

BIOLOGICAL CHEMISTRY

1. INTRODUCTION

THE ramifications of biochemistry are now so extensive that it probably comes as no surprise that one of the topics considered in this year's Report has not been dealt with previously. A considerable amount of work is currently being undertaken on mineral metabolism with special reference to teeth; much of the information available is the result of fruitful co-operation between scientist and clinician and is thus often published in journals not on the reading list of more chemically minded biochemists. A review of this work in *Annual Reports* will remedy this; furthermore, it is important to have available the chemical and biochemical facts on such emotionally charged problems as ^{90}Sr fall-out and fluoridisation of drinking water.

The discovery that β -carotene was converted into vitamin A in the intestinal mucosa of animals and not in the liver was made during 1946—48. When it was further shown that the conversion *in vivo* proceeded easily, one felt that the mechanism of the reaction would soon be revealed. The problem has, however, proved most complex and a stocktaking at the present time after 10 years' endeavours seems appropriate.

Progress in the study of muscular contraction has continued steadily during the past few years, but an important recent development in muscle biochemistry has been the attempt to elucidate the chemistry of relaxation. The characteristic cycle of normal muscle function embraces both contraction and relaxation, and the chemical events associated with these are complementary and equally important aspects of muscle biochemistry. This year's Report discusses modern aspects of both features in relation to earlier observations.

The discovery of new amino-acids is but one aspect of biochemistry which has been enormously aided by chromatography in all its various aspects, and applications of these techniques to a study of higher plants have revealed a number of interesting new compounds. Most of these have now been well characterised chemically and the biosynthesis and metabolism of some are now reasonably clear. However, the biochemistry of many of these new amino-acids remains to be investigated.

T. W. G.

2. CHEMISTRY OF BONE AND TOOTH MINERALS

THERE has been a reawakening of interest in the chemistry of mineralised tissues during the last decade.

The skeleton is not merely a convenient framework upon which muscles can act and from which viscera depend. It is a vast storehouse which provides minerals to the body in times of stress and is replenished in times of plenty. The minerals of bone are distributed in an organic matrix, about 70% by wt. of mature bone being inorganic. The tooth consists of two major tissues, enamel which is the most highly mineralised tissue in the body,

containing less than 1% of organic matter, and dentine, which contains about 78% of minerals. The third mineralised dental tissue, the cementum, covers the roots of teeth in a thin layer and is chemically similar to bone.

Perhaps the most important physical characteristic of the mineral crystals of bone and teeth is their smallness. Early workers,¹ basing their estimates on *X*-ray-diffraction studies, deduced that the length of the crystal was of the order of 10^{-5} to 10^{-6} cm. Measurements based on the determination of total surface area of powdered bone by gas-adsorption techniques² have produced results supporting earlier estimates of size. Such particles are well beyond the range of the light-microscope and it is only with the aid of the electron-microscope that actual crystals have been visualised.³ Using a combination of microdiffraction of *X*-rays and electron-microscopy, Engström and Zetterström⁴ estimated that the crystals were 200 Å in width. Robinson and Watson⁵ produced electron micrographs of intact bone showing crystals 350—400 Å long, almost as wide, and 25—50 Å thick. The crystals appeared to be oriented with their long axes in the direction of the collagen fibres. It seems established, therefore, that bone mineral is crystalline and that the crystals are very small indeed.

Composition of the Crystals.—The main mineral constituents of bone and teeth are calcium, phosphate, carbonate, hydroxyl ions, and water, with smaller amounts of magnesium, sodium, potassium, and chloride.

The main component of the solid phase is undoubtedly some form of basic calcium phosphate. If the major component were tricalcium orthophosphate $\text{Ca}_3(\text{PO}_4)_2$, then the molar Ca : P ratio would be 1.5. Many workers have shown that the Ca : P ratios of samples of human bone are in general higher than 1.5, the most usual values being around 1.66, but values above and below this figure are quite commonly found. Dallemagne and Fabry⁶ are of the opinion that bone salt may be considered as having a fundamental unit which is tricalcium phosphate combined with excess of calcium.

In view of the similarity of the *X*-ray-diffraction patterns of bone and naturally occurring apatites, many workers have concluded that bone mineral is an apatite. This is, however, inconclusive evidence, since any unignited precipitate of calcium phosphate gives the *X*-ray diffraction pattern of an apatite. Neuman and Neuman,⁷ in an excellent review, discuss this problem fully and conclude that the apatite pattern of *X*-ray diffraction is given by almost any sample of calcium phosphate with a molar Ca : P ratio lying between 1.33 and 2.0. Thus, on this basis, any unignited calcium phosphate sample with a Ca : P within 20% of the theoretical value of 1.66 could be classified as hydroxyapatite. It is not justifiable, therefore, to

¹ W. F. De Jong, *Rec. Trav. chim.*, 1926, **45**, 445; W. F. Bale, H. C. Hodge, and S. L. Warren, *Amer. J. Roentgenol.*, 1934, **32**, 369; J. Thewlis, *Proc. Phys. Soc.*, 1939, **51**, 99.

² N. V. Wood, *Science*, 1947, **105**, 531.

³ R. A. Robinson, *J. Bone and Joint Surg.*, 1952, **34**, A, 389.

⁴ A. Engström and R. Zetterström, *Exptl. Cell. Res.*, 1951, **2**, 268.

⁵ R. A. Robinson and M. L. Watson, *Anat. Rec.*, 1952, **114**, 383.

⁶ M. J. Dallemagne and C. Fabry, in Ciba Foundation Symposium on Bone Structure and Metabolism, Churchill, London, 1956.

⁷ W. F. Neuman and M. W. Neuman, *Chem. Rev.*, 1953, **53**, 1.

visualise the main component of bone salts as a compound of fixed composition such as hydroxyapatite, $3\text{Ca}_3(\text{PO}_4)_2\text{Ca}(\text{OH})_2$.

Neuman and Neuman⁷ conclude that the fundamental bone salt is a compound of calcium, phosphate, hydroxyl ions, and water which exhibits a Ca : P ratio of approximately 1.5 and diffracts X-rays to give a characteristic apatite pattern. It represents only one small region of an indefinite series, the transition from one end of which to the other is associated with isomorphic substitution of hydrogen ions and water for calcium. This view is in accord with the concept of the dynamic state of the bone minerals. Fabry⁸ has introduced the term pseudoapatite to describe the fundamental compound of bone salt.

The best estimate of the bone salt therefore seems to be that it consists of a crystal lattice containing mainly calcium, phosphate, and hydroxyl ions, the outer components of which are in equilibrium with a surface hydration shell⁹ containing calcium and other ions and also in equilibrium with the interior ions of the crystal. The hydration shell in turn is considered to be in rapid equilibrium with the tissue fluids in the bone. This concept is not completely irreconcilable with the views of Dallemagne and his colleagues⁸ who have always opposed the idea that bone was essentially hydroxyapatite, nor does it jettison completely the idea of an "apatite-like" structure.

Carbonate of Bone Mineral.—The mineral portion of bone contains about 5% of carbon dioxide. One of the difficulties in the study of bone salts is that the preparation of samples for investigation necessitates destruction of the organic phase; this gives rise to the possibility of producing changes in the mineral fraction. Dallemagne and his co-workers⁶ have investigated the carbonate content of bone salts obtained from whole bone by the removal of the organic material with ethylene glycol. They consider that calcium carbonate has an independent existence in bone salt, and that it is not part of the crystal lattice. It has been shown that the thermal extraction curve for carbon dioxide of bone salts is similar to that for decomposition of calcium carbonate. When bone salts are dissolved in dilute acids, carbon dioxide is released faster than phosphate is dissolved. In young rats,¹⁰ experiments with $^{14}\text{CO}_2$ have shown that skeletal carbon dioxide is in complete equilibrium with blood, which would be unlikely if the carbonate were situated deep in the crystal lattice. These observations provided circumstantial evidence for considering that the carbon dioxide is surface-bound but, as Neuman and Neuman⁷ point out, they do not necessarily prove the independent existence of calcium carbonate. Dallemagne and Fabry⁶ proposed that basic tricalcium phosphate may be bound to carbon dioxide via an additional calcium atom.

Size of Bone Crystal.—Bone crystals are so small that they present a vast surface area. Robinson,³ on the basis of average crystal dimensions, has calculated that the specific surface in autoclaved bone varies from 84 to 106 sq. m./g. Thus in an average man the total bone crystal surface would

⁸ C. Fabry, *Biochim. Biophys. Acta*, 1954, **14**, 401.

⁹ W. F. Neuman, T. Y. Toribara, and B. J. Mulryan, *J. Amer. Chem. Soc.*, 1953, **75**, 4239.

¹⁰ D. L. Buchanan and A. Nakao, *Fed. Proc.*, 1952, **11**, 19.

be of the order of 100 acres. The few litres of body fluid flowing over the surfaces could therefore be in intimate contact with the solid phase, thus facilitating the rapid exchange so often observed in physiological studies.

Tooth Minerals.—The fundamental difference between the tooth of non-persistent growth and bone is that once the tooth is formed it does not undergo the biological remodelling which can take place in bone. Local factors may cause small variations in the composition of teeth during their formation and these variations will then persist throughout the life of the tooth.¹¹

The individual crystals in enamel and dentine are much larger than in bone.¹² The total surface area presented by the crystals is therefore much less, values of 1.8 and 2.4 sq. m./g. have been reported for enamel and dentine, respectively² (cf. 84—106 sq. m./g. for bone). In general, however, the composition of the fundamental tooth salt is believed to be similar to that of bone, but much work remains to be done on minor differences in composition, particularly in relation to the problem of dental caries. There is some evidence to suggest that, in the cotton rat, teeth with a high carbonate content are more liable to decay than those with a lower carbonate content.¹¹

Influence of Diet on Composition of Bones and Teeth.—Severe changes in the mineral content of the diet are reflected in the composition of bone of any age, but are most noticeable in young growing bone. Dietary changes can only influence the tooth during its formative period. An important series of papers have been published by Sobel and his colleagues during the last few years.^{11,12a} They have shown that in the rat and the cotton rat there is a relation between the composition of bone and tooth minerals and the fluid from which they are deposited, and that the composition of the fluid is in turn related to that of the blood serum. Animals reared from weaning on a diet high in calcium and low in phosphorus had a blood-calcium concentration 10% above normal, a blood carbon dioxide level about 10% below normal, and a blood-phosphorus value only 40% of normal. On a diet low in calcium and high in phosphorus the blood-calcium concentration was about 60% of the normal, blood carbon dioxide about 4% above normal, and blood-phosphorus about 17% above normal. These changes in blood composition were reflected in different ways in the different mineralised tissues. In the normal cotton rat the $\text{PO}_4 : 2\text{CO}_3$ ratio is highest in enamel, lower in dentine, and lowest in bones. The $\text{Ca} : \text{PO}_4$ ratio of the tissues shows much less variation, although in enamel it is still slightly higher than in dentine or bone.

In the tibia and femur, and in the enamel and dentine of the incisor and molar teeth, the $\text{PO}_4 : 2\text{CO}_3$ ratio is directly related to that of the serum. In contrast, the $\text{Ca} : \text{PO}_4$ ratio of enamel and dentine is hardly affected by changes in blood $\text{Ca} : \text{PO}_4$ ratio, whereas the ratio in bone varies with that

¹¹ A. E. Sobel, *Ann. N.Y. Acad. Sci.*, 1955, **60**, 713.

¹² R. F. Sognnaes, D. B. Scott, M. J. Ussing, and R. W. G. Wyckoff, *J. Dent. Res.*, 1952, **31**, 85.

^{12a} A. E. Sobel, M. Rockenmacher, and B. Kramer, *J. Biol. Chem.*, 1945, **158**, 475; 1945, **159**, 159; A. E. Sobel and A. Hanok, *ibid.*, 1948, **176**, 1103; A. E. Sobel, A. Hanok, H. Kirschner, and I. Fankuchen, *ibid.*, 1949, **179**, 205; A. E. Sobel and A. Hanok, *J. Dent. Res.*, 1958, **37**, 631.

in the serum. These results refer to growing bones and growing teeth and indicate differences in composition related to differences in dietary intake of calcium and phosphorus. The major changes arising from alteration in diet are in the $\text{PO}_4 : 2\text{CO}_3$ ratios. The changes in $\text{Ca} : \text{PO}_4$ are smaller, though significant, in bone and not significant in teeth. These observations are interpreted by Sobel as indicating the governing influence of "local factors," acting at the site of formation, upon the composition of the bone and tooth. It is also apparent that the "carbonate" fraction of the tooth is more readily susceptible to dietary influence than is the calcium or phosphate portion. This in turn suggests that the "carbonate" of the tooth is not incorporated in the crystal lattice but is situated at the surface or in the hydration cell.

Moisture Content of Mineralised Tissues.—The water content of bone varies with the source of bone and with its age. Human bone from the newborn has been reported to contain 30% of water, compared with 20% in old age.¹³ Other workers have found values of 13—22% of water in human cortical bone and 32—52% in cancellous bone.¹⁴ The water content of powdered bone equilibrated in air for 48 hours and then heated for 24 hours at 105° was found¹⁵ to be 8%. Cortical bone from young dogs contains as much as 54% of water compared with 21% in older dogs.¹⁶

In general, the water content of teeth is less than that of bone. Le Fevre and Manly¹⁷ reported that enamel contained an average of 2·3% of water (range 1—5%) and that dentine had an average water content of 13·2% (range 10·8—15·7%). Deciduous enamel (2·8%) contains slightly more water than permanent enamel (2·3%). Deciduous dentine, on the other hand, contains less water (11·2%) than permanent dentine (13·2%). In a recent study Burnett and Zenewitz¹⁸ found that the maximum water content of freshly extracted whole teeth and dentine was 9·32 and 10·0%, respectively. Rehydration at 98° F and 100% humidity restored some, but not all, of the moisture content.

Earlier work has shown that minerals in fresh untreated bone, although containing considerable amounts of water, are not as highly hydrated as an equilibrated synthetic hydroxyapatite. This has led to the tentative conclusion that not all the crystal surface of intact bone is available for hydration, perhaps owing to its bonding with the organic phase.⁷ From this it may be inferred that with increasing age a larger proportion of adult compact bone may be in less intimate contact with water, either as a hydration shell or as circulating fluid. For example, it is now well established⁷ that the uptake of ^{45}Ca and ^{32}P is greatest in young growing bone. This may depend at least in part on the degree of hydration of the bone crystals.

Association of Mineral Crystal with the Organic Phase.—Electron-micrographs^{3,19} of intact sections of bone show an intimate contact between the

¹³ J. H. Vogt and A. Tonsager, *Acta Med. Scand.*, 1949, **135**, 231.

¹⁴ I. S. Edelman, A. H. James, H. Baden, and F. D. Moore, *J. Clin. Invest.*, 1954,

33, 122.

¹⁵ J. E. Eastoe and B. Eastoe, *Biochem. J.*, 1954, **57**, 453.

¹⁶ R. A. Robinson and S. R. Elliott, *J. Bone and Joint Surg.*, 1957, **39**, A, 167.

¹⁷ M. L. Le Fevre and R. S. Manly, *J. Amer. Dent. Ass.*, 1938, **25**, 233.

¹⁸ G. W. Burnett and J. Zenewitz, *J. Dent. Res.*, 1958, **37**, 581.

surfaces of the crystals and the collagen fibres. The problem arises, what if any is the nature of the linkage between the mineral and organic phase?

Robinson and Watson¹⁹ review existing information and present convincing evidence for the association of mineral crystals with the band regions of collagen fibrils of bone. In human infant bone, a small 100—120 Å period banding is observed. In mature bone the spacing alters and a pair of bands appears at 640 Å intervals. The diameter of the fibril also increases from 150 Å in very young infants to about 800 Å in middle-aged adults. The inorganic crystals are found in association with these bands. The crystals in young bone are very small, ~100 Å in length; in mature bone they are larger, 200—300 Å long, and span the doublet band of the fibril. In senile bone the diameter of the collagen fibril increases, to 1500 Å, and the crystals are large enough to spread over two or more doublet bands, thus obscuring the fibril period. The first appearance of inorganic crystals is associated with the appearance of the collagen fibrils; there does therefore seem to be an intimate link between the two structures. The difference in size of crystals from young and adult bone may be a further factor in the greater isotope-exchange which occurs in newly formed bone.

Role of Citrate.—Bone and dentine contain almost 1% of citrate, and enamel contains nearly 0·1%.²⁰ Little is known of the function of citrate in a mineralised tissue. Dixon and Perkins²¹ suggest that bone citrate is formed in bone cells by normal metabolic activity and is coprecipitated with minerals during calcification of the tissue. This is a reasonable suggestion since a coprecipitate is formed from solutions of inorganic and citrate ions similar to those found in a serum ultra-filtrate.²² Armstrong and Singer²³ believe that at least some, if not the major portion, of bone citrate is of purely adventitious origin owing to the continuous presence of citrate in body fluids. Bellin and Steenbock²⁴ found that administration of vitamin D to previously depleted animals caused an increase in bone citrate but concluded that the amount of citrate in bones was related to the calcium nutrition of the animal rather than to the rachitic state *per se*. Nicolaysen and Eeg-Larsen²⁵ consider that there may be a dual effect, that vitamin D does influence the accumulation of citrate in bones but that over longer periods a defect arising from deficiency of the vitamin may be ameliorated when the diet is rich in calcium and phosphorus.

When whole bone or dentine is treated with dilute hydrochloric acid the portion dissolving contains the minerals, about 5% of the nitrogenous compounds, and all the citrate (0·8—0·9%). Recent work has suggested the

¹⁹ R. A. Robinson and M. L. Watson, *Ann. N.Y. Acad. Sci.*, 1955, **60**, 596.

²⁰ F. Dickens, *Biochem. J.*, 1941, **52**, 260; A. H. Free, *J. Dent. Res.*, 1943, **22**, 477; I. Zipkin and K. A. Piez, *ibid.*, 1950, **29**, 498; M. V. Stack, *Brit. Dent. J.*, 1951, **90**, 173.

²¹ T. F. Dixon and H. R. Perkins, *Biochem. J.*, 1952, **52**, 260.

²² A. C. Kuypers, *J. Biol. Chem.*, 1945, **159**, 411.

²³ W. D. Armstrong and L. Singer, in Ciba Foundation Symposium on Bone Structure and Metabolism, Churchill, London, 1956.

²⁴ S. A. Bellin and H. Steenbock, *J. Biol. Chem.*, 1952, **194**, 311; H. Steenbock and S. A. Bellin, *ibid.*, 1953, **205**, 985.

²⁵ R. Nicolaysen and N. Eeg-Larsen, in Ciba Foundation Symposium on Bone Structure and Metabolism, Churchill, London, 1956.

association of citrate with a peptide.²⁶ When dentine from human teeth is demineralised the solution contains the minerals and the citrate-peptide complex. If minerals are reprecipitated by raising the pH, the citrate complex is adsorbed or coprecipitated with the minerals. Lowering the pH to redissolve the minerals releases the citrate complex. Precipitation of calcium as sulphate at an acid reaction does not cause coprecipitation of the citrate complex. Thus, over the physiological range of pH, citrate is firmly associated with the mineral phase. This linkage of citric acid with mineral and a peptide is of interest since it suggests that not all the citrate present in a mineralised tissue is of adventitious origin. Analysis has shown that the peptide associated with the citrate is highly basic, containing a large proportion of arginine and ammonia, with aspartic acid, valine, leucine, and isoleucine as major components.²⁷ There is, as yet, no evidence that the complex is associated with the collagenous constituents of dentine, unless by ionic linkage.

90Strontium and Mineralised Tissues.—The increased concentration of bone-seeking alkaline-earth nuclides arising from nuclear fission provides a recent alteration in the general environment which requires careful study. Among these baleful products of man's ingenuity are ⁸⁹Sr and ⁹⁰Sr; the latter is widely distributed in human bone and is potentially noxious owing to its comparatively long half-life of 28 years.²⁸

Stable strontium is a normal minor constituent of bone.²⁹ Using an improved method of determination by radioactivation analysis, Sowden and Stitch³⁰ found that samples of bone from normal persons of both sexes and different ages contained about 100 µg./g. of ashed tissue. There is no evidence that quantities of strontium of this order are harmful to bone. The possible hazard to health from ingestion of ⁹⁰Sr would therefore be due to its radiation activity and not to its chemical toxicity. ⁹⁰Strontium is deposited in the United Kingdom approximately in proportion to the rainfall in a given area. The greatest uptake of the nuclide is by vegetation of hill pastures where the soil is acidic and often deficient in calcium and phosphorus.³¹ Hill sheep which graze on these pastures are therefore sensitive indicators of the degree of contamination of a particular area.

Biochemically, strontium behaves very like calcium,³² but there is a definite discrimination against strontium in the presence of an adequate calcium intake. Thus, calcium is preferentially absorbed from the gut, and strontium is more readily excreted *via* the urine than is calcium. There is therefore preferential utilisation of calcium in bone formation and in lactation. Two recent papers are of particular interest; Morgan and

²⁶ R. L. Hartles and A. G. Leaver, *Arch. Oral Biol.*, 1960, **1**, in the press.

²⁷ A. G. Leaver, J. E. Eastoe, and R. L. Hartles, *Arch. Oral Biol.*, 1960, **1**, in the press.

²⁸ J. L. Kulp, W. R. Ecklemann, and A. R. Schubert, *Science*, 1957, **125**, 219; W. R. Ecklemann, J. L. Kulp, and A. R. Schubert, *ibid.*, 1958, **127**, 266.

