (Bonet-Maury, 1955).

c. Temperature coefficient. One can expect, if diffusible free radicals play a part in the indirect effect, that its contribution could be considerably reduced by freezing the solution (Pollard *et al.*, 1955). This is, in fact, what happens. However, irradiation of dry substances at different temperatures shows that the direct effect of ionizing radiations also varies with temperature (Setlow and Doyle, 1953); this makes the use of the temperature coefficient more hazardous, but nevertheless useful. It is possible that, at low temperature, the chemical bonds are more resistant or tend to reconstitute more easily or that the transfer of energy inside the molecule is also temperature-dependent.

III. BIOCHEMICAL EFFECTS

A. Effects on Cellular Constituents

The search for structural damage to important cellular constituents can be made by assaying "immediately," i.e., (as soon as possible) after irradiation, the biological or the physicochemical properties of various cell components whose integrity appears to be important for the economy of the cell. Small molecules like coenzymes or hormones do not appear to be appreciably altered as an immediate result of lethal irradiation (see Errera, 1957), and even coenzyme A concentration is not altered in irradiated tissues (Bacq and Alexander, 1955); when the concentration of these small molecules is altered, it has always been

shown to be the result of the inhibition of synthetic mechanisms or of change in cell wall permeability.

Lipids from UV-irradiated mitochondria are oxidized (Bernheim *et al.*, 1956), and it is possible that small amounts of stable peroxides, perhaps of lipid origin, are formed during X-irradiation of mammals (Horgan and Philpot, 1955); much more, however, is known of the fate of proteins and nucleic acids.

Cellular *proteins* may be studied from several points of view:

1. *SH groups.* The possible oxidation of over-all SH group of irradiated cells has been the object of many studies, and many negative results have been described (see Errera, 1957). However, X-irradiated yeast and the spleen of irradiated mice have been shown to contain a smaller number of SH groups (Bacq and Alexander, 1955; Meissel, 1956); it is, however, very difficult to decide, in these cases, if this results from an immediate or a secondary effect.

2. *Antigenic properties* of proteins whenever assayed have always been found not to be altered for dosages sufficient to kill microorganisms or viruses (see Errera, 1957).

3. *Enzymatic properties.* Many enzymes have been assayed at various times after irradiation, either with cytochemical or with biochemical techniques, and, in a few cases, these were altered soon after irradiation which usually, however, had to be rather violent. Some of these enzymes may be presumed to be associated with "mitochondria," as in the case of rat embryonic lactic dehydrogenase (Holmes, 1935), embryonic grasshopper cytochrome oxidase (Tahmisian and Adamson, 1950), and yeast indophenol oxidase, which are decreased (Bautz, 1954), and adenosine-triphosphatase, which is increased (van Bekkum, 1955), in spleen a very short time after irradiation. Mouse liver catalase (a fraction of which is bound in mitochondria) also decreases within a few minutes (Feinstein *et al.*, 1950) and yeast ribonuclease (Meissel, 1956), yeast hexokinase (Aldous and Stewart, 1952), muscle phosphorylase (Sissakian, 1955) are rapidly inhibited after high X-irradiation.

Some of the enzymes which might be bound to *lysosomes*, as they are in mouse liver, are on the contrary increased after X-irradiation, as they are also when the homogenates are aged; deoxyribonuclease II (acid pH) from spleen and thymus (Goutier-Pirotte, 1956; Kowlessar *et al.*, 1954, 1955) and liver cathepsin (Feinstein and Ballin, 1953) are among these. However, liver ribonuclease increases only at a later stage (Douglass *et al.*, 1953; Roth *et al.*, 1953).

An enzyme known often to be associated with the cell nucleus, like liver arginase, is not affected, but nuclear alkaline phosphatase is affected at a later stage (see Errera, 1957); nothing has yet been reported about the coenzyme-forming enzymes also known to be of nuclear origin.

It is, however, not certain that all these effects are the result of an immediate alteration of the enzymes. In some instances, the interference of a secondary mechanism has been proved, as in the case of liver catalase, where an inhibitor is probably liberated in the blood serum (Mori *et al.*, 1951), or in the case of cathepsin, where a serum inhibitor could on the contrary be destroyed (Ballin and Feinstein, 1952).

Far fewer experiments have been performed with the aid of UV *radiation*, but some enzymes of yeast, *Chlorella*, and mouse ascites cells are inhibited by high UV dosages (see Errera, 1957).

The question of the radiosensitivity of nucleic acids *in vivo* seems still to be controversial, although evidence suggests that nucleoprotein complexes are dissociated in many tissues as a result of moderate irradiation (see Davison *et al.*, 1954; Errera, 1957). It has been calculated, on the basis of *in vitro* measurements, that a dosage of 100 r could damage 1-5 molecules of DNA in a mammalian cell (Butler, 1956), and this figure does not disagree with the data indicating the stability of the transforming activity of *steptococcus* DNA when irradiated *in vivo* (Drew, 1955)—the dose levels used in these experiments not being sufficient to cause any significant inactivation (Guild, 1957). Thus, a very much lower dosage than 1 r would be theoretically sufficient to alter permanently some genetic constituent in a single cell. In this case, not all cells would have one of their DNA molecules affected and we would have to understand the behavior of an altered cell among normal ones to know what effects could be expected at such dosages.

There is no reason to believe that ribonucleoproteins are not as radiosensitive as the deoxyribonucleoproteins, but one knows very little about these and alterations of ribonucleoproteins would affect the cell according to their biological specificity.

B. Effects of Biochemical Mechanisms

1. Energy-forming Systems

More information comes from the study of integrated biochemical reaction chains, like those of *glycolysis* and *respiration* when studied at various times after irradiation: these systems result in the building up of compounds rich in chemical energy which can be used for biosynthetic reactions and cellular work. In radiosensitive organs like bone marrow, spleen, and thymus, such reactions as *aerobic phosphorylations* do seem to be impaired already 30 minutes after irradiation by 50 r (effects on mitochondria); but it cannot yet be stated whether these radiobiological processes are the cause or the result of other biochemical damage (see Errera, 1957; Meissel, 1956).

2. Synthetic Mechanisms

In dividing tissues, the most constant finding is an inhibition of the synthesis of deoxyribonucleic acid (see Errera, 1957; Meissel, 1956; Lebedinsky, 1955; Almeida and Sherman, 1954; Gaulden, 1956). In microorganisms like yeasts or *Escherichia coli*, the homogeneity of the population make experiments more easily interpretable; it has been found that this inhibition is only temporary and synthesis is resumed after various lengths of time (Almeida and Sherman, 1954). The same is true for *E. coli* irradiated with UV during the logarithmic phase (Kellner, 1953; Kanazir and Errera, 1954). In other instances the inhibition of DNA synthesis may not be immediate and may not exceed 50%, as in many mammalian tissues (Hevesy, 1949; Holmes and Mee, 1955).

There remains to be determined the nature of the initial step of this radiation damage: on the basis of bacteriophage inactivation, it has been suggested that the DNA model (template), on which the new molecules are thought to be formed, has been altered in such a way as to make its reduplication impossible. The temporary inhibition of DNA synthesis may lead to abnormal DNA formation, and this is perhaps related to the killing of cells and to mutation, but in what exact manner is not known.

The synthesis of *ribonucleic acid* (RNA) and of *proteins* and *lipids* do not appear to be consistently impaired by radiation, at least primarily, even in sensitive organs such as spleen, and they may even be enhanced; but these compounds are very complex and cannot be studied seriously in bulk form, as they have mostly been up till now. Proteins and RNA can be bound to the chromosomes and other nuclear and cytoplasmic structures; these are probably very complex in themselves, and each fraction will have to be studied independently. It has been shown for instance that the incorporation of simple precursors into proteins and nucleic acids (adenine and phenylalanine) is often more inhibited in the nucleolus (of the UV-treated starfish oöcytes) than it is in the nuclear sap or in the cytoplasm (Errera and Ficq, 1956) and that the incorporation of adenine into RNA and of phenylalanine into the proteins of isolated thymus nuclei is extremely sensitive to UV- or X-radiation (Logan *et al.*, 1958).

