PROFESSOR ALBERT NEUBERGER

Aspects of the Metabolism of Glycine and of Porphyrins

By ALBERT NEUBERGER

THE SECOND HOPKINS MEMORIAL LECTURE

*Delivered to a meeting of The Biochemical Society on 29 June 1960 in
the Anatomy Lecture Theatre, Cambridge University*

An invitation to give a Hopkins Memorial Lecture is the greatest honour which The Biochemical Society can confer upon one of its members and I wish to thank you for awarding me this distinction which I deeply appreciate. The pleasure which such an invitation must necessarily produce in the recipient is mingled, however, with a feeling of humility and inadequacy to perform such a task. Unlike my distinguished predecessor, Sir Rudolph Peters, I was associated with Sir Frederick Gowland Hopkins only for the comparatively short period of three-and-a-half years, and this occurred at a time when my scientific interests had already been defined to a large extent by having worked for six years in the department of Sir Charles (then Professor) Harington. During the time I spent in the Biochemistry Department in Cambridge I was in fairly close and frequent contact with Hopkins and I am conscious of having been deeply influenced by the scientific philosophy and the teachings of that great master. The important part played by Hopkins in the development of British and indeed world biochemistry has been described by others who are better qualified than I am to evaluate his contribution to biological sciences. In choosing as a subject of this lecture the metabolism of glycine, serine and porphyrins, I was actuated by two considerations. It is a field to the knowledge of which my colleagues, past and present, and myself have made some contributions; but this choice also gives me the opportunity to demonstrate the wide sweep of biochemistry covering the whole of living matter from microbes to man, and to show the variety of methods employed, which range from investigations in the whole animal to experiments with a pure enzyme and which include in my opinion most of the techniques used by the organic chemist.

Glycine was first isolated in 1820 by Braconnot from a hydrolysate of gelatin and was first synthesized by Perkin and Duppa in 1858. It is present in all or almost all proteins so far analysed, but, apart from special groups of proteins, its concentration in terms of grams of amino acid/100 g. of protein hardly ever exceeds 6. The exceptions are the keratins, in which glycine contents vary between 5 and 9.5%, the mammalian

collagens, with 26–27% of glycine, and fibroins, which contain between 23 and 44% of glycine. As collagen forms about 20–25% of the total protein of the mammal, and as this protein contains about 26% of glycine, it follows that approximately 60% of the glycine of the organism is bound in collagen linkage. Since the latter is metabolically fairly inert (Neuberger, Perrone & Slack, 1951), it appears that more than half of the glycine is in a state in which it does not take a quantitatively significant part in the relatively fast synthetic and degradative processes in which most other body constituents continuously participate.

It has been known for some time that glycine can be synthesized by mammals, and Rose and his co-workers have shown that the presence of glycine in the diet of the rat is not required for maximum growth; the same is likely to apply to man and probably most other mammals. In the young chick and turkey, growth rate is reduced if glycine is completely absent from the diet (Almquist, Stokstad, Mecchi & Manning, 1940), but glycine can be replaced by serine or aminoethanol.

Table 1. *Biological reactions involving glycine*

Incorporation into proteins
Conversion into carbohydrate
Formation of creatine
Incorporation into purines
Formation of serine
Formation of glutathione
Formation of glycocholic acid
Formation of hippuric acid
Oxidation or transamination to glyoxylic acid
Formation of aminolaevulinic acid and aminoacetone
Reversible transformation into <i>allo</i> threonine (and threonine)

Table 2. *Biological reactions of serine*

Incorporation into proteins
Splitting into glycine and 'formaldehyde' (production of C ₁ units from β -carbon atom)
Deamination to pyruvate
Oxidation or transamination to hydroxypyruvate
Reaction with homocysteine to cystathionine
Decarboxylation to aminoethanol
Incorporation into phospholipid
Reaction with palmitaldehyde to dihydrosphingosine
Condensation with indole to tryptophan

For the adult bird glycine is not required, not even for egg-laying (Johnson & Fisher, 1956). The reasons for the special needs for glycine by the young bird are considered below.

Glycine takes part in a great number of biochemical reactions, as the representative, but incomplete, list (Table 1) shows. It is now well established that glycine and serine are readily transformed into one another, and therefore in an overall assessment of the biochemical importance of glycine it is necessary to take into account the various reactions in which serine participates (Table 2). It would be impossible in the course of this lecture to deal adequately with all the various transformations involving glycine and serine which occur in the different forms of life. I have therefore chosen a few topics which for various reasons seem most appropriate for this occasion.

The enzymic condensation of glycine with acyl-coenzyme A derivatives

The reactions leading from glycine to porphyrins have now in general been well established, mainly owing to the work of the Laboratories of Shemin, Rimington and those of my own group, and the later stages in this sequence of enzymic steps are now being elucidated in the schools just mentioned and in the Laboratories of Granick and Bogorad. In this lecture I wish to confine my attention to the earliest reaction, i.e. the condensation between glycine and succinyl-coenzyme A (CoA).