²⁹ R. M. Hodges, N. S. MacDonald, R. Nusbaum, R. Stearns, F. Ezmirlian, P. Spain, and C. McArthur, *J. Biol. Chem.*, 1950, **185**, 519.

³⁰ E. M. Sowden and S. R. Stitch, *Biochem. J.*, 1959, **67**, 104.

³¹ F. J. Bryant, A. C. Chamberlain, A. Morgan, and G. S. Spicer, *J. Nuclear Energy*, 1957, **6**, 22.

³² H. G. Jones and W. S. Mackie, *Brit. J. Nutr.*, 1959, **13**, 355.

Wilkins³³ analysed the carcass of a yearling sheep reared on hill pasture in an area of high rainfall. The animal was killed in 1957, and analysis showed that the average activity of the whole skeleton was 182 strontium units (1 S.U. = 10^{-12} c per g. of calcium), the level in the teeth was lower (135 S.U.) and that in the pelvis was 203 S.U. Jones and Mackie³² carried out experiments on Scottish Blackface wethers aged 12—15 months; they administered ^{89}Sr and ^{45}Ca simultaneously to their animals and found that the proportion of the dose absorbed and deposited in the skeleton was for ^{45}Ca four times that for ^{89}Sr . They found that the major discrimination against ^{89}Sr was in absorption and urinary excretion. There appeared to be little or no discrimination in rate of excretion into the intestine or in transfer of nuclides from serum to bone.

An interesting experiment is reported by Holgate.³⁴ Rabbits were given a single injection of ^{90}Sr (10^{-4} c per 100 g. of body weight), and the uptake was determined for teeth and femur. Rabbits have teeth which are continuously growing, but they remain constant in length owing to attrition. The ^{90}Sr content of the femur was maximal eight hours after injection and then fell steadily to a minimum after 30 days, this level was then maintained almost constant until the last animals were killed at 180 days. This suggests that most of the ^{90}Sr is rapidly adsorbed on the surface of the bone crystals or stays in the hydration shell. Most of the ^{90}Sr in the bone then returns to the tissue fluids and blood as their content of ^{90}Sr falls. A smaller portion penetrates to the interior of the crystal lattice, possibly by a recrystallisation process, and is only slowly removed as remodelling occurs. In teeth (continuously growing) the picture was quite different. The ^{90}Sr in teeth increased steadily for 30 days after the injection and then fell to a smaller value than in bone after 100 days. This can be explained by the liberation of ^{90}Sr from bones into the blood stream in the days following injection, thus providing a continuously available though decreasing supply of nuclide for incorporation into teeth. Once formed, tooth mineral is much more resistant to change than is bone, and the ^{90}Sr burden remains until the tooth is worn away by normal attrition. Thus the amount of ^{90}Sr in the tooth will increase until the first deposit reaches the biting surface and begins to wear away. In an animal with teeth of non-persistent growth, such as the monkey or man, ^{90}Sr taken up by the developing teeth after exposure to a single high dosage is retained for a very long time. Thus a child exposed to a high level of ^{90}Sr might expect to have radioactive teeth so long as those teeth remained *in situ*, whereas the level in the bones would begin to decrease shortly after exposure.

Information concerning the ^{90}Sr content of human bone is not easily obtained; Holgate³⁴ quotes the annexed data as the latest available figures.

Age	March, 1955	July, 1956
Stillborn	0.44 S.U.	0.55 S.U.
1 month to 1 year	0.70 S.U.	1.1 S.U.
1 year to 5 years	0.83 S.U.	1.2 S.U.
5 years to 20 years	0.25 S.U.	0.45 S.U.
Over 20 years	0.11 S.U.	0.1 S.U.

³³ A. Morgan and J. E. Wilkins, *Biochem. J.*, 1959, **71**, 419.

³⁴ W. Holgate, *Brit. Dent. J.*, 1959, **107**, 131.

The largest rise is in children under 5 years of age. The Medical Research Council suggested that the burden of ^{90}Sr in human bone should not be allowed to rise above 100 S.U., and that if the level reached 10 S.U., then the problem should receive immediate consideration.

Status of Fluoride in Bones and Teeth.—Attention has been focused recently on problems associated with the skeletal deposition of fluoride since many communities are consuming water which naturally contains fluoride, and others are drinking water to which fluoride has been added to bring the concentration up to one part per million (1 p.p.m.). The latter measure has been adopted in certain areas in the United States and in three demonstration areas in the United Kingdom, for it has been shown that the consumption of such a drinking water results in about a 50% reduction in dental-caries experience. The topic of the relation of fluoride to dental caries has been extensively reviewed.³⁵

Fluoride is bone-seeking and all bones appear to contain some fluoride. Its concentration in the skeleton increases with advancing years and in some measure with dietary intake.³⁶ Jackson and Weidmann³⁷ have recently examined the fluoride content of human bone in relation to age and water supply in three areas in the United Kingdom, where the water contained <0.5, 0.8, and 1.9 p.p.m. of fluoride respectively. They found that in all instances the amount of fluoride in bone increased with age up to a maximum at about 55 years. At this point bones from the three areas contained 190, 245, and 400 mg. per 100 g. of dry, fat-free material.

In the case of teeth, fluoride is deposited systematically only during their formation. It has been found that the fluoride content of enamel increases with the fluoride content of the water supply.³⁷ Brudevold and his colleagues³⁸ have suggested that fluoride deposition in enamel takes place in three stages, during enamel formation, after mineralisation is complete but before eruption of teeth, and after eruption during the life span of the tooth. The last two methods of deposition are due to physicochemical changes in the outermost layers of the formed enamel. The maximum concentration of fluoride (3370 p.p.m.) was in the outer enamel from teeth formed in an area where the drinking water contained 5.0 p.p.m. The corresponding innermost enamel contained 570 p.p.m. Comparative figures from areas where the water contained 1.0 and 0.1 p.p.m. were 889 and 129 p.p.m., and 499 and 42 p.p.m., respectively.

The fixation of fluoride is believed to be by exchange with hydroxyl in the crystal lattice or at the crystal surface.³⁹ McCann,⁴⁰ studying the uptake of fluoride by a synthetic hydroxyapatite over a wide range, concluded

³⁵ "The Fluoridation of Domestic Water Supplies in North America," H.M.S.O., London, 1953; H. H. Stones, *Brit. Dent. J.*, 1954, **96**, 173; W. F. Stilwell, N. L. Edson, and P. V. E. Stanton, "The Fluoridation of Public Water Supplies," 1957, Government Printer, Wellington, N.Z.; Wld. Hlth. Org., *Techn. Rep. Ser.*, 1958, No. 146.

³⁶ G. E. Glock, F. Lowater, and M. M. Murray, *Biochem. J.*, 1941, **35**, 1235.

³⁷ D. Jackson and S. M. Weidmann, *J. Path. Bact.*, 1958, **76**, 451.

³⁸ S. Isaac, F. Brudevold, F. A. Smith, and D. E. Gardner, *J. Dent. Res.*, 1958, **37**,

³⁹ W. F. Neuman, M. W. Neuman, E. R. Main, J. O'Leary, and F. A. Smith, *J. Biol. Chem.*, 1950, **187**, 655.

⁴⁰ H. G. McCann, *J. Biol. Chem.*, 1953, **201**, 247.

that at levels of a few parts per million fluorapatite was formed exclusively; with increasing concentration of fluoride a mixture of fluorapatite and calcium fluoride was formed, until at concentrations above 0.2% calcium fluoride is the main product. The uptake of fluoride by the enamel surface has been studied by using ^{18}F , and a method has been developed for the analysis of small quantities of stable fluoride using an isotope-dilution technique.⁴¹

From the dental point of view the important observations have been made that the solubility of surface enamel at pH 4—6 decreases as the fluoride content increases.⁴² Much more information is required concerning the manner in which fluoride is deposited in a mineralised tissue.

Conclusion.—The study of the chemistry of bone and teeth bears a direct relation to three major problems. First, in an ageing population where an increasing number of individuals is surviving beyond three score years and ten, consequences of bone fracture are serious; the chemistry of ageing and senile bone must receive greater attention if the reasons for the slow healing of fractures of aged bone are to be understood. Secondly, the incidence of dental caries in young children is distressingly high; an increased knowledge of the intimate chemistry of the tooth may provide information concerning the decay process. Thirdly, the recently increased concentration of bone-seeking nuclides in the general environment requires that the pattern of their skeletal deposition be studied.

R. L. H.

3. METABOLISM OF β -CAROTENE AND RELATED PROVITAMINS A

ALTHOUGH 30 years have passed since Moore¹ demonstrated that β -carotene (1) was transformed into vitamin A (13) in the animal body, the mechanism of this process represented by route A of Chart 1 is still unknown. The structural relation of the two compounds suggested to Karrer and his co-workers² that β -carotene might undergo hydrolysis of the central double bond to form two molecules of vitamin A, but attempts to elucidate the details of the biological system have been fruitless. Consequently, parallel work on the structural features required for provitamin A and vitamin A activity has received more attention. This has been reviewed by Heilbron *et al.*,³ Zechmeister,⁴ Baxter,⁵ Goodwin,⁶ and, more recently, by Isler and Zeller.⁷

The chemistry and biochemistry of the various carotenoids and related

¹ J. H. Fremlin, J. L. Hardwick, and J. Suthers, *Nature*, 1957, **180**, 1179; J. L. Hardwick, J. H. Fremlin, and J. Mathieson, *Brit. Dent. J.*, 1958, **104**, 47.

² S. B. Finn and C. de Marco, *J. Dent. Res.*, 1956, **35**, 185; S. Isaac, F. Brudevold, F. A. Smith, and D. E. Gardner, *ibid.*, 1958, **37**, 254.

¹ T. Moore, *Biochem. J.*, 1930, **24**, 692.

² P. Karrer, R. Morf, and K. Schöpp, *Helv. Chim. Acta*, 1931, **14**, 1036, 1431.

³ I. M. Heilbron, W. E. Jones, and A. L. Bacharach, *Vitamins and Hormones*, 1944, **2**, 155.

⁴ L. Zechmeister, *Vitamins and Hormones*, 1949, **7**, 57.

⁵ J. G. Baxter, in "Progress in the Chemistry of Organic Natural Products," 1952, Vol. IX, p. 41 (ed. L. Zechmeister), Springer-Verlag, Vienna.

⁶ T. W. Goodwin, "The Comparative Biochemistry of the Carotenoids," Chapman & Hall Ltd., London, 1952.

⁷ O. Isler and P. Zeller, *Vitamins and Hormones*, 1957, **15**, 31.

compounds have also been described in considerable detail by Karrer and Jucker⁸ and Goodwin,^{6,9} respectively. Again, work on the chemical synthesis of vitamin A and various carotenoids and larger homologues has also been reviewed recently,^{5,8,10,11} but detailed knowledge regarding their metabolism is still lacking. By newly developed methods of chemical synthesis, a variety of compounds, intermediate in size between β -carotene and vitamin A, can be prepared which should assist the biochemist in determining the nature of enzymic attack on molecules such as carotenoids which possess long, conjugated double-bond systems. The present Report discusses work carried out during the last few years towards this end with particular reference to the provitamin A-vitamin A transformation.

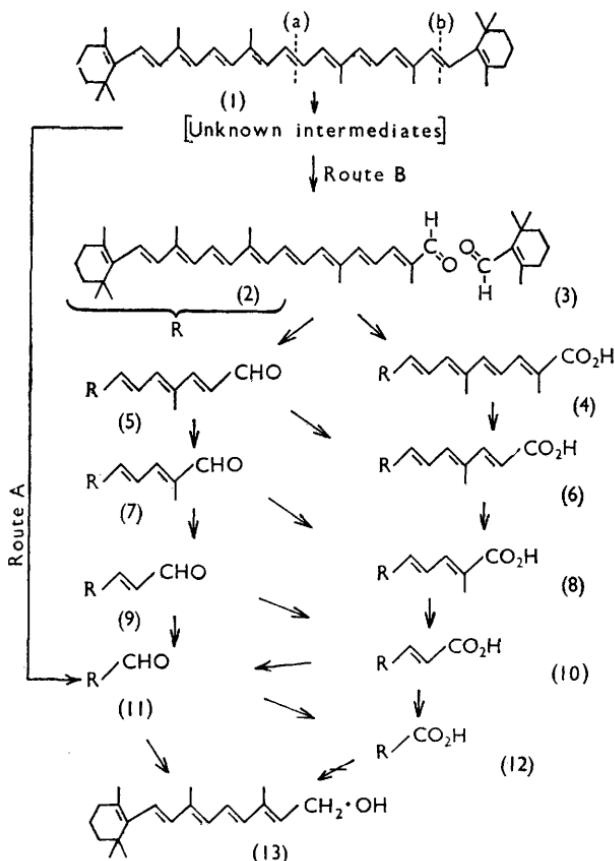


CHART 1. Suggested routes for conversion of β -carotene into vitamin A.

⁸ P. Karrer and E. Jucker, "Carotenoids," transl. E. Braude, Elsevier, Amsterdam, 1950.

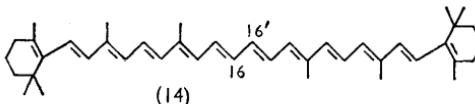
⁹ T. W. Goodwin, *Ann. Rev. Biochem.*, 1955, **24**, 497.

¹⁰ H. H. Inhoffen and H. Siemer, "Progress in the Chemistry of Organic Natural Products," 1952, Vol. IX, p. 1 (ed. L. Zechmeister), Springer-Verlag, Vienna.

¹¹ O. Isler, H. Lindlar, M. Montavon, R. Rüegg, G. Saucy, and P. Zeller, *Chem. Soc. Special Publ.* No. 4, 1956, p. 47.

Provitamin A-Vitamin A Conversion.—Structurally, it would appear that, if β -carotene were oxidised at the central double bond, two molecules of retinene (vitamin A aldehyde) (11) might be formed which could be immediately reduced to vitamin A.¹² On the other hand, if an excentric bond is attacked, only one molecule of vitamin A would result from the further degradation of the larger fragment (see Chart I). The evidence for and against these two views has been outlined previously.^{6,13,14} A major difficulty of the problem is that the conversion of β -carotene into vitamin A takes place only on a small scale in experimental animals and relatively slowly. Further, it has not yet been possible to prepare an enzyme system which will carry out the reaction *in vitro*, so the possibility of obtaining sufficient intermediates to allow their proper characterisation seems remote.

An alternative approach is to synthesise substances closely related to biological intermediates indicated by some hypothetical scheme such as terminal fission followed by β -oxidation (route B in Chart I). Here either the β -apocarotenals (2, 5, 7, and 9) or the related series of β -apocarotenoic acids (4, 6, 8, and 10, respectively) would be possible intermediates. There were several reasons for considering this possible, *e.g.*: (a) Most biological assays¹⁵ suggest that only one molecule of vitamin A is formed per molecule of β -carotene, although perhaps two may be obtained in the presence of optimal amounts of tocopherol.^{16,17} No intermediate values have been obtained. (b) Two yellow pigments were isolated¹⁸ from the lipids of the horse intestine and tentatively identified¹⁹ as β -apo-10'- (5) and β -apo-12'-carotenal (7); these must have resulted from terminal oxidation of β -carotene. They had not hitherto been detected in plant extracts, so it was assumed they were formed in the animal. (c) Chemical oxidation of β -carotene begins at one end of the conjugated double-bond system (see below), and often there is an overall parallelism between chemical and biological oxidations. (d) 16,16'-Bishomo- β -carotene (14) is biologically



active²⁰ (20% as active as all-*trans*- β -carotene) although it does not possess a central double bond. (e) Substances such as α -“vitamin” A²¹ or 3-hydroxy-“vitamin” A²² which would arise in addition to vitamin A from

¹² R. F. Hunter, *Nature*, 1946, **158**, 257; J. Glover, T. W. Goodwin, and R. A. Morton, *Biochem. J.*, 1948, **43**, 109.

¹³ J. S. Lowe and R. A. Morton, *Vitamins and Hormones*, 1956, **14**, 97.

¹⁴ T. Moore, “Vitamin A,” Elsevier, Amsterdam, 1957.

¹⁵ F. M. M. Hume, *Brit. J. Nutrit.*, 1951, **5**, 104.

¹⁶ C. J. Koehn, *Arch. Biochem. Biophys.*, 1948, **17**, 337.

¹⁷ M. J. Burns, S. M. Hauge, and F. W. Quackenbush, *Arch. Biochem. Biophys.*, 1951, **30**, 341.

¹⁸ G. N. Festenstein, Ph.D. thesis, Liverpool, 1951.

¹⁹ E. R. Redfearn, Ph.D. thesis, Liverpool, 1954.

²⁰ H. J. Deuel, jun., H. H. Inhoffen, J. Ganguly, L. Wallcave, and L. Zechmeister, *Arch. Biochem. Biophys.*, 1952, **40**, 352.

²¹ S. R. Ames, W. J. Swanson, and P. L. Harris, *J. Amer. Chem. Soc.*, 1955, **77**, 4136.

²² R. H. Painter, Ph.D. thesis, Liverpool, 1955.

central fission of dietary α -carotene or cryptoxanthin, respectively, and are known to be stored in the liver, have never been detected in rats.

β -Apo-8'-carotenal (2) had been reported²³ to be vitamin-A-active, so the remaining members of the series were prepared by oxidising β -carotene with hydrogen peroxide in the presence of osmium tetroxide.²⁴ More recently they have been elegantly synthesised by Rüegg and his colleagues.^{25,26}

In studying the metabolism of β -apo-8', -10', and -12'-carotenal in the rat, it was observed¹⁹ that small amounts of some were oxidised to the corresponding carboxylic acids; consequently the higher vinylogues of vitamin A acid were also prepared.

Preparation of β -Apocarotenals.—*Oxidation of β -carotene.* When β -carotene is oxidised with chromium trioxide, the double bonds of the β -ionone rings are preferentially attacked and the end products are semi- β -carotenone and β -carotenone.²⁷ Alkaline permanganate, on the other hand, appears to attack the terminal double bonds of the central chain yielding β -apocarotenals (2, 5, 7) having one β -ionone ring intact, but it does not appear to form retinene²⁸ (11). With hydrogen peroxide alone, however, retinene is formed in small yield²⁹ (*ca.* 1%), though with osmium tetroxide as catalyst^{24,30} a moderate yield (*ca.* 30%) is obtained. When a solution of hydrogen peroxide in *t*-butyl alcohol is used,³¹ the progress of the reaction could be followed with time and the β -apo-8', -10', and -12'-carotenal as well as retinene were isolated in chromatographically pure form.¹⁹ Grob and Bütler³² also reported finding these aldehydes. More recently a small amount of the β -apo-14'-carotenal (9) has also been found³³ in the reaction mixture. Dialdehydes, having 3, 4, 5, and 6 ethylenic bonds in conjugation, and corresponding to segments of the central chain of β -carotene, are also present.¹⁹ The pattern of the rates of production of the various aldehydes by osmium tetroxide-hydrogen peroxide pointed to a progressive removal of the ends of the conjugated system rather than random reaction along it.¹⁹ The reagent attacks the penultimate double bond in the conjugated system; semi- β -carotenone or β -carotenone have never been detected among the products. Further, the yields of pure retinene or higher aldehydes from β -carotene were found never to exceed 10—12%.

Direct synthesis. In one procedure (Chart 2) for the synthesis of β -apo-12'-carotenal (C_{25}) (7), Rüegg and colleagues²⁵ used as starting point the

²³ H. von Euler, P. Karrer, and U. Solmssen, *Helv. Chim. Acta*, 1938, **21**, 211.

²⁴ N. L. Wendler, C. Rosenblum, and M. Tishler, *J. Amer. Chem. Soc.*, 1950, **72**, 234.

²⁵ R. Rüegg, H. Lindlar, M. Montavon, G. Saucy, S. F. Schaeren, U. Schwieder, and O. Isler, *Helv. Chim. Acta*, 1959, **42**, 847.

²⁶ R. Rüegg, M. Montavon, G. Ryser, G. Saucy, U. Schwieder, and O. Isler, *Helv. Chim. Acta*, 1959, **42**, 854.

²⁷ R. Kuhn and H. Brockmann, *Annalen*, 1935, **516**, 95.

²⁸ P. Karrer and U. Solmssen, *Helv. Chim. Acta*, 1937, **20**, 682; P. Karrer, U. Solmssen, and W. Gugelmann, *ibid.*, p. 1020.

²⁹ R. F. Hunter and N. E. Williams, *J.*, 1945, 554.

³⁰ G. C. L. Goss and W. D. Macfarlane, *Science*, 1947, **106**, 375.

³¹ N. A. Milas and S. Sussman, *J. Amer. Chem. Soc.*, 1936, **58**, 1302.

³² E. C. Grob and R. Bütler, *Helv. Chim. Acta*, 1954, **37**, 1908.

³³ U. Lüthi, M. J. Fishwick, and J. Glover, unpublished work (1957).