The case of hemoglobin synthesis in bone marrow has been quite attentively studied, and as this is a complex process the hemin and globin moieties have been investigated independently. Richmond *et al.* (1951) found that, when a rabbit is irradiated (800 r) and its bone marrow incubated, during the first few hours there is an increased incorporation of glycine in both moieties; but the relative increase, and its duration, is different and the process in spleen and in bone marrow is not identical. Nizet and his co-workers (1954) have shown a similar

reaction of hemin in dog reticulocytes, the effect being possible after *in vivo* or *in vitro* irradiations of the blood, and it can be partially obtained when nonirradiated reticulocytes are incubated in irradiated plasma. In several experiments, the incorporation of Fe⁵⁹ in red blood cells has been shown to decrease which may mean that there occurs a dissociation of the synthesis of the protoporphyrin and the fixation of Fe on the tetracyclic ring; but, as these are different experiments, one can argue that the timing or other conditions are not quite comparable.

Such a bimodal response to radiation, often found for protein synthesis, makes it difficult to interpret the variations of the serum proteins (see Errera, 1957) in irradiated animals, where a very complex picture is often obtained and when the many investigations available are difficult to compare on account of methods and timing of the experiments.

C. Induced Synthesis

Another type of synthesis which has been shown to be affected by some radiations is induced protein synthesis: in this case, a specific protein is formed as the result of the presence in or around the cell of specific chemical inducers. These may be various metabolites the cell becomes able to utilize in the adaptations of microorganisms to a new substrate (Monod and Cohn, 1952; Spiegelman *et al.*, 1955); tryptophan, which induces an increased activity of tryptophan peroxidase in liver (Gros *et al.*, 1955), as shown in rats and rabbits, or antigens, which, as has long been known, induce the formation of specific antibodies in mammals (Taliaferro and Taliaferro, 1956). All these mechanisms are extremely complex and may not be quite identical, but all respond very similarly to radiation. Although X-rays only exceptionally affect induced synthesis in microorganisms in conditions for which cell division is completely arrested [in the case of hydrogenlyase in *E. coli* (Billen and Lichstein, 1952) and certain staphylococcal enzymes (Craeser, 1955)], one can state, in general, that to be effective, the system has to be irradiated *before* the initiation of synthesis of the protein. Furthermore, it is not the synthetic system itself which is most sensitive, but the process of the organization, under the stimulus of the inductor, of what is necessary for this synthesis to take place; it is almost certain that a particular type of ribonucleic acid is essential for this effect, and it is highly probable that this is, at least, part of the sensitive system (see, for example, Torriani, 1956; Talmage, 1955; Taliaferro and Taliaferro, 1956).

D. Cellular Differentiation

Induced syntheses are perhaps only a particular aspect of the more general process of cellular differentiation in which specific cellular structures or constituents are formed in primitive cells, as a result of which

these and their descendants become endowed with the characters enabling them to perform specific cellular functions. It has been known for a very long time that the embryo in certain specific stages is particularly sensitive to irradiation with, as result, the specific complete or partial inhibition of embryonic development, leading in the latter case to various types of monsters. In grasshoppers (*Melanoplus differentialis*) it is possible to inhibit differentiation specifically if low dosages of X-rays (25,000 r) are given before the embryo has reacted to the inductor stimulus necessary to interrupt diapause, but irradiations in the stages preceding organogenesis are usually extremely effective (Tahmisian *et al.*, 1954); a few dozen rads given to a mammalian fetus may block neural differentiation (W. L. Russell, 1954; L. B. Russell, 1954). It is noteworthy that when gametes are irradiated prior to fertilization, one usually observes recovery of the delay in the first division, but embryonic development will almost certainly be arrested during early gastrulation when differentiation begins (Hertwig, 1911; Daleq and Simon, 1932).

IV. CYTOCHEMICAL EFFECTS

A. Nucleus

In the cell nucleus, the most conspicuous damage lies in the *chromosomes* which are very sensitive and frequently grossly altered; irradiation as low as 25 r or even less is sufficient to induce chromosome aberrations in embryonic nerve cells (Gaulden *et al.*, 1954) or in many plant tissues (Catcheside, 1948; Kaufman, 1954). Irradiation causes the *breakage* of chromosomes, probably during irradiation, followed by normal or abnormal *restitution* of the broken ends; but these may remain free. As not only the molecular integrity, but also the order of the genes on the chromosomes is important, this damage leads to genetical effects (mutations). Point mutations are molecular alterations of genes not accompanied by visible aberrations, and they may perhaps concern only a very few subunits (nucleotides) of genetical material (Benzer, 1957; Demerec, 1956). Two types of mechanisms for chromosome breakage appear to be possible (Wolff and Luippold, 1956); the first would be the result of the breaking of weak ionic bonds, the second the rupture of stronger covalent bonds. In the first case, restitution is possible in the absence of external energy sources; in the second, energy of respiratory origin is necessary. This interpretation is in no way definitive; it is the one which best fits the present experimental data, but its simplicity is obviously a reflection of our total ignorance of the molecular structure of chromosomes and of the dynamic mechanisms of chromosome func-

tion. Irradiation, in order to cause a mutation, must presumably ionize the gene itself or "toxic" molecules in its immediate vicinity.

Less defined damage, making the chromosomes stick to each other, is also observed; the result of this adherence is, as is often also the case for well-defined aberrations, an uneven distribution of chromosomes among the daughter cells, which affects the process of mitosis or the survival of the cells (Carlson and Harrington, 1954; Carlson, 1954). These effects have been observed also in mammalian cells, but owing to the complexity of the chromosome formula, no quantitative observations have yet been made.

The morphology, as well as the number, of *nucleoli* may be altered in insect (Carlson, 1954) or mammalian cells (Scherer and Ringelb, 1954). The total volume may increase as a result of irradiation, as often does the volume of the nucleus, and the nucleoli may also become fragmented or vacuolated. The precise function of the nucleoli in normal cells is far from completely known; but it may be related to such diverse processes as cell differentiation, protein synthesis, and coenzyme synthesis, and their obvious relationship with the chromosomes in many instances, make these organelles of prominent interest (Brachet, 1957).

B. Cytoplasm

Nuclear swelling is often accompanied by cytoplasmic swelling, and giant cells are often observed after irradiation (Bloom, 1948). The fact that the dry weight or total nitrogen increases at the same time, indicates that many synthetic reactions have not been interrupted. Swelling of cells (or elongation of bacteria) appears to be the result of an impaired cytoplasmic cleavage (Klein and Forssberg, 1954; Puck and Fisher, 1956; Meissel, 1956) and has recently been found to be independent of lethal effects when low doses of UV were used in *E. coli* (Deering and Setlow, 1957). This cellular swelling has often been the basis of a misinterpretation: many references to the *stimulation of growth* of irradiated organisms can be cited. Actually, as in the case of seedlings, this is merely the result of the elongation of nondividing cells (Moutschen *et al.*, 1956): the inhibition of one process (cell division) may result in the increase of available energy or building blocks for other reactions, thus merely shifting one steady state to another one. The energy of radiation and its random distribution is such that the chances for obtaining deleterious reactions appear greater than those for specifically removing inhibitory processes, another logical mechanism by which stimulation could be explained.

The cell cytoplasm is known to contain a variety of particulate structures; the exact identity of some of these has not yet thoroughly been

worked out (de Duve and Berthet, 1954). *Mitochondria* have been observed to swell or show abnormal staining in irradiated spleen cells (Scherer, 1956), a finding which has been supported by the biochemical evidence (inhibition of oxidative phosphorylation); see van Bekkum (1956). In many other instances however, mitochondria appear to be quite radioresistant and, in several instances of *in vitro* irradiation, no inhibition of spleen phosphorylations were observed even after doses of 20,000 rads (Ord and Stocken, 1955). If, after irradiation, the behavior of the various biochemical functions which have been attributed to mitochondria were compared, it should be possible to draw a consistent picture of their alterations; unfortunately the experiments have seldom been carried out under comparable conditions.