In the reaction between glycine and acyl derivatives of coenzyme A to yield α -aminoketones and

carbon dioxide, pyridoxal 5-phosphate is required as a cofactor (Laver, Neuberger & Udenfriend, 1958; Gibson, Laver & Neuberger, 1958; Kikuchi, Kumar, Talmage & Shemin, 1958; Kikuchi, Kumar & Shemin, 1959), and it is reasonable to assume that the actual reactant is a Schiff's base or aldimine formed between the amino group of glycine and the carbonyl group of pyridoxal phosphate. As pointed out by Lynen (1959), acyl-CoA compounds can act either as nucleophilic or as electrophilic reagents and in δ -aminolaevulinic acid formation it is likely that the acyl carbon atom of succinyl-CoA functions as electron acceptor to the nucleophilic reagent, which in this case is the anion formed by the removal of a proton from the α -carbon atom of the glycine-pyridoxal phosphate complex. By following largely the suggestions of Braunstein and Snell (see also Metzler, 1957), the initial reaction may be visualized as in Fig. 1.

It is assumed that the proton which dissociates from the α -carbon atom of the glycine moiety migrates to the ring nitrogen atom, the resulting zwitterionic structure (I) being stabilized by resonance with a non-zwitterionic form (II) in which all double bonds including that of the carboxylate group are conjugated. This resonance facilitates dissociation and migration of the proton and the carbanion formation is further assisted, as suggested by Snell, by hydrogen-bond formation between the nitrogen atom derived from glycine and the phenolic oxygen atom. It is further proposed that the complex is linked to protein, probably by ionic forces, and that such linkage

Fig. 1. Initial steps in the synthesis of aminolaevulinic acid.

Fig. 2. Mechanism of the formation of acylaminoketones.

takes place through the phosphate and carboxylate groups. A linkage through the carboxylate group to protein has been suggested for amino acid decarboxylases by Rothberg & Steinberg (1957), and is likely to consist of a Coulomb type of interaction with a positively charged group on the enzyme. The masking of the negative charge of the carboxylate group will also assist the ionization of the proton in the α -position, i.e. the formation of the postulated carbanion. The aminoketone synthetase appears to be fairly non-specific with respect to the acyl group of the acyl-CoA derivative, but the only amino acid which has so far been found to give an aminoketone with the enzyme system is glycine. This specificity suggests that glycine is directly attached to the enzyme protein. With most purified enzymes of animal origin requiring pyridoxal phosphate so far examined, it has been found that the cofactor is fairly strongly bound to the protein. This general finding is supported by observations made on the aminoketone synthetase obtained from bird erythrocytes. Such preparations have, even after prolonged dialysis, considerable activity without addition of pyridoxal phosphate. However, addition of the coenzyme produces a further increase of activity which is not reduced by further dialysis. Enzyme preparations from microbial sources such as *Rhodospseudomonas spheroides* behave differently and even a concentrated extract of bacterial cells shows no activity unless pyridoxal phosphate is added. Another relevant observation is that the inhibition which is caused by cysteine and penicillamine is stereospecific. As suggested by du Vigneaud and his colleagues, this inhibition is almost certainly due to a formation of a thiazolidine ring with participation of the aldehyde group of pyridoxal phosphate. As pyridoxal phosphate itself and glycine are optically inactive, the very specific inhibitory power shown by the L-enantiomorphs of these α -amino β -thiol acids can most readily be explained by the assumption that the coenzyme is combined with an optically active substance which most likely is the enzyme.

Almost all reactions promoted by enzymes can be carried out by the organic chemist with more vigorous conditions or unphysiological catalysts. The enzymic formation of α -aminoketones from

glycine and activated derivatives of carboxylic acids has an apparent parallel in the reaction described by Dakin & West (1928), in which α -acetamidoketones are produced by warming an amino acid with acetic anhydride in the presence of pyridine. Indeed Wiley (1950) has suggested that the α -acetamido acid which is first produced is converted into a carbanion, with simultaneous loss of carbon dioxide, and that this ion then reacts with acetic anhydride to give the acetamidoketone. However, Cornforth & Elliott (1950) have put forward fairly convincing arguments that the more likely reactant is the ion derived from a 2-methyl-5-oxazolone (III), which on reaction with the acid anhydride will give a 4-acyloxazolone (IV or V; R = CH₃) (Fig. 2); the latter, on ring-opening, which occurs readily, will lose carbon dioxide and thus give an acylaminoketone. We ourselves have found that α -acetamido β -oxo acids are fairly unstable. The recent experiments of Crawhall & Watts (1960), however, suggest that the mechanism suggested for acetylative decarboxylation by Cornforth & Elliott may either have to be modified or that there may be a second mechanism.