C_{19} β -aldehyde (15) which is an intermediate in the industrial synthesis of β -carotene. This was condensed with lithium acetylide in liquid ammonia to form the C_{21} acetylenic alcohol (16). The latter without purification was

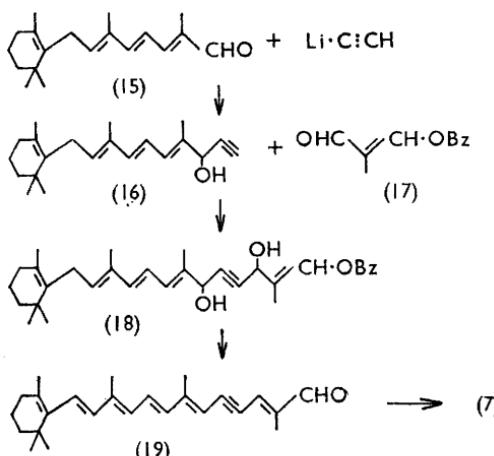


CHART 2. *Synthesis of β -apo-12'-carotenal.*

coupled with methylmalondialdehyde enol-ether (20) in a Grignard reaction, giving the ester (21), which with acetic acid in propan-2-ol under nitrogen yielded 15,15'-didehydro- β -apo-12'-carotenal (C_{25}) (19). Partial hydrogenation with a Lindlar catalyst³⁵ and isomerisation of the 15,15'-

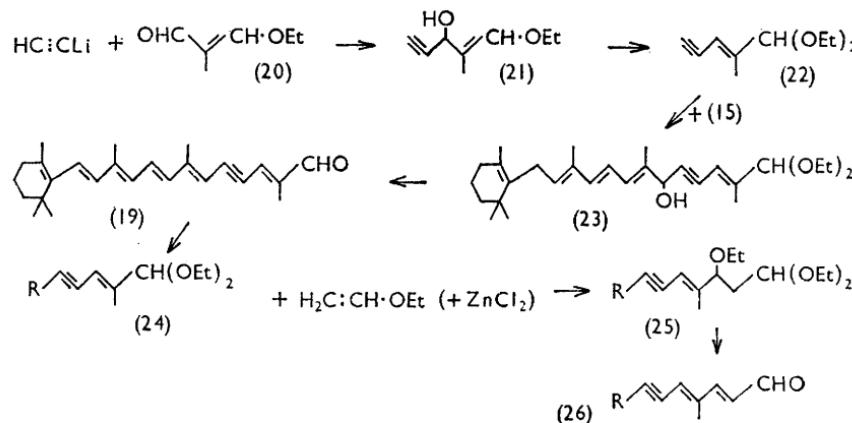


CHART 3. *Synthesis of 15,15'-didehydro- β -apocarotenals.*

cis- β -apocarotenal intermediate produced a good yield of the all-*trans*- β -apo-12'-carotenal (7).

An improved procedure was followed later (Chart 3). Sodium or lithium acetylide was first condensed with the methylmalondialdehyde enol-ether (20) to form the ether (21). This with ethyl orthoformate produced the

³⁴ O. Isler, H. Lindlar, M. Montavon, R. Rüegg, and P. Zeller, *Helv. Chim. Acta*, 1956, **39**, 249.

³⁵ H. Lindlar, *Helv. Chim. Acta*, 1952, **35**, 446.

acetal (22) which is a useful new building unit for branched polyenes. Condensation of C_{19} β -aldehyde and this compound by means of lithium amide in liquid ammonia afforded a C_{25} hydroxy-acetal (23) which was readily converted by acid into the free aldehyde (19).

The higher vinylogues were prepared²⁶ by using this aldehyde as starting material. The diethyl acetal (24) was condensed with ethyl vinyl ether in the presence of zinc chloride, to form the dehydro- β - C_{27} ether acetal (25), which on acid hydrolysis gives the free aldehyde in good yield. Repetition of these steps using alternately ethyl propenyl ether and ethyl vinyl ether for the condensation with the appropriate 15,15'-didehydro- β -apocarotenal acetals enabled Rüegg and colleagues²⁶ to synthesise the higher members of the series up to C_{40} . Reduction of the acetylenic bonds in each of these various 15,15'-didehydro- β -apocarotenals with a Lindlar catalyst, followed by isomerisation of the *cis*-derivatives by heat, gave the all-*trans*- β -apocarotenals which are listed with their ultraviolet absorption maxima in Table 1. Further reduction of the aldehydes with lithium aluminium hydride affords the corresponding series of β -apocarotenols.

TABLE 1. Ultraviolet absorption maxima (μm) of β -apocarotenoids in light petroleum (for R see Chart 1).

Compound	$X = \text{CH}_2\cdot\text{OH}$	CHO	CO_2H	CO_2Me
C_{20}	326	367		350
C_{22}	343, 355	385	376	373 ^a
C_{25}	—	—		325 ^a
	377, 393 376, 393	414 410		400 ^a
	—	—		327 ^a
C_{27}	403, 424	435	430	426 ²⁶
C_{30}	426, 453	457	448	445, 471 ²⁶
C_{32}	443, 471	473	458, 495	464, 491 ²⁶

^a Ref. 36.

Synthesis of β -Apocarrenoic Esters.—Two of this series, ethyl β -apo-14'-carrenoate (28) and β -apo-12'-carrenoate (31) containing 22 and 25 carbon atoms respectively, were synthesised from retinene³⁶ (see Chart 4).

Ester of the C_{22} acid. In a modification of the Reformatsky reaction,³⁷ retinene was condensed with ethyl bromoacetate in refluxing pyridine-benzene (5% v/v) in the presence of zinc dust to form the C_{22} 15-hydroxy-ester (27) in 80—85% yield. Dehydration with freshly prepared aluminium

³⁶ S. Fazakerley, Ph.D. thesis, Liverpool, 1957.

³⁷ R. L. Shriner, "Organic Reactions," John Wiley & Sons Inc., New York, 1942, Vol. I.

phosphate then gave a variable yield (30—60%) of ethyl β -apo-14'-carotenoate.

Ester of the C₂₅ acid. Reduction of the ester (28) with lithium aluminium hydride formed the C₂₂ alcohol (29) which was readily converted into the aldehyde β -apo-14'-carotenal (30) by manganese dioxide.³⁸ A similar condensation of the latter with ethyl bromopropionate followed by dehydration, again with aluminium phosphate, enabled ethyl β -apo-12'-carotenoate (31) to be obtained. Condensation of retinene with ethyl γ -bromotiglate was used in the preparation of the 15-hydroxy-ester (32), but dehydration of this compound always yielded the retro-acid, a difficulty often experienced in chain-lengthening of polyenes.³⁹

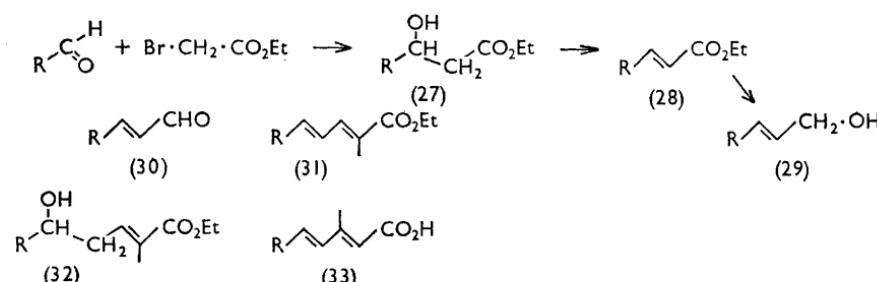


CHART 4. C₂₂ and C₂₅ β -apocarotenoids.

The isomeric C₂₅ acid (33) having a methyl group in the β -position to the carboxyl group was synthesised by Redfearn⁴⁰ using a procedure similar to that of Robeson *et al.*⁴¹ for vitamin A. It involved the condensation of retinene with methyl β -methylglutaconate to form a diester which on hydrolysis and decarboxylation gives the C₂₅ mono-acid (33).

C₂₇ to C₄₀ acids. The higher vinylogues containing 27—40 carbons (*e.g.*, torularhodin) have been recently synthesised by Isler and colleagues⁴² by the Wittig reaction.⁴³ The chain-lengthening was carried out as follows: methyl bromoacetate and triphenylphosphine gave the phosphonium bromide which with sodium methoxide or aqueous sodium hydroxide yielded the phosphorane (34). This compound reacted smoothly with 15,15'-di-dehydro- β -apo-12'-carotenal (C₂₅) (19); the resulting ester (35) was partially hydrogenated and isomerised as described above, giving methyl all-trans- β -apo-10'-carotenoate (36) (C₂₇). Saponification liberates the free acid. The higher members, *e.g.*, β -apo-8'-carotenoic acid (39), were prepared by using the same sequence of reactions with the various 15,15'-didehydro- β -apocarotenals and the appropriate phosphorane (34 or 37) (Chart 5).

³⁸ S. Ball, T. W. Goodwin, and R. A. Morton, *Biochem. J.*, 1948, **42**, 516.

³⁹ O. Isler, W. Huber, A. Ronco, and M. Kofler, *Helv. Chim. Acta*, 1947, **30**, 911; H. O. Huisman, A. Smit, S. Vromen, and L. G. M. Fischer, *Rec. Trav. chim.*, 1952, **71**, 899.

⁴⁰ E. R. Redfearn, 1957, personal communication.

⁴¹ C. D. Robeson, J. D. Cawley, L. Weisler, M. H. Stern, C. C. Eddinger, and A. J. Chechak, *J. Amer. Chem. Soc.*, 1955, **77**, 4111.

⁴² O. Isler, W. Guex, R. Hüegg, G. Ryser, G. Saucy, U. Schwieter, M. Walter, and A. Winterstein, *Helv. Chim. Acta*, 1959, **42**, 864.

⁴³ G. Wittig and U. Schöllkopf, *Chem. Ber.*, 1954, **87**, 1954; G. Wittig and W. Haag, *ibid.*, 1955, **88**, 1654.

The absorption maxima of the series of aldehydes, acids, esters, and alcohols are given in Table 1.

Provitamin-A Activity of β -Apocarotenoids.—The availability of the above compounds has enabled the terminal oxidation hypothesis to be examined. Preliminary results with the β -apocarotenals suggested to Glover and Redfearn⁴⁴ that if oxidative fission of the 7',8'-double bond of β -carotene occurred [i.e., fission at (b) in formula (1)], the larger β -apo-8'-carotenal fragment might be degraded by β -oxidation. The 9'- and 13'-methyl groups lie in α -positions to the potential carboxyl groups; they would slow the process but not stop it. It would, however, be stopped at the central carbon atoms because of methyl substitution in the β -position, at C₍₁₃₎. Again, if the normal β -oxidation were involved, one might also expect the activated forms of the β -apocarrenoic acids to be intermediates.

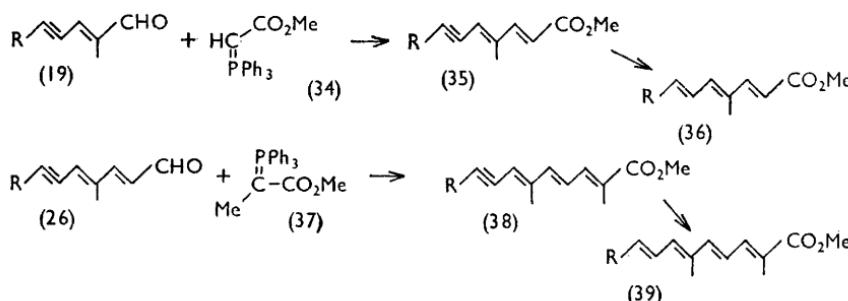


CHART 5. *Synthesis of β -apocarrenoic acids.*

The metabolism of these synthetic compounds in rats has been examined^{44,45} in two ways. First, the minimum daily requirement to promote steady growth of vitamin-A-deficient rats at a rate at least equal to that produced by 1 μ g. of vitamin A was determined under standard dietary conditions. Secondly, lipids mainly from livers and intestines of groups of vitamin-A-deficient rats dosed with the various β -apocarotenoids were examined for metabolites, in particular for vitamin A. It is necessary to do this because growth-activity could mean merely conversion into vitamin A acid and not vitamin A alcohol, which is the main product of β -carotene metabolism. Not all the β -apocarotenoids have yet been tested in this way, but the results for some of the lower members of the series are set out in Table 2, together with those for vitamin A and its aldehyde and acid, as well as for β -carotene.

Recently, the biological activity of the β -apocarotenals containing 25–32 carbons has been determined by Maurisch *et al.*, using the U.S.P. rat curative growth assay.⁴⁶ The results, which are included in Table 2, give a more precise measure of activities in relation to that of β -carotene.

It is clear that all the compounds examined are vitamin-A-active, but

⁴⁴ J. Glover and E. R. Redfearn, *Biochem. J.*, 1954, **58**, xv.

⁴⁵ S. Fazakerley and J. Glover, *Biochem. J.*, 1957, **65**, 38P.

⁴⁶ Personal communication from Dr. M. Montavon (Messrs. Hoffmann-La Roche, Switzerland), regarding results reported by W. Maurisch, E. de Ritter, J. Vreeland, and R. Krukar at an Amer. Chem. Soc. meeting, September, 1959.

to varying degrees, confirming the earlier work of von Euler *et al.*²³ All the compounds, except vitamin A acid, were converted *in vivo* into vitamin A, which was characterised by its chromatographic behaviour on alumina, ultraviolet absorption, and antimony trichloride colour reaction.^{44,45}

TABLE 2. Provitamin A activity of some β -apocarotenoids in the rat.

β -Apocarotenoid *	Ref.	Daily requirement (µg.)	Biological activity				
			Vitamin A formation		Activity (%) ⁴⁶ vs. all-trans- β -carotene	Mean	Range †
			Dose (mg./rat)	Vitamin A (%)			
C_{20}	$R/\text{CH}_2\cdot\text{OH}$ (vitamin A)	<i>a</i>	1	15	40—70		
	R/CHO	12	1	1·7—8·0	50—70		
	$R/\text{CO}_2\text{H}$	44	12	10	0		
	$R/\text{CH}_2\cdot\text{OH}$	—	30	—	—		
C_{22}	$R/\text{CO}_2\text{Me}$	—	50—100	2·5	4		
	R/CHO	19	5—10	2·5	3		
	$R/\text{CO}_2\text{Me}$	44	2	0·9	4	125	86—182
C_{25}	$R/\text{CH}_2\cdot\text{OH}$	—	<2	2·5	21		
	$R/\text{CO}_2\text{Me}$	49	—	1—10	3		
	R/CHO	49	5—10	1—10	0·2		
	$R/\text{CO}_2\text{H}$	19	—	1·5	Trace	5·8	48—70
C_{27}	R/CHO	—	~5 ^c	0·6	3 ^d	72	65—80
C_{30}	R/CHO	—	—	—	—	40	29—56
C_{32}	R/CHO	—	2	1—4	10—15		
β -Carotene							

* For R see Chart 1.

† P = 0·05.

^a E. Le B. Gray, K. C. D. Hickman, and E. F. Brown, *J. Nutrition*, 1940, **19**, 39.
^b I. M. Sharman, *Brit. J. Nutrition*, 1949, **3**, viii. ^c Ref. 23. ^d Ref. 19.

Whether the minimum daily requirements or the activities relative to that of β -carotene are compared, the C_{25} compounds are superior to the other members so far examined and as good as or better than β -carotene in supporting growth; so they could be related to intermediates in the metabolism of β -carotene or of the higher vinylogues. However, the low biological activity of the C_{22} group, varying from one-fifth to one-fiftieth of the C_{25} group, means that they are unlikely to be intermediates in the metabolism of the latter. The absence of any C_{22} compound in the lipids of animals dosed with the various C_{25} compounds tends to confirm this. The lower

biological activities for the higher β -apocarotenals (C_{27} to C_{32}) implies that they also are not intermediates in the main pathway for the formation of vitamin A from β -carotene; however, they could be intermediates in a minor route.

With regard to the ability of the various β -apocarotenoids to produce vitamin A *in vivo* after single doses, only the ester of the C_{25} acid was superior to β -carotene. When, therefore, the growth tests and the speed of conversion into vitamin A are taken into account, only the C_{25} 15-hydroxy-acid really behaves as if it were closely related to an intermediate on the main route of conversion of β -carotene into vitamin A.

β -*Apo-8'-, -10', and -12'-carotenals. All these aldehydes appear to be well absorbed by the rat. A portion of each is immediately reduced to the carotenol in the intestine and another portion is oxidised to the acid; some of the latter then becomes esterified. In the lipids from rats dosed with β -apo-10'-carotenal, a little β -apo-12'-carotenol was found. Its formation must be somewhat analogous to that of vitamin A alcohol from the ester of the C_{22} acid (see below). However, no compound with a carbon skeleton intermediate in size between either β -apo-8'-carotenal or β -apo-12'-carotenal and vitamin A was detected in the lipids from animals dosed with those aldehydes.*

β -*Apo-14'- and -12'-carotenoic esters. Both these esters are well absorbed and are stored in the fat depots as well as in the liver, whereas the free acids tend to be metabolised quickly. This difference was also noted by Redfearn⁴⁰ in studies with the C_{25} β -apo-12'-carotenoic acid isomer (33) and vitamin A acid, and their esters: these three cannot be reduced to the corresponding β -apocarotenols *in vivo*. Some of the C_{22} acid is converted into vitamin A in the intestine during absorption and the process is completed in the liver. If β -oxidation were involved in this step, then vitamin A acid would be the main product and not vitamin A alcohol; so probably a different enzyme system is utilised. Similarly, the C_{22} acid or alcohol has not been detected among the metabolites of the ester of the C_{25} acid.*

Chromatography of the recovered β -apo-12'-carotenoic ester fraction from the tissue lipids resolved it into two zones, both containing esters. One corresponded to the compound administered, having λ_{max} 396 m μ , but the second had λ_{max} 400 m μ . This bathochromic shift is characteristic of the change of a *cis*-compound into the more stable all-*trans*-form,^{5,47} involving the double bond adjacent to the carbonyl group. About 40% of the total ester fraction was in the *trans*-form in the wall of the small intestine, whereas in the liver this proportion had risen to 93—94%, indicating a progressive change to the all-*trans*-form.³⁶ This change probably accompanies hydrolysis and re-esterification of the acid with either glycerol or a higher alcohol. The C_{25} acid isomer behaved similarly. Whether this change is necessary before conversion into vitamin A or merely coincidental is not known. The metabolism of the higher β -apo-10'- and -8'-carotenoic acids or esters has not yet been fully investigated.

⁴⁷ H. O. Huisman, A. Smith, P. H. van Leeuwen, and J. H. van Rij, *Rec. Trav. chim.*, 1956, **75**, 977.

The C₂₅ isomer was studied⁴⁸ to determine the effect of methyl-substitution in the β -position to the terminal carboxyl group on conversion of the compound into vitamin A. Metabolism of this compound by the β -oxidation system is blocked, yet vitamin A alcohol is formed in small amounts. The yield was only slightly less than that from the normal β -apo-12'-carotenoic ester; so another enzyme system must be operative.

15-Hydroxy-esters. Both the C₂₂ (27) and the C₂₅ acid (32) with a 15-hydroxyl group were converted into vitamin A, the latter in a yield (21% of vitamin A in 19 hr. after a single dose) which is greater than can be obtained from a single dose of β -carotene.

As the C₂₅ group of compounds proved the most active of the series tested, they were examined further for participation in the β -carotene-to-vitamin A transformation. β -Apo-12'-carotenal was selected first as representative of the group because it is readily converted into the acid and alcohol and would probably have the best chance to enter the enzyme system responsible for metabolising β -carotene. The aim of the work was to use the aldehyde as a trapping agent for any similar radioactive aldehyde derived from the metabolism of specifically labelled [15,15'-¹⁴C]- β -carotene in the rat.

Metabolism of [15,15'-¹⁴C]- β -Carotene.—Specifically labelled β -carotene was synthesised⁴⁹ and a small sample * was mixed with an excess of β -apo-12'-carotenal and administered to vitamin-A-deficient rats. The animals were killed 5 hr. after dosing, when absorption across the intestine would be optimal, and the lipids from the intestine and liver were examined.⁵⁰ Unchanged β -apo-12'-carotenal and β -apo-12'-carotenoic acid were isolated and purified chromatographically. The fraction containing the aldehyde was treated with hydroxylamine and the oxime crystallised. The acid fraction was too small for crystallisation even as a derivative, but it was purified by quantitative conversion into the ester followed by either rechromatography or reduction with lithium aluminium hydride, the resulting alcohol being isolated chromatographically. The specific activities of the various fractions were measured and compared with those of the original [15,15'-¹⁴C]- β -carotene and the [¹-¹⁴C] vitamin A isolated from the liver. The re-isolated β -apo-12'-carotenal and its metabolite, the corresponding acid, were labelled, indicating that a little β -carotene had been degraded to the aldehyde. As the aldehyde and acid had approximately the same specific activity, the former must have become labelled first since the action of aldehyde-oxidase is irreversible. Again, the agreement in their specific activities suggests that little or no [15,15'-¹⁴C]- β -apo-12'-carotenoic acid could have been derived from a higher homologue by β -oxidation. The aldehyde re-isolated from the lumen was radioactive, so presumably the [15,15'-¹⁴C]- β -carotene was first attacked there. Comparison of the specific activities of the two compounds shows that the amount formed was quite small (<3% of the

⁴⁸ E. R. Redfearn, *Biochem. J.*, 1957, **68**, 39P.

⁴⁹ H. H. Inhoffen, U. Schwieger, C. O. Chichester, and G. Mackinney, *J. Amer. Chem. Soc.*, 1955, **77**, 1053.

⁵⁰ J. Glover and P. P. Shah (1958), unpublished work.