There have been described: (1) An inhibition of respiration and of phosphorylations chiefly in thymus and spleen; but the phosphorylation processes appear to be more sensitive than respiration (Potter and Bethel, 1952; van Bekkum, 1955; Ord and Stocken, 1955). (2) An increase of spleen adenosinetriphosphatase which seems to be independent, at least initially, of the inhibition of phosphorylation (Potter and Bethel, 1952; van Bekkum, 1955). (3) An altered lipid metabolism characterized chiefly by an increased synthesis of the phospholipids of the liver (Chevallier and Bourg, 1954); however in spleen and thymus it is slightly lower or remains normal. It must be emphasized however that lipid synthesis may not necessarily be linked to mitochondrial integrity, as suggested by a number of recent experiments (Popjak and Tietz, 1955).

Thus, three different mitochondrial functions do not appear to respond identically to radiation. This raises the problem of the identity of the mitochondria performing all these three functions. Much better-controlled work, where several properties of the same particles are investigated under identical conditions, might help to solve this important problem, and radiations would perhaps in this instance be useful as an analytical tool: the site of lipid metabolism may be a radioresistant type of mitochondrion. One must, finally, keep in mind that respiratory processes appear, as in yeast, to be controlled by nuclear or cytoplasmic factors (Ephrussi, 1953); the latter may or may not be identical with the cytoplasmic particles carrying the respiratory enzymes themselves. An alteration of these controlling mechanisms could very well be the origin of late radiation effects on these functions.

Microsomes are smaller cytoplasmic structures organized, as seen by the electron microscope, in a reticulum (Palade, 1956; Palade and Siekevitz, 1956). These particles are at present considered to be the major site of protein synthesis (Brachet, 1957). Surprisingly, electron

microscopy has not been much used for the study of the structure of the irradiated cytoplasmic reticulum, and the scanty observations so far performed in the thyroid and in the testes have not revealed any damage to this reticulum (Tahmisian and Devine, 1956). If one considers the microsomes in a dynamic fashion and studies the cellular functions they are related with, several conclusions can be tentatively reached:

In general, *synthesis of bulk proteins* does not appear to be impaired immediately after irradiation; it is on the contrary, often enhanced; however, this increased activity is often followed by a depression, as in the case of the synthesis of the protein moiety of hemoglobin (Richmond *et al.*, 1951). *Cholesterol synthesis* is also related to the integrity of microsomes and is often enhanced after irradiation; when it is inhibited, as in spleen, this only becomes apparent after 24 hours (Cornatzer *et al.*, 1954; Bacq and Alexander, 1955). In most cases, it can probably be stated that the effects of radiation on microsome function does not become apparent immediately after irradiation. The understanding of these late effects will not be possible until the fundamental facts about protein synthesis and their relation to nuclear activity are known. Experiments on enucleated unicellular organisms have shown that the nucleus has a definite but remote control over the cytoplasmic ribonucleoproteins (Brachet, 1957) and that irradiation of nonnucleated cytoplasm in the ameba has demonstrated that at least ultraviolet light affects cytoplasmic ribonucleoproteins quite rapidly (Skreb and Errera, 1957).

Lysosomes are a type of cellular particles chiefly studied in liver; they are intermediate in size between microsomes and mitochondria (de Duve and Berthet, 1954); they are characterized by a high content in iron and by their association with several enzymes, e.g., deoxyribonuclease II, a ribonuclease, a cathepsin, glucuronidase, and acid phosphatase. As the activity of the first three of these enzymes has been found to increase in tissue homogenates or in the blood stream after irradiation (Kowlessar *et al.*, 1955; Okada *et al.*, 1957; Goutier-Pirotte, 1956; Feinstein and Ballin, 1953), it could be suggested that this is a result of damage to the lysosomes; critical experiments in which enzymes are assayed simultaneously in an irradiated animal might be able to prove this hypothesis.

Chloroplasts (Rhoads, 1946; Lesley and Lesley, 1956), the chlorophyll-containing cytoplasmic particles of plant cells, and *kinetosomes* (Lwoff, 1946), the particles related to flagella in protozoa, are both endowed with genetic continuity. Recent work on moderately irradiated grasshopper testes has shown, in the electron microscope, the appearance of supernumerary tail filaments and centrosomes (Tahmisian and Devine,

1956), probably related to the kinetosomes of protozoa. These observations have led their authors to an interesting theory of radiation damage based on the synergistic action of nonspecific molecular displacements leading to the formation of abnormal structures. Extensive work on irradiated plant cells has led to the demonstration that the activity of chloroplast invertase and phosphoglucomutase is enhanced, whereas the phosphorylase (much more strongly bound) is on the contrary inhibited (Sissakian, 1955). No work on the inhibition of chloroplast formation or multiplication has yet appeared.

V. BIOLOGICAL EFFECTS

A. Homogeneous Cell Populations

Cell populations such as microorganisms, protozoa, unicellular algae, cultures and surviving suspensions of cells from multicellular organisms, e.g., fibroblasts, bone marrow cells, gametes, and certain cancerous cells, have been extensively studied (reviews by Hollaender, 1954; Lea, 1947; Errera, 1957; Sparrow and Forro, 1953). Recent techniques make possible the culture in liquid media of almost any type of mammalian cell (Puck and Fisher, 1956); these cells are capable, *in vitro*, of forming organized structures recalling the original tissue they come from (Moscona, 1957). These cell populations have been irradiated under rather comparable conditions, and they have been shown to react in very similar ways.

When fundamental properties of the cells, such as survival, cell multiplication or mitosis, increase in dry weight, differentiation of non-mature cell types, cell movements, permeability of the cell membranes, are studied, one can usually describe a common pattern of reaction to radiation.

On the other hand, cells performing specialized functions may react to radiation in a specific manner related to this function. In multicellular organisms, important interactions between the different tissues have also to be considered.

1. Mitosis

If cells are not killed immediately they often die after having undergone one or several divisions. Mitosis itself is interfered with and is usually delayed if irradiation happens early enough in the mitotic cycle. This has been worked out most elegantly by direct observation on hanging drop preparations of neuroblasts from grasshopper embryos (Carlson, 1954; Gaulden *et al.*, 1953). These experiments have shown the existence of a very critical stage of cell division during the period when the chromosomes condense as visible threads and during which

both the nuclear membrane and nucleolus disappear. Irradiation *before* this critical stage usually makes the whole process stop for a duration depending on dosage; *after* it has passed this stage, the mitotic events do not appear to have been interfered with if dosages are small. It is remarkable that, applied at the right moment before the critical period, dosages as small as 8 to 16 rads will delay the progression of mitosis in this type of cell. These observations are essentially similar to the previous analyses on fibroblast cultures (Spear, 1946-1947; Mayer, 1939); they also fit rather well with the experiments on irradiated gametes of sea urchins, where cleavage of the fertilized embryos obtained by the conjugation of irradiated gametes (either of which or both have been irradiated) is also delayed, if irradiation occurs before early prophase in this case (Henshaw, 1940). If irradiation occurs afterwards, it is the following cleavage which is slowed down. This general picture of mitotic delay may be subject to some alteration when different types of cells are considered; less direct methods of observation than those used by Gaulden may have led in other cells to a different timing of the critical period. Also, in each cell type, although the general course of mitosis is quite similar, the duration of each phase and sometimes the exact denomination of the stage considered may vary to a considerable extent, which makes exact comparisons very difficult. The exact cause of the inhibition of mitotic division is not known. It has been suggested that it is related to the inhibition of DNA synthesis (Errera, 1951, 1957)—which occurs frequently—but some instances where cell division is inhibited with apparently normal DNA metabolism will force us to reconsider this view (Gaulden, 1956). DNA synthesis is a complex process; it is perhaps associated with chromosomal protein or RNA synthesis (Bloch and Godman, 1955; Volkin and Astrachan, 1956). It has, on the other hand, been suggested that an interference of radiation with the oxido-reduction of sulphhydryl compounds known to occur during cell divisions (Rapkine, 1938; Gonzales and Barron, 1956; Stern, 1956), might also be one cause of its inhibition; that mechanisms of cytoplasmic cleavage (Deering and Setlow, 1957) or of spindle formation (Sparrow and Forro, 1953) may be inhibited are other plausible hypotheses.