The close resemblance between the oxazolone structure and the intermediate proposed in the aminoketone-synthetase reaction (structures I $\leftrightarrow$ II) is obvious and in both cases the carbon-nitrogen double bond assists the adjacent carbon atom to lose a proton or to react as a nucleophilic centre. Another closely related example is the reaction used by Attenburrow, Elliott & Penny (1948) in their synthesis of threonine. These workers obtained by condensation of acetic anhydride with hippurate in the presence of base a substance which is in fact the azlactone (IV) or (V) (R = C₆H₅) of α -benzamido- β -oxobutyric acid. It thus appears that the enzymic reaction under consideration is based on the reactivities of the various groups which can be ascertained by studying the behaviour of the compounds in ordinary organic reactions.

The decarboxylation step

When δ -aminolaevulinic acid was discovered to be an intermediate in porphyrin synthesis, both Dr Shemin's group and we assumed that α -amino- β -oxoadipic acid was the first product of the enzymic

reaction and that this compound then lost carbon dioxide either spontaneously or by the action of a decarboxylase. As such compounds had not been synthesized, it seemed desirable to obtain some information about their properties or indeed to ascertain whether they have even a transient existence. It was aimed (Laver, Neuberger & Scott, 1959*a, b*) to obtain the free α -amino β -oxo acid by a synthesis, the last step of which consisted in the liberation of the sensitive carboxy group by the mildest possible means. The various reactions are shown in Fig. 3. Ethyl β -oxoadipate was treated with benzyl alcohol under conditions which led to an ester exchange on the carboxyl group which is in β -position to the keto group. The mixed ester (VI) was then nitrosated and the oximino keto ester (VII) was reduced in acid solution; the acidity was such that only the ethyl ester group was hydrolysed. The difference in the rate of splitting of the two ester groups is caused by the fact that the positive charge of the amino group repels the approach of the proton or hydronium ion to the α -ester grouping to a much greater extent than it does to the more distant δ -ester grouping. The α -benzyl ester of α -amino β -oxoadipic acid (VIII) could thus be obtained in good yield. The free acid was formed by catalytic reduction of the benzyl ester with the use of a large amount of catalyst, but on isolation only the decarboxylation product, aminolaevulinic acid (IX), was obtained. By measuring the volume changes during catalytic reduction and changes in ultra-violet absorption, it appeared that the maximum half-life of the dicarboxylic acid is about 20 min. in ethanol and approximately 8 min. in water at pH 0. At pH 7.0 the half-life is less than 1 min. and it is clear that the likelihood of demonstrating even the transient existence of this aminoketo-dicarboxylic acid in a biological system is at present rather remote.

The observations described render the assumption of a participation of an enzyme in the decarboxylation of aminolaevulinic acid unnecessary. Moreover, the structures postulated above (I $\leftrightarrow$ II) for the intermediate in the condensation of glycine

with succinyl-CoA, i.e. the complex of pyridoxal phosphate, glycine and the enzyme, are identical with those proposed to be concerned in the enzymic decarboxylation of many amino acids not containing a β -carbonyl function. It would thus be reasonable to assume that condensation of the aldimine with succinyl-CoA is followed by a second reaction consisting in the decarboxylation of the pyridoxal phosphate- α -amino-oxoadipic acid complex or that indeed both reactions are simultaneous. It would seem difficult to devise experiments which could decide between these two possibilities, i.e. spontaneous non-enzymic decarboxylation and participation of a pyridoxal phosphate-containing enzyme, and we were prepared to leave this question open, had it not been for other developments in this field which I now wish to relate.

Effect of biotin on porphyrin formation and on synthesis of δ -aminolaevulinic acid

It was shown by Lascelles (1956) that suspensions of the photosynthetic organism *Rhodospseudomonas spheroides* grown in the light and in a medium deficient in biotin do not form coproporphyrin or bacteriochlorophyll from α -oxoglutarate and glycine unless further incubation is carried out in the presence of biotin. The last-named is not required for porphyrin synthesis from aminolaevulinic acid. My colleagues K. D. Gibson and G. H. Tait have extended this work further. They have shown that biotin is also required for the synthesis of aminolaevulinic acid from glycine and succinate, and the latter is presumably transformed into succinyl-CoA, not by the α -oxoglutaric acid dehydrogenase, but by the enzyme succinyl thio-kinase. It seemed therefore possible that biotin is needed for the actual condensation of succinyl-CoA and glycine. It was further found that deficiency of biotin in the medium reduces not only porphyrin formation in whole cells, but also the level of aminolaevulinic acid-synthetase activity in cell extracts, and this decrease is very much greater than that of aminolaevulinic acid-dehydrase activity. Whilst the decrease of porphyrin pro-

Fig. 3. Attempted synthesis of free α -amino- β -oxoadipic acid.

duction in biotin-deficient cells may be caused by a possible participation of biotin in the synthesis of pyridoxal phosphate, or some other reaction not involving the actual synthesis of aminolaevulinic acid, the apparently specific reduction of aminolaevulinic acid synthesis in extracts in which succinyl-CoA is used as substrate in the presence of added pyridoxal phosphate clearly indicates that biotin is either a coenzyme in the actual condensation or participates specifically in the synthesis of the protein without being incorporated into its structure.