* The Reviewer is grateful to Professor G. Mackinney for the gift of this material.

dose). The specific activity of the [$1\text{-}^{14}\text{C}$]-vitamin A was higher than that expected from the specific activities of the [$15,15'\text{-}^{14}\text{C}$]-labelled β -apocarotenoids and so must have been derived mainly by another route. This result confirms earlier observations that, while a small amount of β -carotene may be metabolised *via* carotenals, this is certainly not the main route.

Metabolism of [$U\text{-}^{14}\text{C}$]- β -Carotene and [$U\text{-}^{14}\text{C}$]-Retinene.—An attempt⁵¹ was made to answer the problem of central fission *versus* terminal oxidation by comparing the metabolism of [$U\text{-}^{14}\text{C}$]- β -carotene and -retinene during their absorption across the intestine of the rat. It was considered that oxidative attack on a terminal bond in β -carotene, followed by degradation of the larger fragment to retinene or vitamin A, would lead to small fragments which on entering the various metabolic pools would rapidly release $^{14}\text{CO}_2$ into the respired air. Central fission, on the other hand, would produce [$U\text{-}^{14}\text{C}$]-retinene or [$U\text{-}^{14}\text{C}$]-vitamin A directly, which being quickly absorbed would perhaps not be appreciably metabolised until they reached the liver later. In this event, the pattern of release of $^{14}\text{CO}_2$ would be more gradual.

[$U\text{-}^{14}\text{C}$]- β -carotene has been prepared^{52,53} in two laboratories and its metabolism studied in the rat. It was found to be degraded extensively to small fragments; $^{14}\text{CO}_2$ appeared quickly in the respired air, the amount being maximal 5 hr. after dosing (when absorption across the intestine is optimal) and declining. This was consistent with the above hypothesis. However, when [$U\text{-}^{14}\text{C}$]-retinene was used,⁵⁴ the pattern of release of $^{14}\text{CO}_2$ was almost identical, indicating that this molecule can also be rapidly degraded in transit across the intestine.

Conclusions.—(a) The general conclusion is that terminal oxidation of β -carotene, followed by progressive removal of the side chain of the larger fragment until retinene or vitamin A is left, is not the main metabolic route to vitamin A. (b) When such an oxidation does occur, however, the larger fragment can give vitamin A, but not necessarily by β -oxidation. (c) The very strikingly greater biological activity of the C_{25} β -apocarotenoids than of their homologues indicates that the former best provide the type of substrate which the enzyme system requires for fission. The relatively low yield of vitamin A obtained after single doses of β -apo-12'-carotenal, compared with the biological activity at low dose level, is difficult to understand. It may be that when it is administered in small doses the yield of vitamin A is better; or that vitamin A acid is produced to an appreciable extent. This acid would be difficult to trace chemically *in vivo*, but is biologically active.

J. G.

Note added in proof. An exhaustive study by Worker⁵⁵ with whole organs and tissue preparations to find a suitable system for studying the conversion of β -carotene into vitamin A has had little success.

⁵¹ M. J. Fishwick and J. Glover (1958), unpublished work.

⁵² J. Glover and E. R. Redfearn, *Biochem. J.*, 1953, **54**, viii; J. Glover and P. P. Shah, *ibid.*, 1957, **67**, 15P.

⁵³ J. S. Willmer and D. H. Laughland, *Can. J. Biochem. Physiol.*, 1957, **35**, 819.

⁵⁴ M. J. Fishwick, Ph.D. thesis, Liverpool, 1958.

⁵⁵ N. A. Worker, *Brit. J. Nutrition*, 1959, **13**, 400.

4. THE CHEMISTRY OF MUSCLE CONTRACTION AND RELAXATION

In view of the long interval since any aspect of muscle biochemistry has been reviewed in these Reports¹ the present discussion will refer to recent advances in the field set against the background of relevant earlier work.

Contraction.—*Structure of contractile system.* Like many biological processes the chemical events associated with contraction take place in a heterogeneous phase. Presumably, metabolites and cofactors, both of which are soluble and of relatively low molecular weight, interact with the insoluble contractile system within the cell, causing it to shorten and to bring about contraction of the tissue as a whole. To understand this

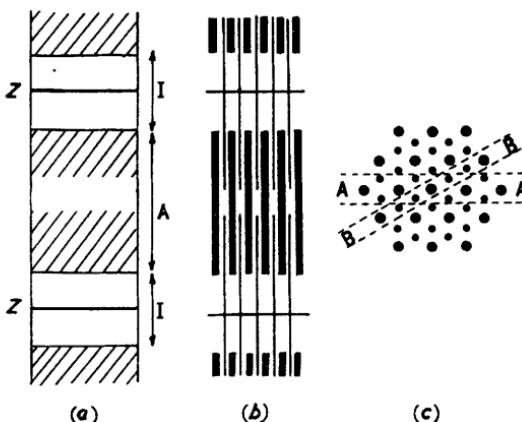


FIG. 1. *Structure of the myofibril.*

- (a) Appearance of myofibril of the striated muscle cell as seen with the ordinary light-microscope. Diameter of myofibril, 1–2 μ .
- (b) Diagrammatic representation of longitudinal section through myofibril in the direction A—A (Fig. 1c).
- (c) Diagrammatic representation of a cross-section of the A band showing the hexagonal array of thick (A) and thin (I) filaments. Note that the appearance of a longitudinal section depends on the angle of sectioning.

process detailed knowledge of the chemistry of the myofibril, the contractile unit, is not in itself enough; an understanding of the ultrastructure down to the molecular level is also required. When examined in the light-microscope the myofibril from striated muscle is characterised by dark and light bands, the A (anisotropic) and I (isotropic) bands respectively, which alternate along its length (Fig. 1). Electron-microscopy has proved an invaluable tool for studies of ultrastructure² and in the case of the striated myofibril two main filament types can be identified. The larger of these, the so-called A filaments, about 100 Å in diameter, are found in the A band arranged parallel to the myofibril axis. I filaments, about 50 Å in diameter, are the

¹ K. Bailey, *Ann. Reports*, 1946, **43**, 280; D. M. Needham, *ibid.*, 1952, **49**, 275.

² S. V. Perry, in "Comparative Biochemistry," ed. M. Florkin and H. S. Mason, Academic Press, New York, 1960, Vol. II, p. 245; *idem*, *Physiol. Rev.*, 1956, **36**, 1; A. F. Huxley, *Progr. Biophysics Biophys. Chem.*, 1957, **7**, 255.

main components of the I band and continue into the A band to form an interlocking hexagonal array with the A filaments^{3,4} (Fig. 1).

Although isolated myofibrils can shorten by about 75—80% of their original length, *in vivo* under physiological conditions contraction rarely exceeds 40% of the resting length. Recent studies⁵ of band changes occurring during contraction over the physiological range have indicated that the A band remains unchanged in length. The whole of the shortening in this range occurs in the I band, which ultimately disappears when the myofibril has contracted by about 35—40% of its resting length. To explain these band changes it has been suggested^{3,5} that the myofibril contracts by virtue of the I filaments' moving further into the A band along the spaces between the A filaments, thus bringing about the shortening and final disappearance of the I band. On relaxation when the myofibril returns to its original length the reverse process takes place.

The problem is to devise a satisfactory physicochemical mechanism which will explain how one protein filament system can be drawn deeper into the other with which it forms an interdigitating hexagonal array. The mechanism must be reversible and be correlated with the chemical changes known to accompany contraction. All workers agree that the myofibril is built of longitudinal filaments, but although the evidence for the two-filament system described above is excellent for rabbit skeletal muscle,⁴ a number of workers have not been able with the electron-microscope to demonstrate such a two-filament system in certain other striated muscles.^{6,7} Likewise in smooth muscle some workers claim that only one type of filament is present.⁸ Contraction is characteristic of all types of muscle and, although the ultrastructure of the muscle tissues so far examined consists of filamentous protein elements, the electron-microscope evidence available to date does not permit us to say that the arrangement of these filaments is identical in every case. It seems, however, that all muscles contain a common specialised biochemical system. The precise proportions and performance of this system depend on the type of muscle, but it might be supposed that the mechanism of contraction at the molecular level is similar in all such tissues.

Chemical nature of contractile system. The muscle tissues so far examined contain three proteins, myosin, tropomyosin, and actin; these proteins are obtained only from muscle and in the limited studies of isolated myofibrils they have been shown to be localised in these contractile elements in the cell and to make up about 85—90% of the myofibrillar dry weight. Certain other components are also said to be present in the myofibril and accordingly presumed to have some special role in contraction. These are usually not well defined and are present in relatively small amounts.² At present nothing is known of their function, nor have any special features in their

³ J. Hanson and H. E. Huxley, *Symposia Soc. Exp. Biol.*, 1955, **9**, 228.

⁴ H. E. Huxley, *J. Biophys. Biochem. Cytol.*, 1957, **3**, 631.

⁵ A. F. Huxley and R. Niedergerke, *Nature*, 1954, **173**, 971; H. E. Huxley and J. Hanson, *ibid.*, p. 973.

⁶ J. L. Farrant and E. H. Mercer, *Exp. Cell Res.*, 1952, **3**, 553.

⁷ A. J. Hodge, *J. Biophys. Biochem. Cytol.*, 1955, **1**, 361.

⁸ C. F. Shoenberg, *J. Biophys. Biochem. Cytol.*, 1958, **4**, 609.

properties which may play a role in contraction yet been recognised. Precise analysis of the myofibril in terms of its protein components is still not possible, owing to difficulties of determination, but provisional figures for the rabbit myofibril are given in the Table.

Approximate protein composition of the isolated rabbit myofibril.

	% Total N
Myosin	50—55
Actin	20—25
Tropomyosin	10—15
Other components	5—10

By selective extraction⁹ and use of fluorescent antibodies¹⁰ it has been shown that myosin is localised in the A band, that actin and tropomyosin are certainly present in the I band, and that both are probably present in the A band. If the model proposed by Huxley *et al.*^{4,5} is accepted and if each filament system is assumed to have a constant protein composition, it

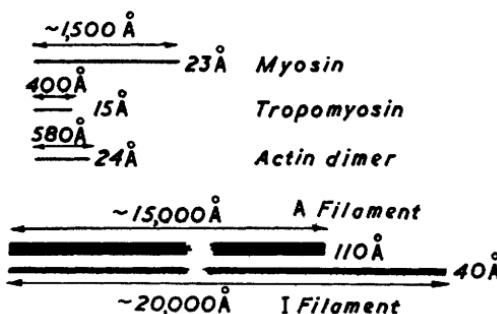


FIG. 2. Diagram to illustrate the relative dimensions of filaments of the rabbit myofibril and the molecules of its three main protein components (vertical and horizontal scales not identical).

follows that myosin is localised in the A filaments and that actin and tropomyosin are associated with the I filaments. There is some electron-microscopic evidence^{4,7} for interfilamentous material in the A band but nothing is known about its nature.

Comparison of the molecular sizes and shapes of the myofibrillar proteins with the dimensions of the contractile system indicate that they are similar. The molecules are presumably aligned along the axes of the filaments which can be at most only a few molecules thick (Fig. 2).

Of the three major protein components, only actin and myosin have physicochemical properties which can be readily related to the contractile process. The role of tropomyosin is not known but, since the original isolation from vertebrate muscle of the soluble form, tropomyosin B,¹¹ it

⁹ W. Hasselbach, *Z. ges. Naturw.*, 1953, **86**, 449; J. Hanson and H. E. Huxley, *Nature*, 1953, **172**, 530; A. Corsi and S. V. Perry, *Biochem. J.*, 1958, **68**, 12.

¹⁰ H. Finck, H. Holtzer, and J. M. Marshall, *J. Biophys. Biochem. Cytol.*, 1956, **2** suppl., 175.

¹¹ K. Bailey, *Biochem. J.*, 1948, **43**, 271.

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has been shown¹² that certain invertebrate muscles contain an insoluble form known as tropomyosin A, in addition to the soluble type. Tropomyosin A is found in large amounts in molluscan adductor muscles (up to 30% of the total protein) and it has been suggested that it may be responsible for the characteristic tonic properties of these muscles.¹³ Although the solubility properties of the tropomyosins are different, the amino-acid analyses of the two types are fundamentally similar and for this reason these proteins are known collectively as tropomyosins.

Myosin. Myosin has been the subject of considerable physicochemical investigation. Although the most highly purified preparations, L- and crystalline myosin, used in these studies appear monodisperse on ultracentrifugation or electrophoresis, evidence is accumulating that they may be far from satisfactory in this respect. Most work has been carried out on preparations of rabbit skeletal-muscle myosin which are well known to be contaminated with 5'-adenylic acid deaminase and small amounts of nucleic acid. According to some workers the standard preparations of L-myosin contain 10—15% of impurities,^{14,15} and very recently Kominz *et al.*¹⁶ succeeded in separating from myosin by dialysis against 0.1M-sodium carbonate a distinct electrophoretic component of molecular weight 29,000 which amounts to about 14% of the original myosin. The relation of this protein to other so-called sub-units of myosin obtained by urea^{15,17} and other treatments¹⁶ is not clear. Treatment with urea would not be expected to destroy covalent linkages but the units obtained in this way are smaller than the L- and H-meromyosin produced by tryptic or chymotryptic digestion. Controlled heat-denaturation of myosin also facilitates the separation of additional components.¹⁸ It is worth speculating whether L- and H-meromyosin¹⁹ represent true covalently bound sub-units rather than components which pre-existed in the myosin molecule and whose separation is facilitated by predigestion.^{20,21} Such a consideration may involve a fine distinction of what constitutes a sub-unit, but recent work on the chromatography of myosin on diethylaminoethylcellulose indicates that myosin preparations contain components of different ATP-ase activity.²¹ In contrast, H-meromyosin appeared to be homogeneous with respect to ATP-ase activity.²² As yet there has been no clear demonstration of the separation of an ATP-ase of high activity from myosin. When fractionation of the protein occurs

¹² K. Bailey, *Pubbl. Staz. Zool. Napoli*, 1956, **29**, 96; *Biochim. Biophys. Acta*, 1957, **24**, 612; D. R. Kominz, F. Saad, J. A. Gladner, and K. Laki, *Arch. Biochem. Biophys.*, 1957, **70**, 16; D. R. Kominz, F. Saad, and K. Laki, Conference on the Chemistry of Muscular Contraction, Japan, 1957, p. 66.

¹³ J. C. Ruegg, *Biochim. Biophys. Acta*, 1959, **35**, 278.

¹⁴ W. F. H. M. Mommaerts and R. G. Parrish, *J. Biol. Chem.*, 1951, **188**, 545.

¹⁵ T. C. Tsao, *Biochim. Biophys. Acta*, 1953, **11**, 368.

¹⁶ D. R. Kominz, W. R. Carroll, E. N. Smith, and E. R. Mitchell, *Arch. Biochem. Biophys.*, 1959, **79**, 191.

¹⁷ A. G. Szent-Gyorgyi and M. Borbiro, *Arch. Biochem. Biophys.*, 1956, **60**, 180.

¹⁸ R. H. Locker, *Biochim. Biophys. Acta*, 1959, **32**, 189.

¹⁹ A. G. Szent-Gyorgyi, *Arch. Biochem. Biophys.*, 1953, **42**, 306; J. Gergely, M. A. Gouveia, and D. Karibian, *J. Biol. Chem.*, 1955, **212**, 165.

²⁰ W. R. Middlebrook, Abs. 4th Internat. Congr. Biochem., Vienna, 1958, p. 84.

²¹ S. V. Perry, *Biochem. J.*, 1960, **74**, 94.

²² H. Mueller and S. V. Perry, *Biochim. Biophys. Acta*, in the press.

the enzymically active portion represents the major part of the original protein.

Notwithstanding these reservations about their nature a considerable amount of precise physicochemical study has been applied to myosin preparations. Up to recently a molecular weight of 840,000 was accepted²³ but with clear evidence now available for aggregation in myosin solutions under certain conditions²⁴ this value has become the subject of controversy.²⁵ Application of the Archibald approach to sedimentation equilibrium has given values in the region of 420,000,²⁶ and similar values have been obtained by the sedimentation-diffusion method at low protein concentration. Such figures would fit well the present views on the sub-unit composition of myosin derived from investigations on the meromyosins.^{26a} Physicochemical study of *N*-ethylmaleimide-poisoned myosin in 5M-guanidine hydrochloride has indicated that the myosin molecule consists of three polypeptide chains each wound in a tight α -helix, these chains together forming a three-stranded rope-like structure.²⁷

Actin. Actin is of particular interest as a myofibrillar component in that it combines with myosin to form the complex, actomyosin, which possesses the special property of responding to the addition of ATP under certain ionic conditions by contraction. Russian workers²⁸ have made the interesting claims (although recently with some reservation²⁹) that this property resides in the myosin alone and that the role of actin is to alter and stabilise the pH-dependence of the ATP-ase. Such claims do not fit in with conventional views, nor are they supported by the observation of Hayashi *et al.*³⁰ that actin is an essential requirement for contraction at both pH 7.6 and 9.0.

Some further work has been carried out on the nature of the process involved in the polymerisation of G(globular)-actin³¹ (considered to be a dimeric form of particle weight 140,000) to the F(fibrous)-form which is extremely viscous in solution and combines with myosin to form contractile actomyosin. Conventional views would regard this as a linear polymerisation of G-actin dimers but polarisation-fluorescence studies³¹ suggest that some caution must be exercised before this model is adopted. Although the polarisation-fluorescence studies give a molecular weight for the polymerised F-actin no greater than that of the dimer, application of the light-scattering technique suggested that it may reach a value of several millions.³²

From a systematic study of the polymerisation of G-actin, Oosawa and

²³ H. H. Weber and H. Portzehl, *Adv. Protein Chem.*, 1952, **7**, 161.

²⁴ A. Holtzer, *Arch. Biochem. Biophys.*, 1956, **64**, 507.

²⁵ H. H. Weber, *Ann. Rev. Biochem.*, 1957, **26**, 667.

²⁶ P. H. Von Hippel, H. K. Schachman, P. Appel, and M. F. Morales, *Biochim. Biophys. Acta*, 1958, **28**, 504; W. F. H. M. Mommaerts and B. B. Aldrich, *ibid.*, p. 627.

^{26a} Cf. S. Lowey and A. Holtzer, *Biochim. Biophys. Acta*, 1959, **34**, 470.

²⁷ W. W. Kielley and W. F. Harrington, *Fed. Proc.*, 1959, **18**, 259.

²⁸ W. A. Kafiani and V. A. Engelhardt, *Doklady Akad. Nauk S.S.R.*, 1953, **92**, 385.

²⁹ W. A. Engelhardt, Conference on the Chemistry of Muscular Contraction, Japan, 1957, p. 134.

³⁰ T. Hayashi, R. Rosenbluth, P. Satir, and M. Vozick, *Biochim. Biophys. Acta*, 1958, **28**, 1.

³¹ T. C. Tsao, *Biochim. Biophys. Acta*, 1953, **11**, 227.

³² J. Gergely and H. Kohler, Conference on the Chemistry of Muscular Contraction, Japan, 1957, p. 14.

his co-workers^{33,34} conclude that it can be regarded as a reversible "fibrous condensation." A critical actin concentration, determined by conditions in the medium, is required before polymerisation occurs. Above the critical concentrations excess of G-actin is converted into the F-form. These investigations suggest that all preparations of F-actin contain both F- and G-actin which are in equilibrium and undergo continuous interconversion. The fact that G-actin preparations contain a small but persistent amount of ATP, which is converted into ADP on polymerisation,³⁵ has stimulated speculation on the role of this nucleotide and the G-F transformation in relation to the contractile process. An implication of these nucleotide changes during G-F interconversion on the theory of Oosawa *et al.* is that dephosphorylation of ATP occurs continuously in F-actin solutions. These authors have claimed that added ATP is slowly dephosphorylated by F-actin solutions.³⁶

As yet there is no direct evidence of participation of the G-F transformation in contraction. It would be attractive to consider the nucleotide which is bound to the myofibril³⁶ as a built-in acceptor for the phosphate bond energy used in contraction, but such a view is not supported by the fact that the phosphorus of this nucleotide only slowly equilibrates with that in the nucleotide pool of the muscle cell after injection of inorganic [³²P]orthophosphate.³⁷ The equilibration is not speeded up significantly by activity of the muscle. Although the bound nucleotide of the myofibril is rather inert so far as enzymes are concerned, Strohman³⁸ has found that the nucleotide associated with isolated actin can participate in reactions involving the creatine-phosphokinase system. Pertinent to the problem are some interesting observations on the localisation of nucleotide metabolism within the I band, as revealed by autoradiography.³⁹

Actomyosin. The interaction of actin and myosin and the effects of ATP on it are perhaps the most striking phenomena of muscle biochemistry. Combination of these two proteins causes a marked increase in viscosity. On addition of low concentrations of ATP this is reduced to a value whose logarithm equals the sum of the logarithms of the viscosities of actin and myosin, measured separately. Szent-Gyorgyi⁴⁰ first explained this effect as a simple dissociation of the complex into actin and myosin, but it has been suggested⁴¹ as a result of light-scattering studies that a change in molecular shape rather than dissociation is responsible for it. This interpretation of the light-scattering data is, however, not supported by all investigators,⁴² and certainly separation of actin and myosin can be demon-

³³ S. Askura, K. Hotta, N. Imai, T. Ooi, and F. Oosawa, Conference on the Chemistry of Muscular Contraction, Japan, 1957, p. 57.