2. *Mutations*

We have stated earlier that, following radiation, cells which do not die after several divisions, are said to recover. This statement is very imprecise, because all we know is that these cells *look* as if they had recovered. However, in certain instances, although they continue to have a quite normal appearance, they have undergone mutation. These have best been found in bacteria, molds, and other unicellular auto-

trophic or heterotrophic organisms; and very recently, the studies of cultures of isolated mammalian cells have suggested that such mutant forms also exist among the survivors (Puck, 1957). These mutations are characterized by the fact that the surviving cells, as well as most of their descendants, have been affected in a way which makes them permanently incapable of performing some biochemical reaction. If this biochemical reaction (for instance, the formation of an essential building block) is necessary for the cell to grow and multiply, it will lead to the arrest of growth and multiplication and, finally, the cells will die if this essential building block is not provided in the culture medium. It is believed that there is a period of time following irradiation during which the process of mutation is not fully established (Demerec and Latarjet, 1946; Witkin, 1957; Luning and Hannerz, 1957; Baker, 1955). What takes place during this time is not known, but it is possible, at least in the case of ultraviolet irradiation of *E. coli*, that the expression of damage depends on the synthesis of some protein. Although this lag gives the possibility of interfering with mutagenesis—a subject we will discuss more thoroughly in Section VII, B—it is generally accepted that this damage once fully established cannot be reversed by nongenetic processes. One always finds in addition to induced mutants a certain number of spontaneous ones, which arise in the absence of any added external agents.

Back mutation, the reversal of the previous mutation and the evolution from dependency to independence of some specific metabolite, may occur spontaneously or by irradiation of the mutant; one apparently has what could be called a true recovery of the cell or, at least, of that part of the cell which had first been altered (Giles, 1954). However, the spontaneous phenomenon has a small probability of occurring and the process of back mutation, unless it could be directed, is not a practical recovery process. Mutations are also induced by *ultraviolet irradiation* and by many chemical substances, some of which are specific metabolic inhibitors and so may help in throwing some light upon the general mechanisms of mutation.

In the case of UV light, it has been possible to study the wavelength dependency for mutagenesis: some evidence obtained fifteen years ago on the induction of endosperm deficiencies in maize (Stadler and Uber, 1942) and genetic alteration in *Sphaerocarpus* (Knapp and Schreiber, 1939) have shown that the wavelengths in the neighborhood of 260 m μ were the most effective, probably indicating that energy absorbed in nucleic acids was most efficient.

The comparison of X- and UV radiation has been made in a certain number of instances. Such a comparison is often rather uncertain on

account of the difficulty of calculating with accuracy the energy absorbed in the cellular structures concerned, especially in the case of UV, where absorption is not a random process. However, an observation of Demerec and Latarjet (1946) has shown that X-rays produce more mutations than UV, but on account of the 200-fold higher killing efficiency of these ionizing radiations, this is only at low doses.

In other than microorganisms comparisons are more difficult, but it is very clear that effects on chromosomes are not qualitatively identical with X- and UV radiation: in *Drosophila* for instance UV does not appreciably produce gross chromosomal rearrangements (Muller and Mackenzie, 1939, see p. 267), and in maize also it has been shown that the ratio of translocation to mutation is very low; it appears then, as concluded by Swanson and Stadler [(1955), see p. 267], that UV can induce mutations without causing chromosome deficiencies very frequently.

Genes presumably control biochemical mechanisms (many of which are located in the cytoplasm) responsible for producing enzymes or other specific cellular constituents. It is possible to imagine that, as a result of irradiation, the block in the reaction chain between gene and enzyme-forming system could occur in some intermediate *cytoplasmic* structure. If this structure is one which, like the chromosome and the genes they carry, has to reproduce itself at each mitosis in order that each daughter cell be identical with its parents, and if damage has rendered the reduplication of the original structure impossible, one will obtain a *cytoplasmic mutation*. Nothing much is known about these, but the induction in yeasts of respiratory-deficient strains by poisons or radiation, and the demonstration that this deficiency is not necessarily of nuclear origin, indicates the existence of heritable cytoplasmic characters (Ephrussi, 1953; Nanney, 1957).

3. Movement

Cell mobility can be stopped by irradiation, but usually very high dosages are needed for such an effect. Irradiation of spermatozoa (Casarett and Cassarett, 1957; Evans *et al.*, 1942) may result in the loss of motion, probably as a consequence of the inhibition of phosphorylation (Kanazir and Errera, 1955); this causes them to become infertile, but the dosages are much larger than the ones required to delay cleavage of the fertilized egg. Nothing specific is known of the effects of radiation on the cellular migrations which occur in the developing embryo. On the other hand, radiation is known to inhibit phagocytosis in mammalian polymorphonuclear white blood cells, but phagocytosis is a complex phenomenon and this effect is not necessarily due to the inhibition of

movements. Alterations of cytoplasmic or nuclear movements inside living cells might also give useful indications, but so far their quantitative measurement is difficult.

4. Membrane Phenomena and Ionic Equilibria

The frequent statement made that radiation alters the cell permeability needs to be specified. The exchange of inorganic or organic molecules and ions between cells and their natural environment is a very complex process because many substances have to be concentrated inside the cell, against a concentration gradient, a process which requires energy (Rothstein, 1954); inhibition of permeability could thus result from the inhibition of energy-forming systems. This is the case for K^+ or carbohydrates; in the case of the latter, complex enzymatic systems, located on the cellular membrane, have been described, and it would not be surprising that this organized structure be upset by radiation, as are other patterns of cellular organization.

It has been shown, in many cases, that potassium leaks out of many irradiated cells, such as erythrocytes (Maizel, 1951; Sheppard and Stewart, 1952), cardiac muscle cells (Coons *et al.*, 1955), but not out of cells of liver or kidney (Denson *et al.*, 1953) or striated muscle (Wilde and Sheppard, 1955).

The entry of glucose or amino acids into cells is also dependent on surface enzymes, and it should be clarified whether an inhibition of these systems might affect secondarily synthetic or energy-forming mechanisms. In microorganisms (*E. coli*, yeasts), as already seen, it is known that the induced synthesis of many enzymes is not inhibited by X-rays (Baron *et al.*, 1953), which indicates that the inductor substrates are still capable of penetrating inside the cells. However, quantitative studies have not been performed. On the other hand it has been definitively proved that, in similar organisms (*E. coli*), irradiation leads to the diffusion of many nucleotides into the outside medium (Billen *et al.*, 1953). In mammals, it has been found that when glucose is injected under the skin immediately after irradiation, its entrance into the blood stream is slowed down (Loureau-Pitres, 1954). The passage of metabolites from the hypodermal region into the blood capillaries could be a more complex phenomenon, because it involves the passing of the molecule through an organized tissue. The same applies to the inhibition of the intestinal absorption of glucose, which is diminished 3 to 6 days after total body irradiation in rats. However, in this case, the inhibition is accompanied by important cytological damage (Detrick *et al.*, 1955).

5. Cell Death

Irradiated cells die either immediately (i.e., during irradiation) or

after a certain delay; in the former case, much higher dosages are needed and death can be attributed to a general denaturation of cellular constituents. Many conflicting results on cell death have appeared in the literature; this can be accounted for by the difficulty one finds in defining cell death: in microorganisms, for instance, death has been defined as the inability to form visible colonies on agar plates. Furthermore, the primary cause of cellular death may differ from one system to another, and it is not necessarily unique; any of the cytochemical, biochemical, physiological, or genetical effects of radiation so far discussed could each participate to kill the cell. A mutation in a microorganism leading to the inability to form an essential building block will be "lethal" *only* in the case where the culture medium does not contain this substance.