Mr Tait has recently shown that addition of a commercial preparation of avidin to a cell extract inhibits synthesis of aminolaevulinic acid from succinyl-CoA and glycine. Avidin is a protein occurring in egg white and known to bind biotin strongly and, so far as is known at present, specifically. This observation was confirmed by using a number of pure proteins isolated from egg white by Dr N. M. Green and Dr R. D. Marshall. Of all the fractions used, inhibition was found only with an avidin fraction which was at least 50% pure. So far it has not been possible to overcome the inhibitory effect of avidin by prior incubation with biotin, and this must necessarily lead to some doubt as to whether the reduction of the synthesis of aminolaevulinic acid by avidin is caused by an interaction with biotin. It is of course not impossible that enzyme-bound biotin displaces free biotin from the complex, and this and other possibilities are being explored at present. We cannot assume that the belief which is generally held at present that avidin interacts only with compounds of the biotin type is securely established. On the other hand, the facts that biotin deficiency results in an apparently specific reduction in aminolaevulinic acid-synthetase activity in cell extracts and that aminolaevulinic acid synthesis is inhibited by avidin may be considered as indications that biotin may play an integral part in the synthesis of aminolaevulinic acid by the synthetase. Lynen (1959) has shown that free biotin combines with carbon dioxide through one of the ureido nitrogen atoms, forming an allophanic acid type of structure. If biotin functions directly in the synthesis of aminolaevulinic acid we may picture the formation of the acid as being a concerted reaction in which the condensation of the enzyme-glycine-pyridoxal phosphate complex with succinyl-CoA is synchronized with the removal of carbon dioxide as a biotin derivative. My colleague Dr J. J. Scott has suggested that the complex formed between α -amino- β -oxoadipic acid, pyridoxal phosphate and the enzyme may be very stable and the intervention of protein-bound biotin is needed to remove the carboxyl group, and this results in the regeneration of the enzyme and pyridoxal phosphate

together with the formation of aminolaevulinic acid. But it should be emphasized that this idea is at present only a somewhat adventurous speculation and that further work is needed with a highly purified enzyme before direct participation of biotin in the actual synthesis of aminolaevulinic acid can be considered established.

In addition to the decrease in the activity of the aminolaevulinic acid synthetase which can be measured in cell extracts by the addition of succinyl-CoA and glycine and pyridoxal phosphate and which is observed in severe biotin deficiency, there is another effect of biotin on porphyrin synthesis which must be more indirect. If the concentration of biotin in the medium is about $2\mu\text{M}$, aminolaevulinic acid-synthetase activity in cell extracts is almost normal, but no porphyrin or bacteriochlorophyll formation is found, in spite of the presence of apparently adequate amounts of enzyme. However, such cells can be induced to produce normal amounts of porphyrin if ethionine is added to the medium at a concentration of 10^{-3} – 10^{-4}M . Porphyrin formation can also be inhibited by a marked increase of the glycine concentration in the medium, and this decrease can be prevented if, together with an excessive amount of glycine, ethionine is added to the medium. We are not at present able to suggest a really satisfactory explanation for these findings, but it seems likely that aminolaevulinic acid synthesis in the cell is regulated by as yet unknown factors in addition to that of the availability of aminolaevulinic acid synthetase. Such factors may be the formation of pyridoxal phosphate, that of succinyl-CoA, the rate of competing reactions or the penetration of glycine into the cell. Biotin may be involved in one or other of these reactions, and the ethionine effect may be caused by an interference with the formation of an inhibitor or by interference with a reaction which competes with the synthesis of aminolaevulinic acid or with its transformation into porphobilinogen.

The possibility occurred to us some time ago that biotin may be required, not by acting as a coenzyme in the actual condensation, but by preliminary activation of glycine through carboxylation to aminomalonic acid. If this were the case, the aminomalonic ion with its high reactivity on the α -carbon atom would function in a manner similar to that of malonyl-CoA in acetoacetate formation. However, Miss M. Matthew in my Laboratory has found that aminomalonic acid was not a substrate in the enzymic condensation with succinyl-CoA. Indeed it proved to be a powerful inhibitor. With a ratio of glycine to aminomalonic acid of 80:1, inhibition amounted to 20%, whilst with a ratio of 15:1 the inhibition was almost complete.