³⁴ F. Oosawa, S. Askura, K. Hotta, N. Imai, and T. Ooi, *J. Polymer Sci.*, 1959, **37**, 323; F. Oosawa, *ibid.*, 1957, **26**, 29.

³⁵ F. B. Straub and G. Feuer, *Biochim. Biophys. Acta*, 1950, **4**, 455.

³⁶ S. V. Perry, *Biochem. J.*, 1952, **51**, 495.

³⁷ A. Martonosi, M. A. Gouveia, and J. Gergely, *Fed. Proc.*, 1959, **18**, 283.

³⁸ R. C. Strohman, *Biochim. Biophys. Acta*, 1959, **32**, 436.

³⁹ D. K. Hill, *J. Physiol.*, 1959, **145**, 132.

⁴⁰ A. Szent-Gyorgyi, *Acta Physiol. Scand.*, 1945, **9**, Suppl. No. 25.

⁴¹ J. J. Blum and M. F. Morales, *Arch. Biochem. Biophys.*, 1953, **43**, 208.

⁴² J. Gergely, *J. Biol. Chem.*, 1956, **220**, 917; H. Nuda and K. Maruyama, *Biochim. Biophys. Acta*, 1959, **30**, 598.

strated⁴³ by ultracentrifugation of actomyosin solutions in the presence of ATP.

Whereas the interaction of actomyosin and ATP can be more readily investigated by physicochemical methods when it occurs in solution, study of the reaction *in vivo*, occurring as it does in a heterogeneous system, presents greater difficulties. The contracting effect of ATP on actomyosin gels in suitable ionic environment can readily be compared with the behaviour of more physiological models such as isolated myofibrils or glycerated muscle preparations.⁴⁴ Any differences which are apparent are probably due to differences in the degree of orientation of the protein filaments in the two types of system. In actomyosin solutions the dissociating action of ATP is reflected in the change of viscosity. Presumably dissociation of the components on addition of ATP also occurs in the gel but is then followed by contraction of the system. Any mechanism proposed for contraction in the myofibril system with its precise orientation of filaments and localisation of protein must apply also to the randomly oriented actomyosin gel precipitated from a solution of the complex.

Protein-protein interactions are not unusual in biological systems but, unlike actomyosin formation, most are non-specific. Myosin thiol (SH) groups are essential for both ATP-ase and actomyosin-forming activity,⁴⁵ and for a number of thiol reagents there is a close correlation between the degree of inhibition of both properties. With several types of inhibitor the general pattern of behaviour suggested that the same active centres were essential for actin-combination and enzyme-substrate complex-formation although there was some indication that with iodoacetamide the ATP-ase activity of myosin was less sensitive to this reagent than was the actomyosin-forming property. Using very high concentrations of the inhibitor, Bárány⁴⁶ succeeded in treating actomyosin with iodoacetamide and isolating from it a myosin component which had no ATP-ase activity but combined normally with actin. These results and those obtained with other thiol reagents are taken⁴⁷ to indicate that different thiol groups on the myosin molecule are necessary for the interaction with ATP and with actin, but that the "pyrophosphate binding" part shares in both activities. Actin also contains thiol groups, which are involved in the G-F transformation,⁴⁸ but the ability of F-actin to combine with myosin is relatively independent of thiol reagents.⁴⁵

Adenosine triphosphatase. The other aspect of the interaction of myosin with ATP which seems important for the contractile process is the hydrolysis of the nucleotide:



⁴³ A. Weber, *Biochim. Biophys. Acta*, 1956, **19**, 345; J. Gergely and A. Martonosi, *Fed. Proc.*, 1958, **17**, 227.

⁴⁴ H. H. Weber and H. Portzehl, *Progr. Biophysics Biophys. Chem.*, 1954, **4**, 60.

⁴⁵ K. Bailey and S. V. Perry, *Biochim. Biophys. Acta*, 1947, **1**, 506.

⁴⁶ M. Bárány, 4th Internat. Congr. Biochem., Vienna, 1958, Abs., p. 84.

⁴⁷ M. Bárány and K. Bárány, *Biochim. Biophys. Acta*, 1959, **35**, 293.

⁴⁸ G. Feuer, F. Molnar, E. Pettkó, and F. B. Straub, *Acta Physiol. Acad. Sci. Hung.*, 1948, **1**, 150; G. Kuschinsky and F. Turba, *Biochim. Biophys. Acta*, 1951, **6**, 426.

The enzyme concerned is perhaps more accurately described as a nucleoside triphosphatase rather than an ATP-ase as it will also split the triphosphates of inosine, guanine, uridine, and cytidine at high rates. Inorganic triphosphate is hydrolysed slowly. Detailed investigation of the enzyme^{2,44,49} has revealed many unusual properties, but despite the mass of experimental facts a satisfactory picture of the mechanism of ATP hydrolysis has yet to emerge.

Myosin ATP-ase is atypical in its activator requirement in that it is activated by calcium and not by magnesium although the latter is usually more effective with enzymes using ATP as substrate. Magnesium, alone or in the presence of calcium, inhibits the enzyme,⁵⁰ and in the latter case antagonism between the ions is apparent.⁵¹ The thiol nature of the enzyme is well established,⁴⁵ and it is thus surprising that under certain conditions low concentrations of specific thiol reagents increase the hydrolysis catalysed by calcium-activated ATP-ase.⁵² Another apparently paradoxical effect is that at high ionic strengths low concentrations of ethylenediaminetetraacetate (EDTA) stimulate the enzyme⁵³ whereas at low ionic strength similar concentrations inhibit it.^{54,55} 2,4-Dinitrophenol also stimulates the calcium-activated ATP-ase of myosin^{52,56} and it is of interest to compare this system with the mitochondrial ATP-ase which is likewise sensitive to dinitrophenol.⁵⁷ Earlier ideas^{57,58} that the mitochondrial ATP-ase might be associated with a contractile system regulating volume changes of mitochondria have recently been discussed further.⁵⁹

Although most of the above observations apply to actin-free myosin preparations, the presence of actin profoundly affects the enzymic behaviour of the system. In strong contrast to their effect on myosin, both magnesium and calcium at low ionic strength markedly activate actomyosin ATP-ase,^{55,60} the ions are no longer antagonistic and under certain conditions may be synergic.⁵⁵ At higher ionic strengths (>0.15) magnesium-activation disappears and the enzymic behaviour more closely corresponds to that of

⁴⁹ D. M. Needham, *Adv. Enzymol.*, 1952, **13**, 151; H. H. Weber and H. Portzehl, *Adv. Protein Chem.*, 1952, **7**, 161; A. G. Szent-Gyorgyi, *Adv. Enzymol.*, 1955, **16**, 313; K. Bailey, in "The Proteins" (eds. H. Neurath and K. Bailey), Academic Press, New York, 1954, Vol. IIB, p. 951; M. F. Morales, J. Botts, J. J. Blum, and T. L. Hill, *Physiol. Rev.*, 1955, **35**, 475.

⁵⁰ I. Banga and A. Szent-Gyorgyi, *Stud. Inst. Med. Chem. Szeged*, 1943, **3**, 72.

⁵¹ M. F. H. M. Mommaerts and K. Seraidarian, *J. Gen. Physiol.*, 1947, **30**, 401.

⁵² G. D. Greville and D. M. Needham, *Biochim. Biophys. Acta*, 1955, **16**, 284; J. B. Chappell and S. V. Perry, *ibid.*, p. 285; W. W. Kielley and L. B. Bradley, *Fed. Proc.*, 1955, **14**, 235.

⁵³ E. T. Friess, *Arch. Biochem. Biophys.*, 1954, **51**, 17; E. T. Friess, M. F. Morales, and W. J. Bowen, *ibid.*, 1954, **53**, 311; G. D. Greville and E. Reich, *ibid.*, 1957, **20**, 440.

⁵⁴ W. J. Bowen and T. D. Kerwin, *J. Biol. Chem.*, 1954, **211**, 237.

⁵⁵ S. V. Perry and T. C. Grey, *Biochem. J.*, 1956, **64**, 184.

⁵⁶ H. C. Webster, Ph.D. thesis, Cambridge, 1953; S. V. Perry and J. B. Chappell, *Biochem. J.*, 1957, **65**, 469.

⁵⁷ S. V. Perry, Conferences et Rapports, 3rd Internat. Congr. Biochem., Brussels, 1955, p. 365.

⁵⁸ J. B. Chappell, Ph.D. thesis, Cambridge, 1954.

⁵⁹ A. L. Lehninger, Symposium on Molecular Biology (ed. R. E. Zirkle), Univ. Chicago Press, Chicago, 1959, p. 122.

⁶⁰ S. V. Perry, *Biochem. J.*, 1951, **48**, 257.

myosin at a similar ionic strength. These effects apply to actomyosin made by combination of the separately isolated proteins and to more physiological systems such as isolated myofibrils. Despite the fact that purified myosin is not activated by magnesium this ion is clearly important for functioning of myosin *in situ* as it is essential for the contraction⁶¹ and relaxation⁶² of isolated myofibrils.

With actomyosin systems marked differences occur in the temperature coefficient of the calcium- and magnesium-activated action of ATP-ase.⁵⁶ Analysis of the Arrhenius plot for the hydrolysis of ATP by myosin in the presence of magnesium (a reaction which can hardly be called activated as the rate is very slow) in the absence and presence of 2,4-dinitrophenol suggests that the enzyme-substrate complex with substrates lacking the 6-amino-group may have a conformation which is particularly sensitive to change at about 16°. Although this phenomenon does not occur with ATP alone, yet if dinitrophenol is added the behaviour now resembles that with ITP, suggesting that the dinitrophenol may be strongly attracted to the group or groups on the protein which normally bind the amino-group in ATP.⁶³

Kinetic analysis of calcium-activated hydrolysis of ATP by L-myosin does not allow a clear distinction between two possible mechanisms, namely, those wherein (i) the substrate is Ca-ATP and free ATP is inhibitory, or (ii) calcium myosinate is the activated form of the enzyme which splits free ATP, and Ca-ATP is inactive. The latter mechanism has been considered more acceptable because of the simpler postulates.⁶⁴ Similar hypotheses have been put forward^{55,65} to explain the experimental findings with magnesium as the activator for actomyosin ATP-ase. Certain features of the latter system, namely, inhibition by EDTA at a concentration one-fiftieth of that of the magnesium,^{55,66} and the relief of inhibition induced by excess of ATP by very low concentrations of calcium,⁵⁵ imply that for the activation by magnesium small amounts of some cation (*e.g.*, Ca^{2+}) are required which can be selectively removed by the binding action either of EDTA or of ATP. Weber's recent study⁶⁷ of the myofibrillar ATP-ase supports such an explanation. This hypothesis can be used as the basis of a plausible theory of the mechanism of inhibition of ATP-ase which occurs during relaxation (see below).

An unusual feature of the hydrolysis of ATP by L-myosin or actomyosin is that the reaction begins with a high initial rate which over the first 10—20 sec. may be up to five times as high as the stationary value reached within 1—2 minutes.^{68,69} EDTA eliminates this effect, whereas both the initial

⁶¹ C. A. Ashley, A. Arasimavicius, and G. M. Hass, *Exp. Cell. Res.*, 1956, **10**, 1.

⁶² J. R. Bendall, *J. Physiol.*, 1953, **121**, 232.

⁶³ H. M. Levy, N. Sharon, and D. E. Koshland, *Biochim. Biophys. Acta*, 1959, **33**, 288.

⁶⁴ L. B. Nanninga, *Biochim. Biophys. Acta*, 1959, **36**, 191; *Arch. Biochem. Biophys.*, 1957, **70**, 346.

⁶⁵ G. Geske, M. Ulbrecht, and H. H. Weber, *Arch. Exp. Pathol. Pharmakol.*, 1957, **230**, 301.

⁶⁶ S. V. Perry and T. C. Grey, *Biochem. J.*, 1956, **64**, 5P.

⁶⁷ A. Weber, *J. Biol. Chem.*, 1959, **234**, 2764.

⁶⁸ A. Weber and W. Hasselbach, *Biochim. Biophys. Acta*, 1954, **15**, 237.

⁶⁹ Y. Tonomura and S. Kitagawa, *Biochim. Biophys. Acta*, 1957, **26**, 15.

and the stationary phase are enhanced by *p*-chloromercuribenzoate and 2,4-dinitrophenol.⁶⁹ If the hydrolysis of ATP is essential for the development of tension this initial phase of high activity may be of physiological significance. It would be of great advantage for the myofibril to hydrolyse ATP very rapidly for a few milliseconds during the initial stage of a muscle twitch.

Some light has been thrown on the mechanism of ATP hydrolysis by studying the reaction catalysed by myosin systems in the presence of H₂¹⁸O. When the activator used with actomyosin is calcium, one atom of ¹⁸O₂ is introduced into each molecule of inorganic orthophosphate produced.⁷⁰ In this respect the mechanism of the splitting of ATP is similar to that occurring in phosphokinase systems.⁷¹ The value of the ratios of the reactivities of water and methanol for enzymic compared with non-enzymic hydrolysis of ATP are of a very different order, which suggests that the mechanism of enzymic hydrolysis involves a specific myosin-water interaction.⁷² However, if the magnesium-activated hydrolysis by L-myosin, intact lobster muscle, or actomyosin is studied, appreciable exchange of ¹⁸O₂ between H₂¹⁸O and the inorganic orthophosphate formed is apparent.⁷³ This finding is interpreted as indicating that a phosphorylated intermediate which is capable of exchanging oxygen with water is formed in these systems. Evidence of another kind, namely, the binding of inorganic orthophosphate by H-meromyosin during ATP hydrolysis, has also been taken as evidence for a phosphorylated intermediate.⁷⁴

When ³²P was used the evidence found for a phosphorylated intermediate was somewhat controversial. No exchange between inorganic ³²P and ATP or between AD³²P and ATP was observed by Koshland *et al.*,⁷⁰ whereas Weber⁷⁵ reported that with "Fuadin"-poisoned myosin such an exchange can be demonstrated. An exchange of ³²P between AD³²P and ATP which is stimulated by magnesium has been demonstrated with preparations of actomyosin extracted as the complex ("natural" actomyosin), and with myofibrils.^{76,77} This exchange also occurred with myosin extracted selectively from whole myofibrils, but not with L-myosin or preparations of "synthetic" actomyosin from purified actin and myosin. The granular ATP-ase of muscle actively catalysed this transfer,⁷⁷ but although this enzyme contaminates "natural" actomyosin and myofibril preparations it was considered that the major part of the exchange studied was catalysed by the actomyosin system. So far as L-myosin is concerned there is general agreement that with this protein alone it has not so far been possible to demonstrate any exchange of ³²P between AD³²P and ATP.

⁷⁰ D. E. Koshland, Z. Budenstein, and A. Kowalsky, *J. Biol. Chem.*, 1954, **211**, 279.

⁷¹ B. Axelrod, *Adv. Enzymol.*, 1956, **17**, 159.

⁷² D. E. Koshland and E. B. Herr, *J. Biol. Chem.*, 1957, **228**, 1021.

⁷³ H. M. Levy and D. E. Koshland, *J. Amer. Chem. Soc.*, 1958, **80**, 3164.

⁷⁴ J. Brahms and C. Rzysko, Abs. 4th Internat. Congr. Biochem., Vienna, 1958, p. 83.

⁷⁵ H. H. Weber, *Conferences et Rapports*, 3rd Internat. Congr. Biochem., Brussels, 1955, p. 356.

⁷⁶ G. Ulbrecht and M. Ulbrecht, *Biochim. Biophys. Acta*, 1957, **25**, 100.

⁷⁷ G. Ulbrecht, M. Ulbrecht, and H. J. Wustrow, *Biochim. Biophys. Acta*, 1957, **25**, 110.

ATP and contraction. Two important aspects of the interaction of ATP with the actomyosin system, namely, the mechanical changes and the hydrolysis of ATP, have to be related to the events occurring *in vivo*. There is little doubt that the contraction which can be induced by ATP in actomyosin systems *in vitro* is the counterpart of contraction in living muscle. Striking confirmation of this is shown by treating myofibrils with ATP under controlled conditions: these structures then contract and exhibit the same band changes as take place in contracting living muscle.^{3,5} ATP is not unique in producing contraction, for other nucleoside triphosphates can bring about this change; nevertheless the latter are present in muscle in concentrations much lower than that of ATP which, if it is not separated from the site of action by some physical barrier, would be expected to be the active agent purely on mass-action considerations.

The question whether ATP is split during contraction, quite apart from the fact as to whether it is the contractile agent, is also controversial. As a consequence of prolonged activity in excess of what the particular muscle is normally called upon to perform, inorganic orthophosphate accumulates, the level of creatine phosphate falls, and subsequently so does the ATP concentration.^{2,78} Some investigators⁷⁹ have felt that to prove that ATP is the primary source of energy for the contractile system it is necessary to demonstrate a fall in the level of this substance during the initial stages of a single twitch, the whole event normally lasting about 100 milliseconds. This is undoubtedly an ideal requirement but the possibility of being able to demonstrate such a change is doubtful. It is estimated⁸⁰ that the orthophosphate liberated would represent a very small fraction of the ATP present. Hydrolysis of ATP would be occurring under conditions where the whole metabolism of the cell is poised ready to rephosphorylate any ADP produced. Certainly the inability to show an appreciable drop in ATP must not in itself be taken to exclude the possibility of ATP splitting during contraction without ensuring that the enzyme systems concerned with rephosphorylation are ineffective.

With *in vitro* systems considerable correlation between contraction and splitting occurs.^{2,23,49} Although there are occasions when correlation is not satisfactory these may arise because the contractile process is more sensitive to the physical state of the systems than is the enzymic activity. Contraction obviously will not be apparent when the actomyosin is in solution, but such conditions certainly favour ATP-ase activity. A more serious objection would be the demonstration that contraction can be induced by ATP in a model system without its being hydrolysed. Evidence of this kind has yet to be obtained.

It may be concluded that studies with *in vitro* systems, in which it is possible to evaluate accurately the ATP level and the rate of its hydrolysis, there is reasonably good correlation between contraction and tension developed on the one hand and simultaneous hydrolysis of ATP on the other.

⁷⁸ W. F. H. M. Mommaerts, "Muscular Contraction," Interscience Publ. Inc., New York, 1950.

⁷⁹ E.g., A. V. Hill, *Nature*, 1949, **163**, 320.

⁸⁰ W. F. H. M. Mommaerts, *Amer. J. Physiol.*, 1955, **182**, 585.

Some exceptions exist, but in the opinion of the Reporter these are not serious objections to this hypothesis.

With intact living muscle, measurement of chemical change during a single twitch presents considerable difficulties. They arise because of the relatively small change which may be expected, its extremely short duration, and problems of fixing the muscle at a given time (measured in milliseconds) to prevent further chemical change. Chemical reactions in the resting muscle approach a steady state and it is impossible to predict the precise effect of a single twitch on this condition. It is conceivable that the nucleoside-polyphosphate level is relatively insensitive to a low level of activity. Within the last 10—15 years a number of attempts have been made to tackle this problem² and, except in two recent investigations,^{80,81} some evidence for ATP breakdown has been presented. It is, however, difficult to compare all the investigations because the conditions and degree of activity to which the muscle was subjected were somewhat variable. The experiments of Fleckenstein *et al.*⁸¹ and of Mommaerts,⁸⁰ involving direct analysis of the phosphate compounds of muscle most likely to be hydrolysed during contraction, have shown that various skeletal muscles of the turtle and frog rectus abdominis can undergo a single twitch without significant change being apparent in the ATP, ADP, or creatine-phosphate levels. On the other hand, indirect determination of nucleotide changes during contractile activity in intact living muscle by spectrophotometric means has provided evidence for a small increase of ADP level after a single twitch. This increase is estimated to be 2% of what would be expected on the assumption that the energy required for the work done by the muscle during contraction was derived from ATP.⁸²

Even if breakdown of ATP in living muscle is masked by the efficiency of rephosphorylation systems, activity should give rise to an increased turnover in the phosphate atoms of creatine phosphate and of the muscle nucleotides. As yet, however, tracer studies have failed to reveal a transfer of phosphate from phosphocreatine to ATP either during drug-induced contracture of frog muscles⁸³ or during tetanic contracture of cat gastrocnemius.⁸⁴ Even more difficult to reconcile with the hypotheses that ATP is dephosphorylated during contraction is the further finding⁸⁵ that after injection of inorganic [³²P]orthophosphate stimulation more than 10,000 times in one hour did not cause a significant change from the normal resting condition in the distribution of ³²P between creatine phosphate, the terminal phosphate of ADP, and the terminal and middle phosphate of ATP. In neither resting nor stimulated muscle were any of the organic phosphates in equilibrium with the total orthophosphate. Difficulties arise in interpreting such studies, for much of the injected isotope is in the extracellular spaces and not in metabolic contact with the nucleotides within the cell. This heavily labelled extracellular phosphate will form part of the orthophosphate

⁸¹ A. Fleckenstein, J. Janke, R. E. Davies, and H. A. Krebs, *Nature*, 1954, **174**, 1081.

⁸² B. Chance and C. M. Connelly, *Nature*, 1957, **179**, 1235.

⁸³ A. Fleckenstein, J. Janke, R. E. Davies, and W. Richter, *Arch. Exp. Pathol. Pharmakol.*, 1956, **228**, 596.

⁸⁴ G. J. Dixon and J. Sacks, *Amer. J. Physiol.*, 1958, **193**, 129.