Delayed death of dividing cells occurs after one or several cellular divisions have taken place (Lasnitski, 1943; Spear, 1946-1947), and it may often be linked to chromosome damage (Carlson, 1954), but it could also be due to nutritional or other deficiencies, such as occur in a nondividing population. Delayed death is caused by much more specific damage than immediate death, and its study is thus of far greater interest. The intensities required for obtaining delayed death may be very different, not only for cells of different species, but also for closely related cells like different strains of the same bacterial species.

Recent experiments on cultures originating from different single mammalian cells have shown a very similar sensitivity (Puck, 1957); this probably results from the fact that, in these abnormal conditions, cells undergo relatively rapid division, whereas in the whole organism this process may be extremely slow and may differ from one tissue to another. When penetrating radiations are used, it can be assumed that each cell of an irradiated population receives the same amount of radiation. In an average-size mammalian cell, submitted to an irradiation of 1 r, several hundreds of ionizations occur, and the probability of a structure being damaged will depend on several factors, including its size and the radiosensitivity of its constituent molecules *in vivo*. It has been calculated that 100 r to a mammalian nucleus, or 1000 to a bacterium nucleus are capable of producing of the order of 1-5 damaged genes per cell from direct hits in the DNA alone, and that every radical which could reach the DNA might damage another gene (Guild, 1957). Alterations to DNA may be one cause of late cellular death but other cellular constituents are also damaged. It can be shown that some cells die, while others recover and apparently behave again like normal ones. This probably results from differences in the distribution of the energy to "critical" and to less "critical" molecules, and it must be remembered that it is the remaining physiological activity of each cell constituent which will determine the final biological effect.

B. Effects on Viruses and Microorganisms

Bacterial viruses (bacteriophage) are the ones most attention has been paid to, and the following fundamental facts have been discovered and have in some cases been confirmed with other viruses. Ionizing or UV radiation applied *in vivo* or *in vitro* inactivates them, i.e., interferes with the self-duplicating mechanism which permits them to multiply inside the cell (Luria, 1955; Pollard, 1953; Stent, 1958). For certain strains, nonirradiated bacteriophages are capable of growing on heavily irradiated (X-rays or ultraviolet bacteria), indicating very clearly that the self-duplicating structure itself has to be affected and that the bacteria remain capable of supporting phage multiplication. Similar experiments have also been done with encephalitis virus (Labaw *et al.*, 1953; Cheever and Smith, 1955). If the conditions of infection are such that there are *several* ultraviolet-inactivated bacteriophages per cell, for certain strains of bacteriophage the intact parts of each virus can *recombine* into a complete new unit, which is again capable of self-reproducing (this is called *multiplicity reactivation*) (Luria and Dulbecco, 1949; Krieg, 1957). This is a crude and probably quite inaccurate way of explaining a complex mechanism of which little is known. This type of reactivation has also been described for X-rays (Watson, 1948).

Cytoplasmic κ particles of Paramecia can also be inactivated by X and UV radiation. (Sonnenborn, 1953, Nanney, 1957).

Effects on lysogenic cells. Certain types of bacteriophages invade their host but do not multiply in the usual way; on the contrary, they appear to become integrated into the bacterial deoxyribonucleoprotein and thus reduplicate simultaneously with the bacterial nuclear material without causing any apparent trouble to the cell. However, extremely low dosages of irradiation as well as a variety of other agents induce the transformation of this "prophage" to a virulent bacteriophage, which will multiply and finally lyse the infected cell (Lwoff, 1953; Bertani, 1958). In certain strains of lysogenic bacteria, a dosage of 0.1 r may give a measurable induction, and the linearity of the dose-response curve for this effect has been demonstrated down to such low dosages (Marcovitch, 1956). What characterizes inductions is that they occur in almost 100% of lysogenic cells, whereas mutations only take place in a small number.

Experiments on infected microorganisms have also shown that a virus is capable of becoming integrated into the genetic material of the host and of transducing some genetic characters from one genetic type of host to another one (Zinder and Lederberg, 1952; Demerec, 1956). It is not unlikely that processes similar to bacterial transformation by DNA or to transduction involving the transfer of genetic material

from one type of cell to another one, also exist in mammals. If such phenomena were discovered, *directed reversed mutations might become possible in mammals.*

C. Differentiating Cell Populations

1. Irradiation of Gametes

We have seen that when either of the gametes is irradiated, the first cleavage of the fertilized egg is delayed; if the embryo is then left to develop, the cleavage divisions usually proceed apparently quite normally up to the blastula stage. However, embryonic development usually comes to a permanent stop before the completion of blastulation or during early gastrulation; this is one of the numerous examples of delayed death (Dalcq and Simon, 1932). The fundamental biological situation is that gastrulation is the first stage of development during which *cellular differentiation* occurs: this process is preceded by a striking increase in the metabolism of ribonucleic acid (both in the cytoplasm and nucleoli), as are most biological processes where intense protein synthesis and differentiation is taking place (Brachet, 1945), and the cell nuclei tend progressively to lose *the general potentialities they had until then* (King and Briggs, 1956). The cause of the death of embryos obtained from oöcytes fertilized with irradiated sperm appears certainly to be related to *nuclear damage*—the sperm cell contains only very little cytoplasm, and the damage can remain hidden, as it may do in mutations, over many cellular generations. Cell division appears to be blocked as a result of incomplete fusion of the maternal chromosomes with the abnormal ones of male origin, a situation leading eventually to chromosome abnormality and uneven distributions among daughter cells (Hertwig, 1911; Dalcq and Simon, 1932; Muller, 1955; W. L. Russell, 1954). It is important to notice that the process of cell division becomes inhibited at a stage of development during which the cell nuclei tend progressively to lose the general potentialities they had until then and during which the genetic material is presumed to initiate differentiation. If, however, the fusion of the abnormal paternal chromosomes with the normal maternal ones is completely prevented (which can be done by using higher dosages of radiation), a situation arises where the abnormal nucleus is eliminated, and in this case an apparently normal embryo will develop if the species studied are capable of parthenogenetic development (Hertwig, 1911; Dalcq and Simon, 1932). This is one example, amongst others, where dosage-effect relationships appear to be non-linear and even paradoxical, *higher dosage producing less final damage than lower ones.* The reason for this is that one observes the complex

mechanisms of development, secondary to the initial damage to the chromatin, which is probably related in a simple way to the amount of irradiation received. A similar paradoxical situation may be found in the experimental inductions in the embryo of certain abnormalities such as microphthalmia (W. L. Russell, 1954), and this can be logically explained by the existence of some competition with other lesions at higher dosage.

In the wasp *Habrobracon* and in the silk worm (Whiting, 1950; Astaurov, 1947) the reverse situation is possible, and the fusion of a normal sperm cell with a highly irradiated egg cell may lead to an androgenic embryo (containing only its *father's* chromatin). Experiments such as this one point again to the very important role of radiation damage to the cell nucleus. Nuclear damage (genetic) is probably also responsible for the various forms of abortion or of malformations of offspring born of parents one of which or both have been irradiated. In this case, the development of the embryo ceases at some stage of organogenesis, very late if the offspring is viable. However, as different stages of gametogenesis have different radiosensitivities, one expects to have different possibilities of abnormal offspring when mating occurs at different times after irradiation (W. L. Russell, 1954). The longer the wait before mating, the smaller the probability of abnormal development, because it has been found that the earlier stages are the least sensitive, at least in mice (Russell and Russell, 1956). With slight irradiation, development may in many cases proceed, and this will result in more or less dramatic expressions of genetic damage visible in the offspring.