*General metabolic importance of
aminoketone formation*

I have referred to the fact that the enzyme aminolaevulinic acid synthetase is not very specific with regard to the acyl group of the coenzyme A derivative, and all aliphatic mono- and di-carboxylic acids which have been examined give rise to aminoketones under the catalytic influence of the rather impure aminolaevulinic acid-synthetase preparations available. As acetyl-CoA and malonyl-CoA are formed in relatively large amounts in the body, we should expect aminoacetone and γ -aminoacetoacetic acid to be synthesized by cells and to be found in Nature. The formation of aminoacetone by *Staphylococcus aureus* has indeed been demonstrated by Elliott (1958, 1960), and Mr Tait and I have recently shown that *Rhodospseudomonas spheroides* can form an aminoketone which was different from aminolaevulinic acid and could be identified as aminoacetone after incubation under certain conditions. Aminoacetone has also been identified as a hydrolysis product of the antibiotic micrococccin P by Mijović & Walker (1960), and the occurrence in urine of a compound having the properties of aminoacetone has also been reported (Mauzerall & Granick, 1956). γ -Aminoacetoacetic acid has not yet been shown to occur in Nature, but it has been synthesized by Neilands, Neuberger & Scott (1959). Interestingly enough, this γ -amino keto acid is much more stable than α -amino- β -oxobutyric acid, and on decarboxylation gives rise to aminoacetone, suggesting a second pathway of its biological formation. The γ -amino- β -oxobutyric acid, if it is formed biologically, might also produce, by reduction of the keto group, γ -amino- β -hydroxybutyric acid, which is presumably the precursor of the betaine carnitine, a substance present in muscle in relatively large amounts and also a growth factor for insects. Finally, there is the possibility that the D-1-aminopropan-2-ol moiety in vitamin B₁₂, which Sprinson and his colleagues (Krasna, Rosenblum & Sprinson, 1957) have shown to be derived biologically from L-threonine, is formed through aminoacetone by reduction. Tait and I have recently obtained an enzyme from *Rhodospseudomonas spheroides* which oxidizes L-threonine to aminoacetone with decarboxylation. This enzyme uses DPN⁺ (diphosphopyridine nucleotide) specifically, no activity being observed if TPN⁺ (triphosphopyridine nucleotide) is employed. The enzyme acts on L-threonine and on D-allothreonine, the two isomers with D-configuration on the β -

carbon atom. D-Threonine and L-allothreonine are not attacked.

This particular enzyme does not act on DL-serine, but the possibility exists that other aminoketones may be formed by a pathway other than the condensation of glycine with acyl-CoA derivatives. Thus succinic semialdehyde may be condensed with glycine-pyridoxal phosphate to give α -amino- β -hydroxyadipic acid, which could be oxidized to aminolaevulinic acid by an enzyme similar to that oxidizing threonine to aminoacetone.

Shemin, in his first paper on the role played by aminolaevulinic acid in porphyrin formation, suggested that this amino keto acid may play a part in the general metabolism of glycine. Shemin and his colleagues (Shemin, 1955; Kikuchi, Kumar & Shemin, 1959; Nemeth, Russell & Shemin, 1957) have extended their scheme, which was originally named the succinate-glycine cycle, from aminolaevulinic acid to aminoacetone, and they have produced evidence showing that the carbon atoms carrying the amino group in the two aminoketones are precursors of C-2 and C-8 of guanine and of uric acid and also of formate. Elliott (1960) has described an enzyme from ox plasma which converts aminoacetone into methylglyoxal, and the Columbia group has shown that $\gamma\delta$ -dioxovaleric acid ('ketoglutaric semialdehyde') produces a similar pattern of labelling of uric acid as does aminolaevulinic acid. The actual enzymic steps involved have not yet fully been elucidated, and, whilst good evidence exists for the occurrence of the reactions postulated, the quantitative significance of this reaction for the overall metabolism of glycine remains still to be established.

The glycine-serine transformation

It was shown by Shemin (1946) that serine is a very effective precursor of glycine in the whole rat, and Sakami (1948) produced evidence for the occurrence of the inverse reaction. The enzymic mechanisms involved have been elucidated by Blakley, who started his work in this field in my laboratory in Mill Hill and continued it in Canberra (see, for example, Blakley, 1955), by Kisliuk & Sakami (1954), by Huennekens, Whiteley & Osborn (1959), and it is now possible to give a complete description of the sequence of reactions concerned. Folic acid, or rather tetrahydrofolic acid, and pyridoxal phosphate have been shown to be required and the C₁ unit involved is of the oxidation state of formaldehyde. Starting with formaldehyde and glycine, the reaction can be represented by the following equations:

- (1) $\text{H} \cdot \text{CHO} + \text{tetrahydrofolic acid} \rightleftharpoons \text{'hydroxymethyl'-tetrahydrofolic acid}$
- (2) $\text{'Hydroxymethyl'-tetrahydrofolic acid} + \text{glycine-pyridoxal phosphate} \rightleftharpoons \text{serine} + \text{pyridoxal phosphate} + \text{tetrahydrofolic acid.}$

Fig. 4. Reversible oxidation and reduction involving methinetetrahydrofolic acid.