⁸⁵ J. Sacks, *Nature*, 1959, **183**, 825.

fraction separated from the muscle. As perfusion of the muscle to remove extracellular phosphate also causes changes in the intracellular organophosphates some workers⁸⁶ consider that it is not possible to determine the absolute turnover rate of any organophosphate compound which has inorganic phosphate as its immediate source of phosphorus. Equilibration between injected inorganic [³²P]phosphate and muscle nucleotides is relatively slow compared with that in other tissues;⁸⁷ even so it has been concluded⁸⁶ that the speed at which the terminal phosphate of ATP is replaced in resting muscle is such as to preclude any attempt to measure differences in rates in working muscle. Nevertheless, if the results obtained by Sacks and other workers are valid, then it is clear that dephosphorylation of ATP is not the direct source of energy for muscle activity.

Some effort has been directed towards a search for alternative sources of energy, but as yet such a compound has not been discovered. It is unlikely that carnosine phosphate is present in muscle in appreciable amounts,^{87a} and the recently discovered phosphorylated guanidino-compounds^{87b} found in some invertebrates are of the phosphagen type rather than substances which directly supply energy for contraction.

Relaxation.—When the stimulus reaches the muscle the electrical changes occurring at the membrane initiate in some way the chemical changes which take place at the myofibril and contraction results. These changes continue for only a limited period after a single stimulus; in a matter of milliseconds the tension in the contractile unit drops. There is good evidence⁸⁸ that relaxation is a passive process and extension to the resting length follows as a consequence of the load on the muscle when the contracting force no longer acts. It seems likely that in resting muscle the enzyme systems which play a part in contraction are in steady-state equilibrium. The electrical changes occurring at the membrane as a consequence of the nerve impulse reaching the cell initiate some slight but significant change, possibly ionic in nature, which completely alters the enzymic balance at the myofibril. A view which has much to commend it is that the myofibrillar ATP-ase is greatly activated above the low basic level characteristic of the resting myofibril, and that shortening then ensues. The initiating electrical changes at the membrane are of short duration and, once they are over, the tendency is for the system to return to the condition characteristic of resting muscle; tension falls and the myofibril extends to its resting length.

In earlier work with actomyosin model systems and glycerated fibres^{2,23,49} conditions were devised in which previously contracted actomyosin systems could be made to relax. Usually this occurred in the presence of ATP and various enzyme inhibitors such as EDTA, "Salyrgan," etc., which brought the ATP-ase activity of the system to a low level. In some cases relaxation could be obtained in the absence of ATP but in the presence of

⁸⁶ E.g., A. H. Ennor and H. Rosenberg, *Biochem. J.*, 1954, **56**, 308.

⁸⁷ K. K. Tsuboi, *Arch. Biochem. Biophys.*, 1959, **88**, 445.

^{87a} D. F. Cain, A. M. Delluva, and R. E. Davies, *Nature*, 1958, **182**, 720.

^{87b} N. V. Thoai, J. Roche, Y. Robin, and N. V. Thiem, *Compt. rend. Soc. Biol.*, 1953, **147**, 1241; N. V. Thoai and Y. Robin, *Biochim. Biophys. Acta*, 1954, **14**, 76; G. E. Hobson and K. R. Rees, *Biochem. J.*, 1955, **61**, 549.

⁸⁸ A. V. Hill, *Proc. Roy. Soc.*, 1949, **B**, **136**, 420.

pyrophosphate and triphosphate²³ which dissociate actomyosin but are not themselves split. From such studies with model systems the concept has emerged of the dual role of ATP, *i.e.*, it is able to act, depending on the conditions, as both a contracting and a relaxing agent. When ATP-ase activity is high, ATP acts as a contracting agent; when it is low, ATP plasticises the system, presumably by breaking the link between actin and myosin, the tension drops, and the actomyosin fibre relaxes.

An important step towards more physiological systems was made when it was shown that addition of ATP to crude muscle-cell fragments⁸⁹ and to partly washed glycerated fibres⁹⁰ produced an elongation rather than a contraction. If the systems were more highly purified, addition of ATP produced the well-known contraction. The implication was that these crude preparations contained some factors which modified the effect of ATP on the actomyosin system. This factor (known variously as the Marsh factor, Marsh-Bendall factor, or relaxing factor) is considered to be effective in living resting muscle and prevents contraction from taking place, although the ATP concentration *in situ* in the absence of such a factor would cause the myofibrils to contract.

Ideally, the relaxing factor should be studied in systems in which its effect on the tension of the contracted system can be measured directly. This involves using fibres with which, unless they are extremely thin, it seems impossible to maintain the ATP concentration constant throughout the fibre. ATP is diffusing into the fibre and at the same time being broken down by the ATP-ase activity of the actomyosin component. For this reason certain phosphorylase preparations such as creatine phosphokinase⁹¹ and myokinase⁹² have been claimed to possess relaxing-factor activity. It seems possible that these phosphokinases may act by keeping the ATP at a sufficiently high concentration throughout the system.* Pertinent to this question are the findings of Japanese workers⁹³ that in addition to the phosphokinase system another muscle fraction, which appeared to be related to the granular fraction of muscle sarcoplasm, was necessary. Portzehl⁹⁴ also found the relaxing factor in the granular fraction which could be sedimented completely from sarcoplasm by high-speed centrifugation. Apart from causing relaxation of fibre models, relaxing-factor preparations inhibit in a parallel manner the ATP-ase activity of myofibrils⁹⁴ and of actomyosin,⁹⁵ and the syneresis of actomyosin gels.⁹⁶ Whenever relaxation occurs *in vitro* the evidence so far indicates that ATP-ase activity is low, which suggests that inhibition of the enzymic activity brings about the loss in

⁸⁹ B. B. Marsh, *Nature*, 1951, **167**, 1065; *idem.*, *Biochim. Biophys. Acta*, 1952, **9**, 247.

⁹⁰ E. Bozler, *Amer. J. Physiol.*, 1951, **167**, 276.

⁹¹ M. C. Goodall and A. G. Szent-Gyorgyi, *Nature*, 1953, **172**, 84; L. Lorand, *ibid.*, p. 1181; E. Bozler, *J. Gen. Physiol.*, 1954, **37**, 63.

⁹² J. R. Bendall, *Proc. Roy. Soc., 1954*, **B**, **142**, 409.

⁹³ H. Kumagai, S. Ebashi, and F. Takeda, *Nature*, 1955, **176**, 166; S. Ebashi, F. Takeda, M. Otsuka, and H. Kumagaia, *Symposia on Enzyme Chem.*, Japan, 1956, **11**.

⁹⁴ H. Portzehl, *Biochim. Biophys. Acta*, 1957, **26**, 373.

⁹⁵ D. J. Baird and S. V. Perry, *Biochem. J.*, in the press.

⁹⁶ H. Mueller, *Biochim. Biophys. Acta*, in the press.

* At high ATP concentrations the magnesium-activated ATP-ase is inhibited.

tension and resulting relaxation of the system.⁹⁷ Portzehl⁹⁸ concluded that if sufficiently thin fibres were used there was no requirement for a phosphokinase system as well as the granular preparation of the relaxing factor, either to bring about relaxation in fibres or to inhibit the ATP-ase activity of myofibrils. More recently, however, Molnar and Lorand⁹⁹ have provided further evidence for the potentiating action of pyruvic phosphokinase with relaxing-factor preparations. Nevertheless, other workers¹⁰⁰⁻¹⁰² consider the phosphokinase function in the system to be non-specific, but their experiments suggest that there is a requirement for a dialysable cofactor in the system.

The precise centrifugal force required to sediment granules (or possibly reticular material) with relaxing-factor activity from muscle homogenates depends somewhat on the species and type of muscle used.⁹⁵ In the literature the activity is often considered to be associated with the microsome fraction, but studies on the distribution in rabbit skeletal muscle render this less certain. The bulk of the activity in rabbit skeletal muscle is sedimented below 20,000 g, and the fraction rich in oxidative activity is also the most concentrated with respect to relaxing factor. The factor is less easily sedimented from pigeon breast-muscle homogenates than from similar preparations of rabbit skeletal muscle, and there is a sharper distinction between it and the fraction rich in oxidative activity. In any case muscle is poor in a granular fraction rich in nucleic acid and corresponding to the conventional microsome fraction of other tissues.¹⁰³

The factor preparations are lipoprotein in nature and possess ATP-ase activity^{95,101,104} which is not derived from myosin and is now known to be associated with the granular components of sarcoplasm.¹⁰⁵ Both ATP-ase and relaxing-factor activity are destroyed by phospholipase, but under certain conditions the relaxing-factor activity can be preferentially destroyed.¹⁰⁶ As yet there has been no report of the preparation of the relaxing factor in a soluble form. A significant finding is that relaxing-factor activity of these preparations is readily abolished by low concentrations of calcium.^{62,107} When assayed in the presence of 5-millimolar sodium oxalate, preparations are effective in extremely low concentrations, which suggests that the function of oxalate is to remove some substance (perhaps calcium) present in the preparation and inhibiting their activity.⁹⁵ For example, with preparations of granules from rabbit muscle 50% inhibition of the myofibrillar ATP-ase associated with 1 mole of myosin

⁹⁷ J. R. Bendall, *J. Physiol.*, 1953, **121**, 232; E. Bozler and J. T. Prince, *J. Gen. Physiol.*, 1953, **37**, 53.

⁹⁸ H. Portzehl, *Biochim. Biophys. Acta*, 1957, **24**, 474.

⁹⁹ J. Molnar and L. Lorand, *Nature*, 1959, **183**, 1032.

¹⁰⁰ F. N. Briggs, G. Kaldor, and J. Gergely, *Biochim. Biophys. Acta*, 1959, **34**, 211.

¹⁰¹ J. Gergely, G. Kaldor, and F. N. Briggs, *Biochim. Biophys. Acta*, 1959, **34**, 218.

¹⁰² G. Kaldor, J. Gergely, and F. N. Briggs, *Biochim. Biophys. Acta*, 1959, **34**, 224.

¹⁰³ S. V. Perry and M. Zydow, *Biochem. J.*, 1959, **72**, 682.

¹⁰⁴ L. Lorand, J. Molnar, and C. Moos, Conference on the Chemistry of Muscular Contraction, Japan, 1957, p. 85.

¹⁰⁵ W. W. Kielley and O. Meyerhof, *J. Biol. Chem.*, 1948, **176**, 591; S. V. Perry, *Biochim. Biophys. Acta*, 1952, **8**, 499.

¹⁰⁶ S. Ebashi, *Arch. Biochem. Biophys.*, 1958, **76**, 410.

¹⁰⁷ E. Bozler, *Amer. J. Physiol.*, 1952, **188**, 760.

(molecular weight 420,000) is obtained when 20 kg. of total microsomal protein is present, of which the relaxing factor probably represents only a small part.⁹⁵

In the absence of oxalate much larger amounts of granules are required to bring about inhibition and under such conditions activity falls off on ageing at 0°.⁹⁵ Such a loss in activity is evident only when assays are carried out in the absence of oxalate. These results are compatible with the slow liberation, in relaxing-factor preparations, of an ion such as calcium which inactivates the preparation unless chelating agents are present. Pyrophosphate behaves in a similar manner to oxalate and to the cofactor reported by Kaldor *et al.*¹⁰²

If speculation is justified at this stage the following mechanism appears plausible. In view of the inhibition of the magnesium-activated myofibrillar ATP-ase with low concentration of EDTA or higher concentrations of ATP,^{55,66} it is possible that traces of calcium or of a similar cation are essential in some way for the enzyme.⁶⁷ The relaxing factor may inhibit myofibrillar ATP-ase in a similar way to these substances by binding traces of a cation (perhaps calcium) essential for enzymic activity. Further support for this hypothesis would come from the demonstration that relaxing factor preparations have a strong affinity for calcium. It is striking that the insoluble granular preparations can exert their influence on the splitting of ATP which occurs on another insoluble system, the myofibril. This suggests that the relaxing factor brings about inhibition, not by direct interaction at the active centres of the enzyme, but rather by changing the common soluble environment of the two systems to one which is unfavourable for ATP hydrolysis by the magnesium-activated enzyme. The actions of oxalate and the soluble cofactor^{100–102} are sufficiently similar to suggest that their function is to bind the cation (perhaps calcium) present, which would otherwise inactivate the relaxing factor. As a physiological counterpart of oxalate the cofactor could have this function in resting muscle, but could lose it momentarily when muscle is stimulated so that the myofibril can hydrolyse ATP at a high rate and hence contract.

Conclusions.—The theories which have been proposed (see Perry² for a review) to explain the mechanism of contraction usually involve two main assumptions about the ultrastructure of the contractile unit. These are that it consists of a single-filament system which shortens by folding in some way or that it is a two-filament system in which one type of filament moves along the other.^{3,5,108} The latter mechanism has much to commend it; but, although the evidence is good for its occurrence in rabbit skeletal muscle, conclusive evidence for a similar system in other types of muscle has yet to be produced. It seems reasonable to suppose that the mechanism of contraction at this level of organisation is common to all muscle tissues. Both actin and myosin (and possibly other myofibrillar proteins) appear to be active participants in contraction, and the two-filament mechanism utilises the interaction between the two proteins in a convincing way to explain both contraction and relaxation. ATP is usually given a role in modern theories

¹⁰⁸ H. H. Weber, "The Motility of Muscle and Cells," Harvard Univ. Press, Cambridge, Mass., 1958.

but not all workers consider its dephosphorylation to occur simultaneously with contraction. Present views on enzymic activity during relaxation strengthen the case for this hypothesis; and, in addition, ATP has unique properties as a relaxing agent compared with other nucleoside triphosphates.¹⁰⁹ Much of the work at the growing points of muscle biochemistry may seem imprecise by purely physicochemical standards, but this is a consequence of the complexity of the systems studied and the problems involved. It can be said, however, that progress in this field towards a molecular biology—the integration of the chemical events and the ultrastructure of a complex biological system—is certainly as well advanced here as anywhere in biochemistry.

S. V. P.

5. NEW AMINO-ACIDS FROM PLANTS

DURING the last decade about fifty amino- or imino-acids have been newly identified as components of higher plants. About twenty more have been recognised either as constituents of micro-organisms or as fragments of antibiotics excreted by them. As a group, these newly discovered acids have no striking chemical or physiological properties, and their rapid discovery is the result of the application of paper and ion-exchange chromatography to the examination of plant extracts. Brief accounts of the chemistry of some of the newly recognised acids have appeared in earlier Reports,¹ and their biological importance has been considered in other reviews.² Only a few of the acids are distributed widely in plants; the majority are found only in occasional plant species. Their distribution follows no rules. Certain compounds are characteristic of particular plant families, e.g., azetidine-2-carboxylic acid for the Liliaceae. The distribution of many others is haphazard, i.e., they are accumulated in high concentration by only a few species that are botanically quite unrelated. The random distribution has favoured the idea that they are unimportant and perhaps "accidental" products of metabolism; however, this concept may prove unacceptable when more plant species have been examined and when more crucial information is available concerning their metabolic relationships. This Report will consider recent contributions to our knowledge of the chemistry and biochemistry of each of the main types of acid.

Dicarboxylic Acids.—The only new dicarboxylic amino-acid identified as a constituent of higher plants since the last Reports¹ is α -m-carboxyphenylglycine,³ which was isolated from the acid fractions of an extract of *Iris* bulbs. Comparison with synthetic material prepared from m-carboxybenzaldehyde by a Strecker reaction proved its identity.

Two acids have been identified as products of microbial metabolism. β -Methylaspartic acid was formed as an intermediate in the reversible anaerobic conversion of glutamate into mesaconate and ammonia by *Clostridium*.

¹⁰⁹ W. Hasselbach, *Biochim. Biophys. Acta*, 1956, **20**, 355.

¹ *Ann. Reports*, 1955, **52**, 271; 1957, **54**, 276.

² F. C. Steward and J. K. Pollard, *Ann. Rev. Plant. Physiol.*, 1957, **8**, 65; L. Fowden, *Biol. Rev.*, 1958, **33**, 393.

³ C. J. Morris, J. F. Thompson, S. Asen, and F. Irreverre, *J. Amer. Chem. Soc.*, 1959, **81**, 6069.

dium tetanomorphum extracts.⁴ The acid was provisionally assigned the L-threo-configuration. After growth of *Streptomyces rimosus*,⁵ an actinomycete better known as the producer of the antibiotic terramycin, substantial quantities (1—2 g./l.) of (+)- $\alpha\alpha'$ -diaminosuccinic acid were isolated from the fermentation medium. New syntheses of meso- and racemic $\alpha\alpha'$ -diamino-succinic acids have been published.⁶

Synthetic γ -hydroxyglutamic acid has been resolved into its four optical isomers.⁷ One pair of diastereoisomers formed a lactone very easily and this provided a basis for their separation. After conversion into chloroacetyl derivatives, the resolution of each pair of diastereoisomers into D- and L-forms was undertaken by using a hog renal L-acylase preparation. The specific rotations of all the isomers were given. By comparison of specific rotations in water and 5N-hydrochloric acid, the natural acid isolated from *Hemerocallis* was shown to be L-allohydroxyglutamic acid.⁸

A new synthesis for DL- γ -methyleneglutamic acid has been published and the racemic mixture has been resolved.⁹ The synthetic method of Hellmann and Lingens¹⁰ has been shortened⁸ by substituting ethyl α -iodomethylacrylate (prepared from α -iodomethylacrylic acid¹¹) for the analogous bromo-compound. The modified synthesis was used to prepare DL- γ -methylene[α -¹⁴C]glutamic acid by condensation of the iodo-intermediate with diethyl acetamido[α -¹⁴C]malonate.⁸

The metabolism of these glutamic acid derivatives has been studied recently. When plants assimilate ¹⁴CO₂ photosynthetically, glutamic acid normally becomes labelled rapidly, but when ¹⁴CO₂ was supplied to leaves of *Phlox decussata*,¹² *Adiantum pedatum*,¹² *Lilium regale*,¹³ or tulip¹⁴ little incorporation of the carbon-14 into γ -hydroxyglutamic acid, γ -methyleneglutamic acid, or γ -hydroxy- γ -methylglutamic acid was observed. Experiments with [carboxy-¹⁴C]pyruvate have provided more definite information about the biosynthetic pathways leading to the acids. After labelled pyruvate had been supplied to the fern, *Asplenium septentrionale*, the specific activity of γ -hydroxy- γ -methylglutamic acid was higher than that of other free amino-acids;¹⁵ a similar result was obtained for γ -methyleneglutamic acid and γ -methyleneglutamine in ground-nut leaves.¹⁶ These facts suggest that the basic carbon skeleton may be produced by condensation of two molecules of pyruvate (De Jong¹⁷ observed an analogous slow chemical

⁴ H. A. Barker, R. D. Smyth, E. J. Wawszkiewicz, M. N. Lee, and R. M. Wilson, *Arch. Biochem. Biophys.*, 1958, **78**, 468.

⁵ F. A. Hochstein, *J. Org. Chem.*, 1959, **24**, 679.

⁶ H. McKennis and A. S. Yard, *J. Org. Chem.*, 1958, **23**, 980.

⁷ L. Benoiton, M. Winitz, S. M. Birnbaum, and J. P. Greenstein, *J. Amer. Chem. Soc.*, 1957, **79**, 6192.

⁸ L. Fowden, unpublished result.

⁹ Y. Nakagawa and T. Kaneko, *J. Chem. Soc. Japan*, 1957, **78**, 232; T. Kaneko and Y. Nakagawa, *ibid.*, p. 1216.

¹⁰ H. Hellmann and F. Lingens, *Chem. Ber.*, 1956, **89**, 77.

¹¹ K. N. Welch, *J.*, 1930, 257.

¹² G. E. Hunt, *Plant Physiol.*, 1958, **33**, suppl., xii.

¹³ M. E. Wickson and G. H. N. Towers, *Canad. J. Biochem. Physiol.*, 1956, **34**, 502.

¹⁴ L. Fowden and F. C. Steward, *Ann. Bot. N.S.*, 1957, **21**, 69.

¹⁵ P. Linko and A. I. Virtanen, *Acta Chem. Scand.*, 1958, **12**, 68.

¹⁶ L. Fowden and J. A. Webb, *Ann. Bot. N.S.*, 1958, **22**, 73.

¹⁷ A. W. K. De Jong, *Rec. Trav. chim.*, 1900, **19**, 259.

condensation of pyruvic acid in the presence of gaseous hydrogen chloride). Certainly one molecule of pyruvate must enter γ -hydroxy- γ -methylglutamic acid and γ -methyleneglutamic acid intact and not after conversion into acetyl-CoA. γ -Hydroxyglutamic acid may be formed by condensation of pyruvate with glycine or glyoxylic acid, but negative results were obtained when labelled substrates were supplied to *P. decussata*.¹⁵

When γ -methylene[α - ^{14}C]glutamic acid was supplied to leaves of pea, tulip, or peanut, carbon-14 was incorporated fairly quickly into a variety of amino-acids, sugars, and organic acids.⁸ The relative constancies of the concentrations of γ -methylene-glutamic acid and -glutamine present in excised tulip leaves during storage¹⁴ must then be maintained by dynamic equilibria and not by the metabolic inertness of the two substances.