2. Irradiation after Fertilization

If irradiation is given at different stages of embryonic development, the inhibition of cell division and differentiation and cell death may cause the development to be either completely or partially arrested. In the mouse, the pattern of response to irradiation (200 r) of the embryo is as follows: Irradiation of the mother after fertilization, but during the preimplantation period, leads to a high incidence of prenatal death; however, the survivors have very few major abnormalities; this means that only the slightly affected embryos survive. In contrast, if irradiation occurs after the embryo is implanted *in utero*, during the period of organogenesis, death usually occurs only after birth—but it is much less frequent; on the other hand, there is a very marked increase of malformations of the embryo. During early embryonic development (if irradiation takes place during the formation of the neural folds), malformations may occur in the eyes, brain, and medulla, but also in the

kidney and liver. Irradiation at a slightly later stage of organogenesis gives rise chiefly to various types of abnormalities of the skeleton. There appear to be short critical periods of development during which certain types of abnormalities arise with very great frequency (L. B. Russell, 1954). The exact mechanism of all these effects is far from being well understood and may be a result of the impairment of *induction*, of *morphogenetic movements*, or of *genetic expression*, that is, the mechanism by which one single cell is capable of becoming differentiated into a multitude of daughter cells performing a variety of functions. *Dosage-effect relationships* have been studied in certain cases and for most bone abnormalities they have been found to be of the *sigmoid type* (multihit) (L. B. Russell, 1954). In the case of the decreased weight of the fetus at birth, the dosage relationship is *linear* (L. B. Russell, 1954), and litter size appears to fall off logarithmically with dosage to the gametes (Snell, 1933). A constant finding is that a higher dosage not only increases the incidence, but also the degree of malformation and the length of the sensitive period during which a specific response can be induced (L. B. Russell, 1954). It has been shown that dosages as small as 25 r to the mouse embryo have led to the induction of abnormalities, discrete perhaps, but nevertheless well defined. Response to lower dosages still could probably be expected if a greater number of animals and more refined tests were used. The case of human leukemia, which can be induced by irradiating the fetus, the child, or the adult organism is being attentively studied, and its induction may have a linear relationship with dosage although this is still highly controversial. (Lewis, 1957; Stewart *et al.*, 1956; Court-Brown and Doll, 1957).

D. Adult Organisms

1. Differentiation and Cell Division

As in the case of isolated cells, experimental evidence points to the particular radiosensitivity (survival experiments or histological alterations) not only of rapidly dividing cells, but also of the embryonic or stem cells which are still due to undergo cellular differentiation (Patt and Brues, 1955). The mature lymphocyte, however, which does not belong to either of these classes is an exception to this rule; its great sensitivity to radiations is not well understood but may be related in some way to the fact that the nucleus is surrounded by unusually little cytoplasm—a condition which probably disfavors spontaneous recovery mechanisms—or to the fact that it is a cell with a very short life expectancy. It is also sensitive to many other stimuli. The situation is different from that in the spermatozoon, whose haploid nucleus plays an

important role both in cell division and in differentiation processes which do not occur in the case of the lymphocyte, whose diploid nucleus may be more resistant than the sperm nucleus.

2. Mutations in Multicellular Organisms

Genetic mutations are found when gametes or the cells they originate from have survived irradiation and happen to undergo fertilization (Auerbach, 1957; Welshom and Russell, 1957). Many mutations are not lethal, and genetic abnormality of one of the gametes is believed to be the cause of many forms of congenital pathology: in this case embryonic development is only very locally arrested, and this leads to abnormalities such as harelip, cleft palate, spina bifida, or many other malformations. Well-defined biochemical deficiencies are also known to occur in mammals and in a few instances they have been quite thoroughly analyzed: in man the missing enzyme has sometimes been identified, as in galactosemia (Kalckar, 1957) and in phenylpyruvic oligophrenia (Fruton and Simmonds, 1953), a form of mental deficiency related to abnormal phenylalanine metabolism.

3. Mutations in Somatic Cells

Mutations in somatic cells are those which affect the lineage of these cells and will not be carried to the offspring. These mutations have been shown to take place at a frequency of the same order as that found in the germ cells before meiosis (gonia) (Muller, 1954; L. B. Russell, 1954; Russell and Major, 1957; Naville, 1955) and have been found to occur in irradiated tissue cultures (Puck, 1957; Puck *et al.*, 1956). Such mutations might play an important part in the determination of malignant growth. Many somatic effects may have their origin in such mutations or chromosome damage of nongerminal cells either as a result of death or loss of specific cell functions.

VI. VARIABLES IN RADIATION EFFECTS

A. *Physiological Conditions*

1. Mitosis and Meiosis

During cell division there are different phases of radiosensitivity, and attempts have been made, not too successfully so far, to link them to the different phases of new chromosome formation and nucleic acid synthesis which occur during these events. The survival of cells, the incidence of mutation, the alterations of chromosomes, all undergo a striking change in radiation response depending on the stage of the division cycle during which the organisms are irradiated; but it is diffi-

cult to generalize as to which is the critical stage, since it can change from one effect or from one organism to another (Giese, 1947; Sparrow and Forro, 1953; Errera, 1957). The induction of abnormalities or of lethal effects in developing embryos after irradiation of immature gametes of either sex, is strongly dependent, as we have seen, on the stage of gametogenesis during which irradiation takes place: the *first meiotic* division is the most sensitive period for the mouse oöcyte if one scores dominant lethals (Russell and Russell, 1955). In the case of the male, irradiation of the testes kills spermatogonia with greatest efficiency and it seems that the degeneration occurs during the interphase or the first prophase following irradiation. The period of greatest sensitivity for various effects induced during embryonic development need not be identical.

2. Oxygen Tension

The irradiation of water solutions in the presence of oxygen results in the formation of O_2H^\bullet radicals, in addition to H^\bullet and OH^\bullet . There is no reason to believe that a similar situation does not exist *in vivo*. The result is that, when the *oxygen tension is diminished*, a lower response to irradiation occurs (Bacq and Alexander, 1955): this is true for the survival of mammals (Dowdy *et al.*, 1950; Stender and Hornykiewytsch, 1955; Stearner *et al.*, 1954), for certain mutations (Russell and Major, 1953; Baker, 1955), but not all (Anderson, 1951), for chromosome damage (Giles, 1954), for various effects on embryonic development (L. B. Russell, 1954), and for certain biochemical reactions dependent on oxygen. Chemical metabolites or poisons, whose presence in tissues reduces the oxygen tension, may have a similar effect. Lowering the oxygen tension may reduce the response to irradiation by a factor of 3 to 5 in the case of high-energy radiation having a low ionizing density (X - and γ -rays); when the oxygen tension is increased, these effects are not enhanced, which indicates that, in air, the oxygen tension is such that one reaches a leveling-out point for the oxygen effect. In the case of the densely ionizing α -particles or neutrons, there is no oxygen effect (Bacq and Alexander, 1955). In the case of UV variable responses have been observed (see Errera, 1957).

3. Other Physiological Conditions

The *age* of the organism may affect its radiosensitivity: in mammals, the sensitivity (to survival) is greater during embryonic development; it then decreases after birth until adult life, but usually it increases again toward the last months of life: in certain strains of mice, 200 rads may be lethal to all embryos irradiated on the 9th day of gestation, but

670 rads kill only 50% of the adults (Kohn and Kallman, 1956; Sacher, 1957). *Malnutrition* and *anemia* are also thought to increase mammalian sensitivity.

B. Secondary Effects

One important problem is that of knowing whether irradiation applied to one site of a cell or organism can induce an effect in another part.