Probable structures of the amino acid-pyridoxal phosphate complexes have been proposed by Snell and his co-workers and also by Braunstein, and are very similar to those discussed above in connexion with the aminolaevulinic acid-synthetase reaction. For the 'active formaldehyde' Blakley and I suggested a structure in which a methylene group joins N-5 and N-10 in tetrahydrofolic acid to form

formate. Acetone has been shown to be a precursor of C_1 units, but it is doubtful whether this can represent a readily available store of 'active' formaldehyde. It must be pointed out that not all the radioactivity found in serine on administration of labelled glycine to an animal may represent a net synthesis, and at least part of it may result from an exchange reaction:

a new ring (Fig. 4; XI). N^5N^{10} -Methylenetetrahydrofolic acid can be oxidized to N^5N^{10} -methine-tetrahydrofolic acid (X) with the aid of a TPN-requiring dehydrogenase and thus give rise to N^{10} -formyltetrahydrofolic acid and further to the C-2 and C-8 atoms in purines (for review see Huennekens *et al.* 1959). The C_1 unit can also be reduced to a methyl group reacting with adenosylhomocysteine to give methionine or yield the methyl group in thymine. The first reaction mentioned above, the condensation of formaldehyde with tetrahydrofolic acid, occurs at a reasonable rate on just mixing the two reactants, but Osborn, Vercamer, Talbert & Huennekens (1957) have shown that pigeon liver contains an enzyme which catalyses this reaction.

On the basis of these enzymic reactions it is easy to see how the organism can synthesize glycine from serine, if the latter can be made from substances involved in either the tricarboxylic acid cycle or glycolysis. But it appears that the reverse reaction, the formation of serine from glycine, also occurs readily and the origin of the formaldehyde required cannot be indicated with certainty. There are of course present in the body a number of compounds containing active methyl groups, such as methionine and choline, but these are not made or are not present in large amounts in the body, and the same applies to substances such as histidine or tryptophan, whose catabolism gives rise to

Secondly, the α -carbon atom of glycine itself may be a precursor of C_1 units. Sakami (1949) showed that, on administration of large amounts of α -labelled glycine, serine could be isolated which contained the label in almost equal amounts in the α - and β -positions of the molecule, indicating that the α -carbon atom of glycine can give rise to a C_1 fragment. Arnstein & Neuberger (1953b) showed that this labelling pattern was observed only if the glycine content of the diet was 2%; with 0.5% of glycine, the specific radioactivity of the β -carbon atom of serine was only 20% of that of the α -carbon atom. However, it appears that glycine may make, under conditions of normal nutrition, a significant contribution to the supply of C_1 units in the body. There are two known metabolic pathways to account for the formation of C_1 units from glycine. As already discussed, the condensation of glycine with various acyl-CoA derivatives leads to δ -aminolaevulinic acid and aminoacetone, both of which have been shown by Shemin and his colleagues to produce C_1 units which are ultimately derived from the α -carbon atom of glycine. It is still unknown whether the aminoketone condensation takes place on a quantitatively important scale outside the cells specifically concerned with erythropoiesis, and further work is required to assess the significance of this type of reaction for the general metabolism of glycine. The second

possibility is an oxidation of glycine to glyoxylic acid or the formation of the latter by transamination (Nakada & Weinhouse, 1953). Glyoxylic acid will give rise to formate by known pathways. Arnstein (1954) has pointed out that if glyoxylic acid were an important metabolite of glycine, it would be expected (see Weinhouse & Friedmann, 1951) that feeding of glycine would yield oxalate. But this is not the case. It seems possible, however, that this type of argument is not valid. Sanadi & Bennett (1960) have recently described a particulate enzyme system from the mitochondrial fraction of chicken liver which brings about a net synthesis of serine from glycine, and this reaction does not require the addition of a folic acid derivative or of glyoxylic acid; moreover, added formaldehyde does not participate in serine formation in this system. It is conceivable that the reactions involved are those known already, i.e. formation of glyoxylic acid, followed by decarboxylation, reduction of formate to formaldehyde and addition of the latter to a second glycine molecule to give serine. But the reactions might take place inside the organized mitochondrial structure and the intermediates would then not be in equilibrium with external glyoxylic acid formaldehyde.

*Quantitative aspects of the formation
of glycine and serine*

Some years ago, Arnstein and I (Arnstein & Neuberger, 1953*a, b*) carried out some experiments in rats which were intended to give information about the rates of synthesis in the intact animal of glycine and serine, the availability of the various carbon atoms of these two amino acids for the formation of labile methyl groups and the effect of vitamin B₁₂ on these processes. Dr Arnstein studied the effect of deficiencies of other vitamins on the biosynthesis of glycine (Arnstein & Stanković, 1956) and compared alanine and glucose as precursors of serine and glycine (Arnstein & Keglević, 1956). In all these experiments, known amounts of isotopically-labelled amino acid were fed as a component of a diet of known composition over long periods extending to 39 days to animals which in most cases were growing. At the end of the experiments, amino acids were obtained from the protein of the viscera after hydrolysis and the specific radioactivity of individual carbon atoms was measured after application of the appropriate chemical techniques. Comparison of the radioactivities of the fed and of the isolated amino acid and knowledge of the total amount of labelled amino acid eaten by the animal allowed an estimate to be made of the amount formed by the animal from precursors. It was assumed that at the end of the experiments all the amino acids in the protein had been replaced by amino acids derived from the