The degradation of γ -hydroxyglutamic acid in plants has not been investigated, but when γ -hydroxy[α - ^{14}C]glutamic acid was supplied to rats, substantial amounts of the original ^{14}C activity appeared rapidly in glutamic and aspartic acid.¹⁸ Enzymes present in extracts of animal livers convert hydroxyproline into γ -hydroxyglutamic acid via its γ -semialdehyde in a manner analogous to that involved in the interconversion of proline and glutamic acid. It is possible that the proline-glutamic acid enzymes act in a non-specific manner by catalysing the conversion of the hydroxy-compounds.¹⁹

γ -Hydroxy- and γ -methylene-glutamic acid readily donate their amino-group to α -oxoglutarate in the presence of extracts of *Phlox*²⁰ and peanut²¹ leaves respectively. Ellis²² has shown that both transamination reactions are catalysed by a purified aspartate-glutamate transaminase prepared from cauliflower buds. Since this plant material is not known to contain either γ -hydroxy- or γ -methylene-glutamic acid, the reactions catalysed by extracts of *Phlox* and peanut may be the result of non-specific enzyme action.

Both γ -methylglutamic and α -amino adipic acid occur in certain higher plants. The structural relation existing between the two acids is the same as that between β -methylaspartic and glutamic acids, and therefore interconversion of the six-carbon acids may be shown ultimately to proceed by a mechanism similar to that observed for the five-carbon acids in *C. tetanolomorphum*.⁴

Imino-acids.—Three new derivatives of proline have been isolated from seaweeds. 3-Carboxymethyl-4-isopropenylproline occurs in two forms (*L*- α -kainic acid, and *L*- α -allokainic acid; I) in *Digenea simplex*.²³ In *L*- α -kainic acid, the 2- and 3-substituents are *trans* to one another and those at C₍₃₎ and C₍₄₎ are *cis*; both configurations are *trans* in *L*- α -allokainic acid. The substances have been synthesised.²⁴ 3-Carboxymethyl-4-(2-carboxy-

¹⁸ L. Benoiton and L. P. Bouthillier, *Canad. J. Biochem. Physiol.*, 1956, **34**, 661.

¹⁹ E. Adams, R. Friedman, and A. Goldstone, *Biochim. Biophys. Acta*, 1958, **30**, 212.

²⁰ A. I. Virtanen and P. K. Hietala, *Acta Chem. Scand.*, 1955, **9**, 549.

²¹ L. Fowden and J. Done, *Nature*, 1953, **171**, 1068.

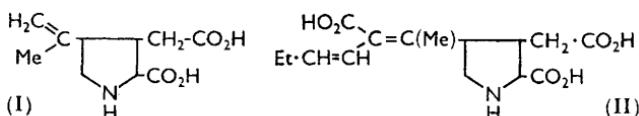
²² R. J. Ellis, personal communication.

²³ S. Murakami, T. Takemoto, and Z. Shimuzu, *J. Pharm. Soc. Japan*, 1953, **73**, 1026.

²⁴ Y. Ueno, K. Tanaka, J. Ueyanagi, H. Nawa, Y. Sanno, M. Honjo, R. Nakamori, T. Sugawa, M. Uchibayashi, K. Osugi, and S. Tatsuoka, *Proc. Japan Acad.*, 1957, **33**, 53; K. Tanaka, M. Miyamoto, M. Honjo, H. Morimoto, T. Sugawa, and M. Uchibayashi, *ibid.*, p. 47.

1-methylhexa-1,3-dienyl)proline (domoic acid; II) was obtained from *Chondria armata*.²⁵ The three substances have useful anthelmintic properties.

Hydroxypipeolic acids have received considerable attention in the past two years. 4-Hydroxypipeolic acid was isolated first from *Acacia pentadena*.²⁶ A hydroxypipeolic acid, isolated from *Armeria maritima*,²⁷ was provisionally assigned the 3-hydroxy-configuration. Comparisons of the two materials showed them to be very similar, if not identical. Subsequent isolations from *Acacia willardia* and *Lysiloma bahamense*²⁸ have provided additional support for the 4-hydroxy-structure. More recently a hydroxypipeolic acid was isolated from *A. ecelsa*, and evidence supporting a *trans*-



4-hydroxy-configuration was obtained.²⁹ 4-Oxopipeolic acid has been shown to occur in the antibiotic, staphylocin;³⁰ on catalytic hydrogenation *L-cis*-4-hydroxypipeolic acid was formed. *cis*-3-Hydroxypipeolic acid has been prepared³¹ and shown to be separable from the imino-acid of *Armeria* by paper chromatography.⁸ The position regarding the natural occurrence of the 3-hydroxy-compound is now uncertain. However, 3-hydroxypicolinic acid occurs in the antibiotics etamycin³² and staphylocin.³¹

The *trans*-configuration assigned to 4-hydroxypipeolic acid brings this compound into line with natural 5-hydroxypipeolic acid isolated from dates; the *trans*-configuration of hydroxyl and carboxyl groups in the latter compound is established unequivocally.³³ A new method of obtaining the diastereoisomeric mixture of (\pm)-5-hydroxyallo(*cis*)- and (\pm)-5-hydroxypipeolic acid from kojic acid is available.³⁴ (\pm)-5-Hydroxypipeolic acid was prepared essentially free from the allo-isomer by reduction of 5-oxo-piperidine-2-carboxylic acid (obtained from glutamic acid) with sodium borohydride.³⁵ The related dehydro-derivative, baikiaiin (1,2,3,6-tetrahydropyridine-2-carboxylic acid), has also been synthesised by a new method. After hydrolysis of the benzoyl group from *cis*-5-benzamido-1-bromopent-3-yne-1-carboxylate, base-catalysed elimination of hydrogen bromide and ring closure gave baikiaiin.³⁶ Unexpected difficulties were

²⁵ K. Daigo, *J. Pharm. Soc. Japan*, 1959, **79**, 353, 356.

²⁶ A. I. Virtanen and S. Kari, *Acta Chem. Scand.*, 1955, **9**, 170.

²⁷ L. Fowden, *Biochem. J.*, 1958, **70**, 629.

²⁸ A. I. Virtanen and R. Gmelin, *Acta Chem. Scand.*, 1959, **13**, 1244.

²⁹ J. W. Clark-Lewis and P. I. Mortimer, *Nature*, 1959, **184**, 1234.

³⁰ H. Vanderhaeghe and G. Parmentier, Symposium on Peptide Antibiotics, 17th, Congr. Pure Appl. Chem., 1959, p. 56.

³¹ H. Plieninger and S. Leonhäuser, *Chem. Ber.*, 1959, **92**, 1579.

³² J. C. Sheehan, H. G. Zachau, and W. B. Lawson, *J. Amer. Chem. Soc.*, 1958, **80**, 3349.

³³ B. Witkop and C. M. Foltz, *J. Amer. Chem. Soc.*, 1957, **79**, 192.

³⁴ H. C. Beyerman, *Rec. Trav. chim.*, 1958, **77**, 249.

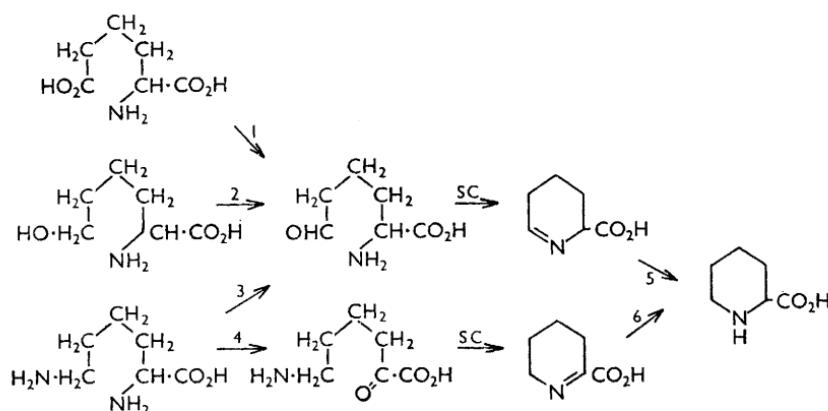
³⁵ H. C. Beyerman and P. Boekee, *Rec. Trav. chim.*, 1959, **78**, 648.

³⁶ N. A. Dobson and R. A. Raphael, *J.*, 1958, 3642.

found when attempts were made to cause the cyclisation of other 1,5-substituted *cis*-pent-3-ene-1-carboxylic acids.

The biogenetic pathways leading to the heterocyclic ring systems of proline and pipecolic acid have common features. The possible pathways by which pipecolic acid may be formed are illustrated in the annexed chart.

The conversion of lysine into pipecolic acid has been demonstrated by isotopic tracer experiments with higher plants, *Neurospora*, and intact rats. Reaction (4) is probably the primary one occurring in *Neurospora*³⁷ and



Reactions: (1) +2H. (2) -2H. (3) Transaminase or amine oxidase. (4) α -Amino-acid oxidase. (SC) Spontaneous cyclisation. (5) +2H. (6) +2H.

rats.³⁸ Loss of the ϵ -amino-group of lysine [reaction (3)] is catalysed by a plant amine oxidase from peas;³⁹ transamination could also yield α -amino-adipic δ -semialdehyde. The reduction of 1-amino-5-hydroxyhexanoic acid (hexahomoserine) by extracts of *N. crassa* [reaction (2)] also produced the aldehyde; DPNH acted as a better hydrogen donor than TPNH.⁴⁰ α -Amino- δ -hydroxyvaleric acid is reduced to glutamic γ -semialdehyde under similar conditions. As yet, reaction (1) does not appear to have been demonstrated with certainty, but the analogous conversion of glutamic acid into its γ -semialdehyde is well established.

Enzymic reduction of 3,4,5,6-tetrahydropyridine-2-carboxylate into pipecolic acid [reaction (6)] has been observed.⁴¹ Extracts of rat organs, of *N. crassa*, and of seedlings of pea and green gram (*Phaseolus radiatus*) are good sources of the dehydrogenase, which can use either DPNH or TPNH. Reaction (5) is not proved but the corresponding reduction of Δ^4 -pyrrolidine-2-carboxylic acid has been demonstrated for *N. crassa*⁴² and crude extracts of rat organs.⁴¹ Ultimately, reaction (5) may be shown to occur in higher

³⁷ R. S. Schweet, J. T. Holden, and P. H. Lowy, *J. Biol. Chem.*, 1954, **211**, 517.

³⁸ M. Rothstein and L. L. Miller, *J. Amer. Chem. Soc.*, 1954, **76**, 1459.

³⁹ P. J. G. Mann and W. R. Smithies, *Biochem. J.*, 1955, **61**, 89; A. J. Clark and P. J. G. Mann, *ibid.*, 1959, **71**, 596.

⁴⁰ T. Yura and H. J. Vogel, *J. Biol. Chem.*, 1959, **234**, 339.

⁴¹ A. Meister, A. N. Radhakrishnan, and S. D. Buckley, *J. Biol. Chem.*, 1957, **229**, 789.

⁴² T. Yura and H. J. Vogel, *J. Biol. Chem.*, 1959, **234**, 335.

plants since the necessary substrate, 2,3,4,5-tetrahydropyridine-2-carboxylic acid, is presumably produced by the action of plant amine oxidase.

The degradation of pipecolic acid has not been studied extensively. After [¹⁴C]- and [³H]-pipecolic acid had been supplied to phyllodes of *Acacia homalophylla*, radioactivity appeared quickly in various amino-acids, sugars, and organic acids. 4-Hydroxypipecolic acid and a compound presumed to be 5-amino-1-hydroxyhexanoic acid were amongst the first compounds to become labelled in the tritium experiments.⁸ α -Aminoadipic acid and lysine later became labelled and so some of the above reactions are probably reversible.

Two general mechanisms can be postulated for the biogenesis of the hydroxy-imino-acids. The first requires that the heterocyclic ring is formed only after the hydroxy-group has been introduced into an appropriate open-chain amino-acid. γ -Hydroxyglutamic acid and δ -hydroxylysine, both of which occur naturally, could then yield hydroxyproline and 5-hydroxypipecolic acid by reactions analogous to those involved in the synthesis of proline and pipecolic acid. However, there is no evidence that the conversion of hydroxyproline into γ -hydroxyglutamate (see above¹⁹) can be reversed, even in animal tissues. The metabolism of δ -hydroxylysine has not been investigated.

According to the second mechanism the hydroxyl group is introduced after the formation of the heterocyclic ring. This mechanism for hydroxyproline synthesis has now been shown to occur in animal and plant tissues. Hydroxyproline is a rare component of plant proteins but occurs as a characteristic component of the protein present in abnormally growing cells, e.g., in tissue cultures and plant tumours. By using cultures of carrot root, it has been shown that hydroxyproline is produced by hydroxylation of proline only after the latter imino-acid has been incorporated into the cell protein.⁴³ Free hydroxyproline causes inhibition of growth by suppressing the incorporation of proline into protein.

The experiments in which labelled pipecolic acid was supplied to *Acacia*⁸ (see above) indicated that 4-hydroxypipecolic acid was formed directly from the parent imino-acid. When [¹⁴C]lysine was supplied, labelled pipecolic acid was formed very rapidly; the carbon-14 was introduced subsequently into the 4-hydroxy-compound. No active hydroxylysine could be detected.

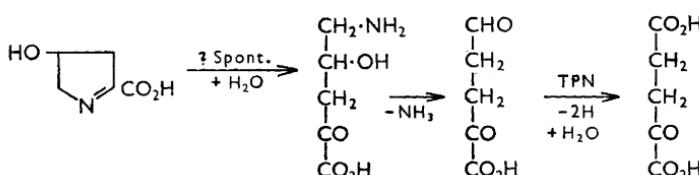
Degradation of hydroxyproline has been reported for two systems. The D-amino-acid oxidase of sheep kidney converted either D-hydroxyproline or D-allohydroxyproline into pyrrole-2-carboxylic acid; presumably 4-hydroxy- Δ^1 -pyrrolidine-2-carboxylate is an intermediate which then spontaneously loses water.⁴⁴ A strain of *Pseudomonas*, isolated from soil, also produced pyrrole-2-carboxylic acid from L-hydroxyproline or D-allohydroxyproline, which were interconverted by an epimerase.⁴⁴ An alternative degradative pathway was present in *Pseudomonas*; the Δ^1 -pyrrolidine ring could open and, after loss of ammonia and subsequent oxidation, α -oxoglutarate was formed, as shown opposite.⁴⁵

⁴³ J. K. Pollard and F. C. Steward, *J. Exp. Bot.*, 1959, **10**, 17.

⁴⁴ A. N. Radhakrishnan and A. Meister, *J. Biol. Chem.*, 1957, **226**, 559.

⁴⁵ E. Adams, *J. Biol. Chem.*, 1959, **234**, 2073.

The biosynthetic mechanism leading to azetidine-2-carboxylic acid is still not clear. Aspartic acid, $\alpha\gamma$ -diaminobutyric acid, and homoserine are possible precursors, but when these acids were supplied as ^{14}C -labelled compounds to leaves of *Convallaria majalis* (lily-of-the-valley), which contained considerable amounts of azetidine-2-carboxylic acid, only slight incorporation of radioactivity into the imino-acid was observed.⁴⁶ Diaminobutyrate gave a labelled compound that could be catalytically hydrogenated to yield azetidine-2-carboxylic acid; by analogy with proline and pipecolic acid metabolism, this compound may be Δ^1 -azetidine-2- or -4-carboxylic acid. Later work showed that roots, and not leaves, may be the main site for synthesis of azetidine-2-carboxylic acid in these plants.⁴⁷



Only slight degradation of $[^{14}\text{C}]$ azetidine-2-carboxylic occurred in *C. majalis* leaves in 48 hours.⁴⁶ In contrast, a soil yeast degraded azetidine-2-carboxylic acid rapidly with initial formation of γ -amino- α -hydroxybutyric acid; this was subsequently converted into β -alanine, possibly *via* γ -amino- α -oxobutyrate.⁴⁸

Diamino- and Basic Acids.—Recently several additions have been made to this group of naturally occurring acids. $\alpha\beta$ -Diaminopropionic and $\alpha\gamma$ -diaminobutyric acid, both previously recognised only as components of antibiotics, have now been identified as products from higher plants. The former was isolated from seeds of *Mimosa palmeri*;⁴⁹ the latter was present in traces in the rhizome of *Polygonatum multiflorum*.⁵⁰ New chemical syntheses are available for diaminopropionic acid⁵¹ and for diaminobutyric acid.⁵²

Derivatives of $\alpha\beta$ -diaminopropionic acid and the homologous acids, ornithine and lysine, have been isolated. L-(—)- α -Amino- β -ureidopropionic acid (albizzine, the lower analogue of citrulline) occurs in large quantities in seeds of various *Albizzia* species and those of other Mimosaceae.^{49,53} Albizzine was converted into diaminopropionic acid by treatment with boiling 48% hydrobromic acid. The isomeric L- β -amino- α -ureidopropionic acid has been synthesised.⁵³

δ -Acetyltornithine occurs in ferns, grasses, and many members of the

⁴⁶ L. Fowden and M. Bryant, *Biochem. J.*, 1959, **71**, 210.

⁴⁷ L. Fowden, *Biochem. J.*, 1959, **71**, 643.

⁴⁸ H. Vinson and L. Fowden, unpublished result.

⁴⁹ R. Gmelin, G. Strauss, and G. Hasenmaier, *Z. physiol. Chem.*, 1959, **314**, 28.

⁵⁰ L. Fowden and M. Bryant, *Biochem. J.*, 1958, **70**, 626.

⁵¹ H. Hellmann and G. Haas, *Chem. Ber.*, 1957, **90**, 1357.

⁵² G. Talbot, R. Gaudry, and L. Berlinguet, *Canad. J. Chem.*, 1958, **36**, 593; M. Fraenkel, Y. Knobler, and T. Sheradsky, *Bull. Res. Council Israel, Sect. A. Chem.*, 1958, **7**, 173; M. Zaoral, *Chem. Listy*, 1958, **52**, 2338.

⁵³ A. Kjaer, P. O. Larsen, and R. Gmelin, *Experientia*, 1959, **15**, 253.

Fumariaceae.⁵⁴ The flagellar proteins of the bacterium, *Salmonella typhimurium*, yield ϵ -N-methyl-lysine on hydrolysis;⁵⁵ this amino-acid was not detected in the protein component of the remainder of the bacterial cell.

γ -Guanidinobutyric acid is present in a variety of plant tissues;⁵⁶ from 1 to 20 $\mu\text{g}./\text{g}$. fresh weight occur in the fruit of many species. The acid may be formed by transamidination known to occur in animal tissues; canavanine, arginine, or guanidinoacetic acid can donate their amidine group to ornithine, canaline, glycine, or hydroxylamine.⁵⁷ The formation of γ -guanidinobutyrate from γ -aminobutyrate and arginine is catalysed by extracts of rat or pig kidney.⁵⁸ Glycine was better than, and β -alanine inferior to, γ -aminobutyrate as an acceptor of the transferred amidine group. A deguanidinase, differing in its properties from arginase, has been found in various fish livers; this enzyme hydrolytically splits γ -guanidinobutyric acid, yielding urea and γ -aminobutyrate.⁵⁹

Canavanine, an important constituent of jack-bean seeds (*Canavalia ensiformis*), has now been shown to occur in a wide variety of other leguminous seeds.⁶⁰ A new type of cleavage of canavanine to homoserine and hydroxyguanidine occurs in a pseudomonad.⁶¹ Kalyankar *et al.*⁶¹ also summarise the other known degradative reactions of canavanine that yield a variety of products including *O*-ureidohomoserine, guanidine, canaline, and α -oxo- δ -guanidinoxybutyric acid.

Another guanidino-compound, γ -hydroxyarginine, has been isolated from the marine animal, *Polycheira rufescens* (the sea-cucumber).⁶² Alkaline hydrolysis produced γ -hydroxyornithine, an amino-acid whose natural occurrence would not be unexpected, and which bears the same structural relationship to hydroxyproline as δ -hydroxylysine does to 5-hydroxy-pipeolic acid.

Acids derived from Alanine.—Amino-acids containing a benzenoid or heterocyclic ring attached to the β -carbon atom of an alanine residue feature as protein components (*e.g.*, phenylalanine, tyrosine, tryptophan, and histidine). Recently several additional alanine derivatives have been isolated from higher plants; they appear to occur only as free amino-acids.

2,4-Dihydroxy-6-methylphenylalanine (III) has been obtained from seeds of *Agrostemma githago* (corn cockle).⁶³ The acid was synthesised by the annexed route.

An amino-acid (stizolobic acid) containing a γ -pyrone nucleus has been isolated from *Stizolobium hassjoo*,⁶⁴ a shrubby legume; the plant also contains 3,4-dihydroxyphenylalanine. The new acid, β -(3-carboxy- γ -pyron-

⁵⁴ A. I. Virtanen and P. Linko, *Acta Chem. Scand.*, 1955, **9**, 531; L. Fowden, *Nature*, 1958, **182**, 406; R. H. S. Manske, *Canad. J. Res.*, 1937, **15**, B, 86; G. Reuter, *Flora*, 1957, **145**, 326.

⁵⁵ R. P. Ambler and M. W. Rees, *Nature*, 1959, **184**, 56.

⁵⁶ F. Irreverre, R. L. Evans, A. R. Hayden, and R. Silber, *Nature*, 1957, **180**, 704.

⁵⁷ J. B. Walker, *J. Biol. Chem.*, 1957, **224**, 57.

⁵⁸ J. J. Pisano, C. Mitoma, and S. Udenfriend, *Nature*, 1957, **180**, 1125.