1. Nuclear Cytoplasmic Relationships at the Cellular Level

Such secondary effects can be expected on account of the close physiological relationship between the different cell organelles and because irradiated media could have lethal or mutagenic effects on bacteria growing on it (Wyss *et al.*, 1948). It is known that if the isolated cytoplasm of an ameba which has previously been irradiated is injected with a normal nucleus, mitosis is inhibited in the reconstituted ameba, with cytoplasmic dosages only three times those producing the same effect in a normal organism (Ord and Danielli, 1956). It has also been shown that unspecific chromosome damage can be induced in an intact frog oocyte nucleus introduced into the irradiated cytoplasm of another oocyte (Duryee, 1949); and ultraviolet irradiation of the cytoplasm of the giant unicellular alga *Acetabularia mediterranea* induces very rapidly some cytochemical alterations in the nucleolus which had been shielded during irradiation (this last effect is hardly apparent in the case of X-rays) (Errera and Vanderhaeghe, 1957). However, nuclear damage to *Acetabularia* is also demonstrated if only the nucleus is irradiated. In the course of experiments on eggs of *Drosophila*, the much greater sensitivity of the nucleus when directly irradiated is evident: it takes much more energy to kill the offspring by irradiating the cytoplasm of the egg alone than by irradiating the nucleus (Ulrich, 1955); the same holds true when one attempts to induce chromosome damage by micro-irradiating other parts of the cell (Zirkle and Bloom, 1953). Primary nuclear damage appears to play a prominent role in processes where nuclear activity is important, as in cell division, mutations, or many lethal effects. However, this does not mean that the cytoplasm does not contribute to radiation damage: in some cells where no divisions occur, cytoplasmic processes may become effectively inhibited; this is the case in nonnucleated cytoplasms of *Acetabularia* and *Amoeba* which survive for shorter periods than if they contain a nucleus (Hämmerling, 1956; Six, 1956; Bacq *et al.*, 1957; Mazia and Hirshfield, 1951; Skreb and Errera, 1957). In this case, the role of the nucleus could be linked to some repair processes which cannot take place as efficiently in its absence, perhaps

on account of the fact that the synthesis of cytoplasmic ribonucleic acid becomes seriously impaired in cytoplasm which has been deprived of its nucleus for some time (Brachet, 1957).

2. *Peroxide Formation in Irradiated Cells*

One of the possible agents for these secondary effects could be organic or other peroxides arising during irradiation; it has been shown that the irradiation of bacterial culture media has lethal or mutagenic effects on bacteria (Wyss *et al.*, 1948). It has furthermore been found that bone marrow cells incubated *in vitro* produce peroxides when the cells originate from an irradiated rabbit (Bernheim *et al.*, 1956). The significance of such a finding is difficult to understand on account of the fact that many tissues from nonirradiated rabbits (but not bone marrow) also produce peroxides *in vitro*. Not much is known of effects these peroxides might have on other cellular populations. It has, on the other hand, been demonstrated that many lysogenic bacteria show a diminished response when put in the presence of catalase (catalase reactivation) after UV and X-irradiation (Latarjet and Caldas, 1955). Another argument for the formation of peroxides in irradiated organisms is that even with small dosages (17,000 r) to yeasts grown in anaerobiosis, these organisms synthesize catalase or peroxidase when kept in anaerobiosis, a condition during which they normally only have traces of the enzymes (Sels and Chantrenne, 1957). This synthesis of new enzymes is believed to be induced by peroxides formed during irradiation.

3. *In Multicellular Organisms*

It has been found repeatedly that the *nucleic acid metabolism* of a carcinoma is temporarily decreased as a result of irradiation of the animal bearing it, although it had been completely shielded during the irradiation (Ahlström *et al.*, 1945; Kuzin and Budilova, 1953). It has also been demonstrated that tumors originating from nonirradiated thymus cells can develop if these cells are grafted on a totally irradiated host from which the thymus had previously been removed (Kaplan *et al.*, 1956); damage (by radiation or other means) or removal of the thyroid may lead to pituitary cancer (Furth, 1955). No final explanation can be given for effects of this type; they could be due to diffusible organic peroxides produced during irradiation, and very small quantities of peroxides have in fact been found in irradiated mice (Horgan and Philpot, 1955).

On the other hand, normal regulatory processes located in the irradiated part of the animal, like those mediated by hormones, can be

affected (Lebedinsky, 1955). Stimulation of the pituitary mentioned above as a result of thyroid dysfunction is probably the cause of the pituitary tumor.

VII. ALTERATIONS OF RADIATION EFFECTS BY FOREIGN AGENTS

A. Chemical Protection

1. Theoretical Considerations

Protecting agents are those whose *presence during irradiation* results in a smaller response. Many experiments reported earlier constitute a basis for finding chemicals capable of protecting living organisms against radiations. The idea of protecting organisms against radiations arose about a decade ago as a result of the discovery of the indirect nature of radiation effects on dilute solutions. However, as we have stated earlier, it is very much doubted at present that effects of radiation on organisms occur necessarily through indirect mechanisms. It can be expected that the relative contribution of direct and indirect mechanisms will vary for different biological effects, and in each case the possibility of protection may thus be different (Bacq and Alexander, 1955; Patt, 1953; Bond and Cronkite, 1957). There are many possible ways by which radiation damage might be diminished: (a) Loading the organism with chemicals capable of reacting with H^{\bullet} , OH^{\bullet} , and O_2H^{\bullet} radicals may divert these from reacting with important biological centers. (b) Protecting agents could also act by covering the sensitive site of cell constituents, and this type of mechanism could be operative both for direct and indirect effects (Eldjarn and Pihl, 1956). (c) All agents capable of decreasing the intracellular oxygen tension can be expected to protect against effects (direct or indirect) which are oxygen dependent (Gray, 1957). (d) One could, finally, imagine that a protector could give more chemical stability to a macromolecule and favor the rejoining of broken bonds or divert energy from it. It is, however, very difficult at present to choose among any of these possibilities or to decide which are the relatively more important of these mechanisms.

2. Experimental Evidence

a. *The survival* of uni- and multi-cellular organisms has been quite considerably increased by the use of various agents. *SH* and *amino reagents* (cysteine, cysteamine or cystamine, glutathione) or the methyl derivative, methionine, as well as thiourea have been used successfully on microorganisms and mammals (Bacq and Alexander, 1955; Patt, 1953; Bond and Cronkite, 1957). Very similar possibilities have been found with *S-β-aminoethylisothiouronium · Br · HBr* (AET) (Hollaender and Stapleton, 1956), which is less toxic and may thus be used in many

mammals including monkeys and dogs (Overman, 1957). We are not aware of any attempts to use this compound in man. These protecting agents appear to have a greater efficiency in promoting recovery processes than in preventing the initial damage observed: this is most striking in the case of the white blood cells and of the metabolism of spleen nucleic acid, which seem to follow a similar pattern of response (Bacq and Alexander, 1955).

b. The number of chromosome aberrations (Mikaelson, 1955; Riley, 1955) and, in some instances, the *number of mutations* (Hollaender and Stapleton, 1956) have also been found in smaller amounts when similar protective agents were used during irradiation. Successful experiments on plant cells have been reported, but cysteine does not reduce chromosome aberrations in mouse thymus (Devik, 1956), although nucleic acid integrity does appear to be protected by thiourea and cysteamine (Gros *et al.*, 1953; Limperos and Mosher, 1950) in the same organ. In *Drosophila*, however, and in microorganisms, mutations have not always so responded to the protective action of these agents (Hohne *et al.*, 1955). In microorganisms, furthermore, it is often difficult to interpret the experiments because in many instances mutations *increase* when death rate is decreased as a result of protection (Hollaender and Stapleton, 1956).

These agents have in common the properties of having an amino group and a sulfur atom (which often is in the form of a sulphydryl group), and both these functions are believed to be important (Bacq and Alexander, 1955). However, they can act independently because many amines are also found to be satisfactory protectors in the absence of a sulphydryl group, and agents carrying only a sulphydryl group may be efficient (Bacq and Alexander, 1955; Hagen, 1956). It has often been suggested that the sulphydryl group decreases the intracellular oxygen tension, and this has been found to be the case in a few living systems protected with cysteine or cysteamine (Gray, 1956).