diet or made by the animal, but this assumption may not have been completely correct. But a more doubtful hypothesis underlying this approach is that the fed amino acid had mixed completely with the chemically identical compound made in the body. This assumption is certainly grossly incorrect in some cases; for example, aminolaevulinic acid, if administered to an animal, behaves almost like a foreign substance; it is not used effectively for haem synthesis and is quickly excreted by the kidney. It is unlikely that amino acids arising from the hydrolysis of dietary protein behave in this extreme manner, but the assumption of a quantitative equivalence of dietary glycine or serine and the same amino acids formed in the body is not proven. This fundamental difficulty arises from the fact that the body or even the cell is not homogeneous, but consists of compartments which are semi-isolated. Thus the concentrations of different enzymes differ not only in different organs, but even in different parts of the same cell, and the rates of transfer of amino acids and other compounds across cell membranes or intracellular barriers cannot be neglected in comparison with the rates of the chemical changes under consideration (Henriques, Henriques & Neuberger, 1955). Thus a substance B, which results from the action of enzyme 1 on substance A, may be further changed into substance C by an enzyme 2 present within the same mitochondrial or microsomal structure. Substance B may normally never diffuse into the cytoplasm or be transferred into the extracellular fluid or blood plasma. If the substance B is fed to an animal or injected into an animal, its fate is likely to be completely different from that of the same substance produced and further metabolized within a particular cell structure. This difficulty is likely to be met in all experiments on whole animals, and is particularly marked if an attempt is made to interpret labelling patterns in terms of known enzyme reactions. In the present case conditions are likely to be somewhat more favourable. α -Amino acids, unlike aminolaevulinic acid, are present in cells in the extracellular fluid and in the plasma in significant amounts and they appear to enter the cells of the liver, kidney and intestinal tract relatively easily. This assumption that the rate of mixing of dietary and endogenous amino acids is fast compared with the rates of metabolic reactions is likely to be incorrect, but it is probably a reasonable, if somewhat crude, approximation. The calculations made from the experimental findings indicate that the rat synthesizes about 3.5 m-moles of serine/100 g. of rat/day, whilst the corresponding value for glycine synthesis is about 2.5 m-moles. These results were critically considered by White (1958), who, using the mathematical method of Reiner (1953), obtained some-

what lower values than those given above. But the values quoted are likely to be of the correct order of magnitude. The combined daily requirements for these amino acids under conditions of maximum growth may be about 2.0–2.5 m-moles, and results obtained on effects of different doses of benzoate on growth indicate that the animal does not produce amounts of glycine greatly in excess of these requirements. In some of the experiments just discussed, labelled glycine or serine was fed as a component of diets containing homocystine, but no methionine and no choline. Growth was slow, but a comparison of the specific radioactivity of the methyl carbon atom of methionine with that of the β -carbon atom of serine allowed the conclusion to be drawn that the hydroxymethyl group of serine provides between 60 and 70 % of the methyl groups other than those already preformed in the diet. The hydroxymethyl group of serine is also likely to be the most important precursor of formate for purine synthesis.

There has been a considerable amount of discussion on the source of serine in the body, but it now seems clear that this amino acid is synthesized, not from pyruvic acid by a reversal of the serine-dehydrase reaction, but from 3-phospho-D-glycerate or glycerate through hydroxypyruvate or phosphohydroxypyruvate (Ichiara & Greenberg, 1955, 1957; Dickens & Williamson, 1959). The likely or possible reactions are shown in Fig. 5; not all enzymic steps postulated have been fully established.

Glycine is, as already discussed, formed from serine; it can be obtained from threonine by the specific aldolase enzyme and it may also arise from choline. Neither of these two last pathways is likely to be important quantitatively. The possibility has been discussed that glycine is formed from a C_2 precursor such as glyoxylate or glycollic aldehyde. The latter is transferred as a unit in the transketolase reaction, but there is no evidence that it ever appears as a free molecule in this reaction. Glyoxylic acid is formed from glycine, but its main origin in plants and micro-organisms is from D-isocitrate, where it functions in the glyoxylic acid cycle. The enzyme *isocitratase*, which cleaves *isocitrate* into succinate and glyoxylic acid, has never been found in animal tissues, and there is at present no suitable C_2 compound known which could form glycine by known pathways and which is made by animal cells. We must therefore assume for the present that almost all glycine synthesized is made from serine. There are thus

two main groups of non-essential amino acids: the first group consists of aspartic acid, glutamic acid, asparagine, glutamine, proline, hydroxyproline and arginine, which are formed directly or indirectly from non-nitrogenous substances participating in the tricarboxylic acid cycle, namely oxaloacetate and oxoglutarate. The second group, which comprises serine, glycine and cystine, arises from 3-phosphoglycerate or D-glycerate, substances formed during glycolysis. Alanine may be considered to be a member of both groups, since pyruvic acid, its precursor, is formed in glycolysis and is also closely related to acetyl-CoA and oxaloacetate, substances which participate in the tricarboxylic acid cycle. It is of interest to note that the daily rate of alanine or pyruvate formation in the rat amounts to about 38 m-moles/100 g. (Arnstein & Grant, 1957), compared with 3.5 m-moles/100 g./day for serine, and, whilst the absolute figures are not very reliable, it is clear that the amounts of alanine, and probably those of the carbon skeletons of glutamic acid and aspartic acid, synthesized daily are about ten times those of serine and glycine.