⁵⁹ R. Baret and M. Mourgue, *Compt. rend. Soc. Biol.*, 1957, **151**, 1561.

⁶⁰ E. A. Bell, *Biochem. J.*, 1958, **70**, 617.

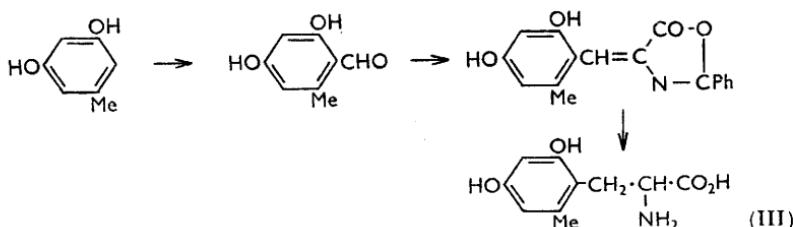
⁶¹ G. D. Kalyankar, M. Ikawa, and E. E. Snell, *J. Biol. Chem.*, 1958, **233**, 1175.

⁶² Y. Fugita, *Bull. Chem. Soc. Japan*, 1959, **32**, 439.

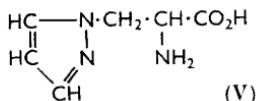
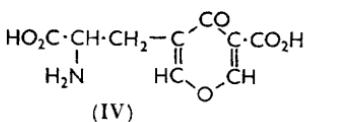
⁶³ G. Schneider, *Biochem. Z.*, 1958, **330**, 428.

⁶⁴ S. Hattori and A. Komamine, *Nature*, 1959, **183**, 1116.

5-yl)alanine (IV), is only a minor component and was isolated from the exudate flowing from cut stems of seedlings. Ozonolysis of stizolobic acid give a mixture of aspartic, oxalic, and formic acid.



Another heterocyclic ring (the pyrazole nucleus) is present in β -pyrazol-1-ylalanine (V), an amino-acid isolated from seeds of *Citrullus vulgaris* (watermelon). This is the first report of a naturally occurring pyrazole derivative. Smaller quantities of the acid are present in seeds of related cucurbitaceous plants. The acid was synthesised by refluxing silver

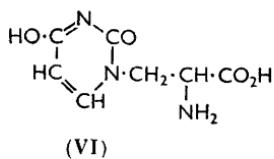


pyrazole with ethyl α -amino- β -chloropropionate. The infrared spectrum of the synthetic material was identical with that of the natural acid after its racemisation.⁶⁵ The compound isolated from watermelon juice, and provisionally identified as β -imidazol-1-ylalanine,⁶⁶ was probably the above pyrazole derivative.

The genus *Albizia* is a rich source of newer amino-acids. A new heterocyclic alanine derivative recently isolated from *A. willardia* has been identified as L- β -uracil-3-ylalanine (willardiine; VI).^{66a} The new acid is an isomer of mimosine (leucanol), first isolated from *Mimosa pudica*.^{66b} Willardiine and mimosine may both be regarded as derivatives of $\alpha\beta$ -diaminopropionic acid.

The biosynthesis and biodegradation of these alanine derivatives have not been investigated.

Hydroxy-amino-Acids.—Certain hydroxy-amino-acids have been described under earlier headings. Two further hydroxy-acids have been obtained from higher plants. γ -Hydroxyvaline was isolated from *Kalanchoe daigremontiana*; its presence in other *Kalanchoe* species could not be confirmed,⁶⁷ but by paper chromatography small amounts of its lactone were



⁶⁵ F. F. Noé and L. Fowden, *Nature*, 1959, **184**, B.A. 69.

⁶⁶ S. Shinano and T. Kaya, *J. Agric. Chem. Soc. Japan*, 1957, **31**, 759.

^{66a} R. Gmélén, *Z. physiol. Chem.*, 1959, **316**, 164.

^{66b} J. Renz, *Z. physiol. Chem.*, 1936, **244**, 153; R. Adams and J. L. Johnson, *J. Amer. Chem. Soc.*, 1949, **71**, 705.

⁶⁷ J. K. Pollard, E. Sondheimer, and F. C. Steward, *Nature*, 1958, **182**, 1356.

identified in extracts of *K. daigremontiana*. γ -Hydroxyvaline was synthesised by catalytic hydrogenation of β -methyl- α -oxo- γ -butyrolactone to give its α -hydroxy-lactone; this was converted into the α -chloro-derivative by treatment with thionyl chloride in pyridine, and γ -hydroxyvaline was then obtained after treatment with concentrated ammonia. The stereoisomeric composition of the product was not investigated.

The second compound isolated was *O*-acetylhomoserine;⁶⁸ like homoserine, it was isolated from pea plants. The function and metabolism of the two hydroxy-acids have not been studied.

β -Hydroxyleucine has been identified as a component of a peptide-type antibiotic produced by a strain of *Paecilomyces*.⁶⁹

Aminobutyric Acids.— γ -Aminobutyric acid is present in the soluble-nitrogen fraction of almost all plants. It may be formed from glutamic acid by the action of glutamic decarboxylase, an enzyme widely distributed in plants. γ -Aminobutyrate is produced by other pathways in micro-organisms, e.g., by transamination from succinic semialdehyde in *Pseudomonas fluorescens*,⁷⁰ and *Torulopsis utilis*,⁷¹ by hydrolysis of the lactam ring of 2-pyrrolidone in *P. aeruginosa*,⁷² and by oxidation of pyrrolidine or putrescine via γ -aminobutyraldehyde in *P. fluorescens*.⁷⁰ It is doubtful whether these mechanisms are of any importance for the synthesis of γ -aminobutyric acid in higher plants, but putrescine, and possibly succinic semialdehyde, have been identified in some plants.

The suggestion has been made that γ -aminobutyric acid may be re-carboxylated to yield glutamic acid. Comparison of the metabolism of γ -amino-[¹⁴C]butyrate and [¹⁴C]glutamine in tissue culture of carrot root has lent support to this idea,⁷³ but only slight reversal of normal glutamic-decarboxylase activity has been demonstrated.⁷⁴ However, when the enzymic re-carboxylation of γ -aminobutyric acid was studied in the presence of an anion-exchange resin, larger proportions of glutamic acid were produced (the glutamate formed was absorbed by the resin and rendered unavailable to the enzyme).⁷⁵ A similar situation may be operative in living tissues where cellular organisation may ensure that newly formed molecules of glutamic acid are removed immediately from the site of enzyme action.

It is more probable that γ -aminobutyric acid is converted into succinic semialdehyde by a transamination and that the aldehyde is then oxidised to succinic acid. Many micro-organisms, including yeast, bacteria, and unicellular algae, contain transaminases capable of catalysing the reaction between γ -aminobutyrate and α -oxoglutarate. The properties of the enzyme present in *P. fluorescens* have received detailed study.⁷⁰ The same

⁶⁸ N. Grobbelaar and F. C. Steward, *Nature*, 1958, **182**, 1358.

⁶⁹ G. W. Kenner and R. C. Sheppard, *Nature*, 1958, **181**, 48.

⁷⁰ E. M. Scott and W. B. Jakoby, *J. Biol. Chem.*, 1959, **234**, 932; W. B. Jakoby and J. Fredericks, *ibid.*, p. 2145.

⁷¹ R. Pietruszko and L. Fowden, unpublished result.

⁷² F. F. Noé and W. J. Nickerson, *J. Bact.*, 1958, **75**, 674.

⁷³ F. C. Steward, R. G. S. Bidwell, and E. W. Yemm, *J. Exp. Bot.*, 1958, **9**, 11.

⁷⁴ R. Koppelman, S. Mandelis, and M. E. Hanke, *J. Biol. Chem.*, 1958, **233**, 73.

⁷⁵ J. K. Pollard, personal communication.

authors studied the properties of succinic semialdehyde dehydrogenase, isolated from this organism.⁷⁶

The metabolism of γ -amino[carboxy-¹⁴C]butyric acid has been studied in the organism, *Bacillus pumilus*,⁷⁷ which produces and excretes large amounts of glutamic acid into the culture medium. If glutamic acid was formed primarily by direct carboxylation from this specifically labelled γ -aminobutyrate, then the ω -carboxy-group would have carried the heaviest labelling. But over 99% of the activity present in the glutamate was associated with the α -carboxy-group, which is in accord with its formation from the carbon skeleton of γ -aminobutyric acid *via* the reactions of the tricarboxylic acid cycle. When the organism was grown in the presence of some of the organic acid intermediates of this cycle, the glutamate excreted had, as expected, a lower specific activity.

γ -Amino[¹⁴C]butyric acid was metabolised quite readily when supplied to pea leaves; aspartic acid, alanine, and glutamic acid became radioactive in succession,⁷⁸ as would be expected if the carbon skeleton was metabolised *via* the tricarboxylic acid cycle. This sequence of labelling suggests that the initial degradation of γ -aminobutyrate involved a transamination; direct carboxylation would give glutamic acid as the primary labelled compound.

Convincing demonstrations of a γ -aminobutyrate-transaminase in higher plant tissues are, however, rare. A transaminase, catalysing a reaction between γ -aminobutyrate and α -oxoglutarate, was found in extracts of nodulated pea roots,⁷⁹ but further experiments have shown that the enzyme activity is confined to the nodules.⁷⁸ Since the microbial symbiont, *Rhizobium*, contains an active γ -aminobutyrate-transaminase, the transamination obtained with nodulated roots may have been caused entirely by the infective organism. A similar transaminase has been reported in a potato tuber extract,⁸⁰ but this observation is of doubtful value because microbial contamination was not excluded during the long, 24 hour, reaction period. With mitochondria from peanut seedlings, definite transamination between γ -aminobutyrate and either α -oxoglutarate or pyruvate has been obtained in 3 hours.⁷⁸ Pyruvate was approximately five times more efficient than α -oxoglutarate as an amino-group acceptor.

Oxidative degradation of γ -aminobutyric acid has not been demonstrated as yet in plants, but oxidative mechanisms operate in brain tissue and lead to the formation of γ -amino- β - and - α -hydroxybutyric acid.⁸¹ Eventually these mechanisms may be shown to be widely distributed. By the production of these β - and α -hydroxy-acids, γ -aminobutyrate metabolism uses intermediates common to degradation of hydroxyproline⁴⁴ and azetidine-2-carboxylic acid,⁴⁸ respectively. Mutant strains of *Escherichia coli* also produce γ -amino- α -hydroxybutyric acid by decarboxylation of γ -hydroxy-

⁷⁶ W. B. Jakoby and E. M. Scott, *J. Biol. Chem.*, 1959, **234**, 937.

⁷⁷ T. Tsunoda and I. Shioi, *J. Biochem. (Japan)*, 1959, **46**, 1011, 1227.

⁷⁸ R. O. D. Dixon and L. Fowden, unpublished result.

⁷⁹ J. K. Miettinen and A. I. Virtanen, *Acta Chem. Scand.*, 1953, **7**, 1243.

⁸⁰ T. Suzuki, A. Maekawa, T. Hasegawa, M. Ito, H. Honda, T. Nagano, S. Saito, and Y. Sahashi, *Bull. Agric. Chem. Soc. Japan*, 1958, **22**, 39.

⁸¹ K. Inui, *Med. J. Osaka Univ.*, 1959, **11**, 681; S. Sao, *ibid.*, 1957, **7**, 833.

glutamic acid,⁸² and the β -hydroxy-acid similarly from allo- β -hydroxy-glutamate.⁸³

γ -Amino- α -hydroxybutyric acid has been synthesised only recently. One method used γ -aminobutyrate which, after *N*-acetylation, was brominated on the α -carbon atom. The bromine atom was replaced by a hydroxyl group and acid-catalysed de-acetylation yielded the required acid.⁸⁴ It has also been prepared by treating $\alpha\gamma$ -diaminobutyric acid with nitrous acid.⁴⁸

α - and β -Aminoisobutyric acid are both known as plant products. The β -amino-compound was isolated recently from the bulbs of *Iris tingitana*;⁸⁵ synthetic and racemised natural material gave identical infrared spectra. A new synthesis of the acid from glycine involving Wolff rearrangement of the diazoethyl ketones has been described.⁸⁶ α -Aminoisobutyric acid is a component of the antibiotic produced by *Paecilomyces*.⁶⁹ The metabolism of these acids has not been investigated.

Acids derived from Cysteine.—The isolation of (+)-S-methyl-L-cysteine sulphoxide from cabbages and turnips⁸⁷ has been followed by that of its probable biological precursor (−)-S-methyl-L-cysteine. The latter acid was isolated from seeds of *Phaseolus vulgaris* (kidney beans),⁸⁸ and occurs as a metabolite of *N. crassa*. It can act as a sole source of nutrient sulphur for this organism.⁸⁹ A transmethiolase, isolated from yeast, has been partly purified and shown to catalyse the formation of S-methyl-L-cysteine from L-serine and methanethiol.⁹⁰ No other amino-acid tested could substitute for L-serine as an acceptor of the MeS group. Ethanethiol reacted at about 60% of the rate observed for methanethiol. γ -L-Glutamyl-S-methyl-L-cysteine has also been obtained from kidney-bean seeds;⁹¹ and the corresponding sulphoxide occurs in the Lima bean.⁹²

The seeds of *Albizzia julibrissin* contain S-2-carboxyethyl-L-cysteine in amounts equal to 0·3% of their dry weight.⁹³ The acid has been synthesised from L-cysteine and β -bromopropionic acid.⁹⁴ A crude enzyme preparation from *A. lophantha* seeds causes hydrolysis of S-2-carboxyethyl-L-cysteine to ammonia, pyruvic acid, and β -mercaptopropionic acid.⁹³ S-2-Carboxypropyl-L-cysteine has been identified tentatively as a component of *A. willardia*.^{86a}

Cycloalliin (5-methyl-1,4-thiazan-3-carboxylic acid 1-oxide) (VII), isolated recently from onion bulbs (*Allium cepa*),⁹⁵ may be regarded as a cyclised

⁸² A. I. Virtanen and P. K. Hietala, *Acta Chem. Scand.*, 1955, **9**, 549.

⁸³ W. W. Umbreit and P. Heneage, *J. Biol. Chem.*, 1953, **201**, 15.

⁸⁴ A. Mori, *J. Biochem. (Japan)*, 1959, **46**, 59.

⁸⁵ S. Asen, J. F. Thompson, C. J. Morris, and F. Irreverre, *J. Biol. Chem.*, 1959, **234**, 343.

⁸⁶ K. Balenović, I. Jambrešić, and I. Ranogajec, *Croat Chem. Acta*, 1957, **29**, 87.

⁸⁷ R. L. M. Syngle and J. C. Wood, *Biochem. J.*, 1956, **64**, 252; C. J. Morris and J. F. Thompson, *J. Soc. Chem. Ind.*, 1955, 951.

⁸⁸ J. F. Thompson, C. J. Morris, and R. M. Zacharius, *Nature*, 1956, **178**, 593.

⁸⁹ J. B. Ragland and J. L. Liverman, *Arch. Biochem. Biophys.*, 1956, **65**, 574.

⁹⁰ E. C. Wolff, S. Black, and P. F. Downey, *J. Amer. Chem. Soc.*, 1956, **78**, 5958.

⁹¹ R. M. Zacharius, C. J. Morris, and J. F. Thompson, *Arch. Biochem. Biophys.*, 1959, **80**, 199.

⁹² H. Rinderknecht, *J. Soc. Chem. Ind.*, 1957, 1384.

⁹³ R. Gmelin, G. Strauss, and G. Hasenmaier, *Z. Naturforsch.*, 1958, **13b**, 252.

⁹⁴ A. Schöberl and A. Wagner, *Z. physiol. Chem.*, 1956, **304**, 97.

⁹⁵ A. I. Virtanen and E. J. Matikkala, *Acta Chem. Scand.*, 1959, **13**, 623.

Some properties of recently isolated amino- and imino-acids.

Amino- and imino-acid	M. p.*	[α] _D in H ₂ O	Approx. R _F in phenol-H ₂ O †	Approx. <i>R</i> _{Asahie} butan-1-ol-acetic acid-water ‡	Colour with ninhydrin ¶
<i>m</i> -Carboxyphenylglycine	215° d	—	—	—	Y → Br or RV
L- <i>threo</i> -β-Methylaspartic acid	240—290 d	-10°	+13.3° (5N)	0.20(+)	BP
(+)- <i>ex</i> -Diaminosuccinic acid	187 d	-12	+59 (1.5N)	0.12(+)	RBr
L-γ-Allohydroxyglutamic acid	195—197 d	-12	+3 (5N)	0.13(+)	BP
L-γ-Methyleneglutamic acid	195 d	+14 (3N)	0.29(+)	0.78	YBr
L-α-Kainic acid	251 d	-15	—	—	Y
L-α-Allokainic acid	237 d	+8	—	—	—
Domoic acid	217 d	-109.6	—	—	B
L-5-Hydroxybipeolic acid	235 d	-23.1	+0.34 (N)	0.78(+)	Y → G → B
L- <i>trans</i> -4-Hydroxybipeolic acid	294 d	-13.1	+0.34 (N)	0.82(+)	0.92
L- <i>xy</i> -Diaminobutyric acid dihydrochloride	197—198	+13.3	—	0.33(—)	0.04
L- <i>ab</i> -Diaminopropionic acid monohydrochloride	237 d	—	+25 (N)	0.31(—)	0.04
Albizzine	217 d	-67	-22.2 (N)	0.43(—)	0.44
δ-Acetylornithine	266 d	+13.1	—	0.84(+)	BP
γ-Hydroxyarginine monohydrochloride	190—191 d	—	+5.4 (5N)	0.86(+)	1.0
β-(2,4-Dihydroxy-6-methylphenyl)alanine	252 d	—	+19.7 (N)	0.56(—)	BP
Stizolobic acid	231—233	—	—	0.16(—)	Or
β-Pyrrozol-1-ylalanine	236—238 d	-73	—	0.84(+)	BP
1-β-Uracil-3-ylalanine	204—205 d	—	-12.1 (N)	0.52(—)	BP
γ-Hydroxyvaline	228	+10	—	0.69(—)	V
β-Acetylhomoserine	—	—	—	1.27	V
β-Aminoisobutyric acid	183	-13	—	1.26	V
S-Methyl-L-cysteine	169—170	-26	—	0.82(—)	BP
S-Methyl-L-cysteine sulphoxide	220 d	+125	—	1.8	BP
γ-Glutamyl-S-methyl-L-cysteine	165—173 d	-21	—	0.71(—)	BP
S-2-Carboxyethyl-L-cysteine	218 d	—	-9 (N)	0.62(—)	BP
Cycloallin	—	—	—	0.47(—)	0.50
			—	0.35(—)	1.0
			—	0.85(+)	1.1
			—	2.2	V
			—	Y	Y

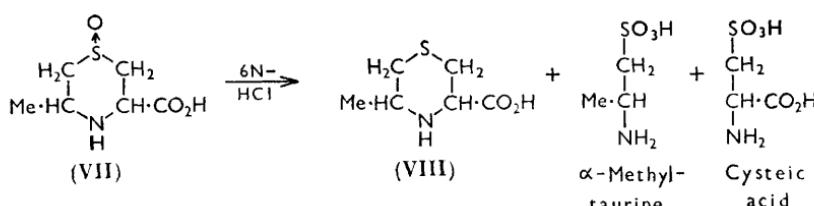
* M. p.'s associated with decomposition are indicated as d.

† Phenol-H₂O mixtures normally contain 75—80% (w/v) of phenol. + or — in parentheses indicates *R*_F value determined in presence or absence of NH₃, respectively.

‡ Composition of solvent mixture used varies slightly between different laboratories.

¶ Colour symbols: Y, yellow; Br, brown; R, red; V, violet; B, blue; P, purple; G, green; Or, orange.

form of alliin, S-allyl-L-cysteine sulphoxide, obtained earlier from garlic (*Allium sativum*).⁹⁶ Cycloalliin was synthesised by the following stages:⁹⁵ L-cysteine, S-allyl-L-cysteine, S-2-bromopropyl-L-cysteine, 5-methyl-1,4-thiazan-3-carboxylic acid (VIII) (by ring closure involving elimination of hydrogen bromide in pyridine), cycloalliin [formed from (VIII) by treatment with hydrogen peroxide]. Cycloalliin was decomposed by refluxing it with 6*N*-hydrochloric acid, and oxidised and reduced products were obtained (see formulæ).



The biosynthesis of cycloalliin may proceed by hydration of alliin to yield S-2-hydroxypropyl-L-cysteine sulphoxide; then elimination of water from the hydroxyl and α-amino-groups may cause ring closure.

Summary of Properties.—The Table annexed lists some of the physical properties of amino- and imino-acids isolated recently. Unfortunately, complete data are frequently not published, sometimes owing to lack of material. To the biologist, *R*_F values are often more useful than m. p.'s and data are given here for two commonly used solvents. Differences in the experimental procedures used in various laboratories cause minor variations in these values, and, in order to minimise these variations, *R*_F values obtained with butan-1-ol-acetic acid-water mixtures are based upon that of alanine (*R*_{alanine}). The colour produced from each amino-acid spot on paper chromatograms treated with ninhydrin is somewhat variable; as far as possible those given in the Table are quoted from the original literature.

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L. FOWDEN.
J. GLOVER.
T. W. GOODWIN.
R. L. HARTLES.
S. V. PERRY.

⁹⁶ A. Stoll and E. Seebeck, *Experientia*, 1947, **3**, 114.