Many other agents have been used with a variable amount of success and the mechanism of action of some of these does seem to be dependent on the decrease of cellular oxygen, as in the case of the protection of microorganisms with hydrosulfite (Hollaender and Stapleton, 1956). A certain number of natural metabolites (succinate, glucose, alcohol) have protecting properties, probably exercised by consuming the cellular oxygen in the course of their normal enzymatic oxidation (Hollaender and Stapleton, 1953). Anoxia can also be obtained with a certain number of drugs such as morphine, which depress the respiratory centers, in which case a protective effect also ensues (Kahn, 1951). The protective action of cyanide is still very puzzling because, being a strong inhibitor of respiratory enzymes, it would tend to increase the intracellular tension of oxygen (Herve and Bacq, 1949). The effect of this agent is indeed

very complex: seeds irradiated in its presence show a greater mutation rate when it is used in low concentrations, but a smaller one when the concentration is increased (D'Amato and Gustafsson, 1948). However, in these conditions an increased number of chromosome breakages is observed (Mikaelson, 1954). It is not clear at present to what extent the protection is complete, because although damage is not lethal, it may well persist and become apparent only at a later stage. It has been shown that rats, protected during irradiation, develop a high number of tumors (Maisin *et al.*, 1956; Kaplan, 1953; Meeuwissen and Brucer, 1957); these might have developed in the nonprotected animals, as in the case of mutation in microorganisms, had they lived, and it is difficult to know if the primary events of induction of cancer have or have not been diminished. Nothing much is known on the protection against other late damage or against the early aging of irradiated organisms.

Protecting agents are much less efficient in the case of alpha rays or neutrons. As we have seen, in these cases no reduction of the oxygen tension is expected to have any effect (Forssberg and Nybom, 1953; Patt *et al.*, 1953).

B. Recovery

When organisms are irradiated, many processes, inhibited at first, recover. The synthesis of deoxyribonucleic acid is often decreased immediately after irradiation, but only temporarily; other biochemical effects which appear later are also temporary and apparently recovery occurs. In mammals, one finds a cellular depletion in the bone marrow or in the gonads during the days following irradiation; but these tissues recover and the depleted cells are replaced by new ones which, having originated from irradiated cells, may, however, have undergone some irreversible alteration, leading for instance to more rapid aging, to an increased radiosensitivity, or to the development of cancer, if irradiation has not been fatal in the first few days. As there is a lapse of time between irradiation and the biological expression of the primary damage, an opportunity exists of preventing the development of the lesion or enhancing the spontaneous recovery processes. *Recovery agents* are those which are effective when given *after irradiation*. Various methods for promoting the recovery of irradiated organisms have been described and can roughly be classified into two groups, those having for object the "destruction" or removal of damage and those based on the replacement of damaged molecules or cells.

1. Removal of the Damage

Among the methods of restoration whose object is to destroy some intermediate compound before the damage is definitively established:

photorestitution of a great number of effects of ultraviolet light (Dulbecco, 1955; Errera, 1957; Jagger, 1958) or *catalase restoration* of lysogenic bacteria treated with ultraviolet light (Latarjet and Caldas, 1955) or, in one instance, X-rays (Miletic, 1954) are of this type. The first of these processes, in the case of ultraviolet irradiated bacteriophage, is only possible if illumination takes place in the presence of extracts of normal bacteria; the second appears to lead (exceptional in the case of X-rays) to the destruction of organic peroxides formed during irradiation. Restoration obtained in some instances by cooling or heating the irradiated cells (see Errera, 1957) may favor the elimination of injury before it is definitively established, but none of these mechanisms is properly understood.

2. *Replacement of Damaged Molecules or Cells*

The case of providing nutrients to microorganisms which have lost the capacity of synthesizing them could be considered as one possible mechanism of recovery: recovery is however only apparent, because in this case the fundamental damage has not been removed.

True recovery would depend on the possibility of replacing the damaged molecules or cells by nonirradiated ones. Experiments on bacterial transformations or on genetic recombinations in microorganisms have shown the possibility of controlling some alteration of their genetic characters. The mechanism of the greater radioresistance of diploid compared to haploid cells may well have its origin in closely related mechanisms. On these grounds, the use of intact deoxyribonucleic acid to replace the irradiated compound inside the chromosome becomes a possibility: one successful experiment of saving ultraviolet irradiated *Salmonella* with intact DNA has been reported (Kanazir, 1957).

It is possible to replace whole cell populations of irradiated animals and thus promote their survival; this can be done by injecting intact bone marrow from an unirradiated donor into the circulation of a lethally irradiated one. This type of experiment was at first performed as a consequence of the demonstration that the death incidence of mice was considerably decreased when hematopoietic organs (like bone marrow of the hind limb, spleen, or liver) are shielded during irradiation. Protection by bone marrow is also successful in dogs, hamsters, monkeys (Congdon, 1956). Only tissues containing cells capable of forming granulocytes (mostly polymorphonuclear white blood cells), red blood cells, or platelets are capable of this activity. These cellular suspensions are effective in preventing acute death from X- or γ -rays, but apparently death caused by neutrons is much more difficult to prevent (Barnes and Loutit, 1956; Congdon, 1957; Duplan, 1956; Comsa and Gros, 1956). As a result, after injection of bone marrow, the

blood cells and platelets tend to reach normal values again, the weight of the body and of the thymus and spleen increase, and immunological defense, which had disappeared, also becomes functional again. However, many of the lesions caused by radiation are not diminished after bone marrow injection: the graying of hair is not influenced and the fertility of gametes is not restored (Habermayer *et al.*, 1957), tumors develop with greater frequency in protected or parabiotic animals (Brecher *et al.*, 1953; Finerty *et al.*, 1953; Binhammer *et al.*, 1957), and the normal life expectancy of the animal remains decreased (Cole *et al.*, 1956). All these facts seem to demonstrate that only acute death has been prevented by the graft.

Important immunological problems are brought up by such experiments, as they were in the case of the first blood transfusions: it is well known that mammals are able to accept definitively only grafts from subjects belonging to the same genetic strains (isologous grafts), and it has long been known that grafts from one human being to another (homologous grafts) are usually eliminated rather rapidly, as in the case of skin grafts; this is also the case when different species of animals like rats and mice are used (heterologous grafts). We have seen that the immunological response of mammals is strongly inhibited in the days following total body irradiation, and it has, in fact, been found, owing to these circumstances, that both homologous grafts (from other strains of mice) and heterologous grafts (from rats) of bone marrow are capable of saving lethally irradiated mice. Cells of the donor animal have unquestionably been characterized in the receptor animal by specific genetical or immunological identification (Barnes and Loutit, 1956; Makinodan, 1956); the repopulation of the myeloid and of the lymphoid tissue has been demonstrated. In the case of the thymus, although at first the cells appear to be exclusively of rat origin, in the following days the appearance of an agglutination reaction with specific mouse antisera indicates that thymus cells of mouse origin may be recovering (Urso *et al.*, 1957). The survival of the animals injected with bone marrow becomes, however, dangerously compromised after a certain time, because when homologous or heterologous grafts are used, the incompatibility between these and the cells from the receptor animals reappears: discussion has arisen as to whether the recovered cells from the irradiated organisms are again able to synthesize antibodies against the injected cells or whether these are making antibodies against the cells of the irradiated host (van Bekkum, 1957). Experiments of cell transfer have been prompted in attempts to replace leukemic cells, which can be destroyed by high dosages of irradiation, by normal marrow tissue with the hope of preventing further development of leukemia.

It has been shown that such a treatment is capable of increasing considerably the survival time of experimental leukemic mice (Barnes *et al.*, 1956). The multiplication of donor cells in the irradiated host has been unquestionably established; however, this does not necessarily exclude a possible effect of subcellular fractions. The idea of the possible recovery capacity of bone marrow or spleen nucleoproteins was put forward a few years ago but later abandoned on the basis that a small number of intact cells were present in the fractions injected (Cole and Ellis, 1954). One cannot at present exclude the possibility that subcellular fractions are not also capable of inducing these recovery phenomena and, on account of the tremendous importance of proving or disproving this hypothesis, both for fundamental and applied purposes, work on the biological activity of nucleoproteins in normal or irradiated mammals is of great interest and should certainly be very actively pursued.

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