Importance of glycine and serine in uric acid synthesis

The last problem I want to discuss is the importance of amino acids in the synthesis of uric acid as compared with that of urea. In urea synthesis carbon dioxide combines with ammonia, which is mainly derived from glutamic acid, to give carbamoyl phosphate; this combines with ornithine to give citrulline, which reacts with aspartic acid; the resulting compound is split into arginine and fumaric acid and the arginine finally yields urea and ornithine. The only two amino acids used up are aspartic acid and glutamic acid, but these can be readily regenerated by reactions which we can assume to occur at high rates in the body. Uric acid formation is likely to occur by the same mechanism as that established for inosinic acid, which has so brilliantly been elucidated in the Laboratories of J. M. Buchanan and G. R. Greenberg. In this series of reactions, two carbon atoms (C-2 and C-8) come from formate through a tetrahydrofolic acid derivative and two carbon atoms (C-4 and C-5) originate from glycine, whilst C-6 is derived from carbon dioxide. Of the four nitrogen atoms, one (N-7) is specifically derived from glycine, whilst the other three originate from aspartic acid and glutamine. The semi-essential character of glycine for the young chick suggests that the capacity of the bird to synthesize glycine is, like

Fig. 5. Possible pathways of the biosynthesis of serine (following Greenberg).

that of the rat, not very great, at least not as great as the ability to form glutamic acid or aspartic acid. Moreover, it is likely that in the chicken, as in the rat, C_1 units are mainly derived from the β -carbon atom of serine, and it appears therefore that in the bird one or two molecules of serine or glycine have to be supplied in the diet or synthesized in the body in order to excrete three nitrogen atoms in the form of uric acid. It follows therefore that nitrogen excretion in uricotelic animals is dependent on the rate of synthesis of glycine and serine, substances formed from intermediates in glycolysis and probably only available in limited amounts. Urea synthesis, on the other hand, depends on the availability of aspartic acid and glutamic acid, substances which can be made by the organism in large amounts, and which are derived from tricarboxylic acid-cycle intermediates. Uric acid synthesis also differs from urea synthesis in the demands made by the former on an adequate supply of folic acid. These considerations may help to explain some of the specific nutritional requirement of uricotelic animals.

An attempt has been made in this lecture to weave together results obtained by methods of organic chemistry and observations made with isolated enzymes, and I have tried to use such results to interpret experiments done with the whole animal. I am conscious that I have not fully achieved my aim, but I hope to have given at least some indication as to how further progress can be made.

REFERENCES

- Almquist, H. J., Stokstad, E. L. R., Mecchi, E. & Manning, P. D. V. (1940). *J. biol. Chem.* **134**, 213.
- Arnstein, H. R. V. (1954). *Advanc. Protein Chem.* **9**, 1.
- Arnstein, H. R. V. & Grant, P. T. (1957). *Progr. Biophys. biophys. Chem.* **7**, 165.
- Arnstein, H. R. V. & Keglević, D. (1956). *Biochem. J.* **62**, 199.
- Arnstein, H. R. V. & Neuberger, A. (1953a). *Biochem. J.* **55**, 259.
- Arnstein, H. R. V. & Neuberger, A. (1953b). *Biochem. J.* **55**, 271.
- Arnstein, H. R. V. & Stanković, V. (1956). *Biochem. J.* **62**, 190.
- Attenburrow, J., Elliott, D. F. & Penny, G. F. (1948). *J. chem. Soc.* p. 310.
- Blakley, B. J. (1955). *Biochem. J.* **61**, 315.
- Cornforth, J. W. & Elliott, D. F. (1950). *Science*, **112**, 534.
- Crawhall, J. C. & Watts, R. W. E. (1960). *Biochem. J.* **74**, 514.
- Dakin, H. D. & West, R. (1928). *J. biol. Chem.* **78**, 91, 757.
- Dickens, F. & Williamson, D. H. (1959). *Biochem. J.* **72**, 496.
- Elliott, W. H. (1958). *Biochim. biophys. Acta*, **29**, 446.
- Elliott, W. H. (1960). *Nature, Lond.*, **185**, 467.
- Gibson, K. D., Laver, W. G. & Neuberger, A. (1958). *Biochem. J.* **70**, 71.
- Henriques, O. B., Henriques, S. B. & Neuberger, A. (1955). *Biochem. J.* **60**, 409.
- Huennekens, F. M., Whiteley, H. B. & Osborn, M. J. (1959). *Symposium on Enzyme Reaction Mechanisms, Oak Ridge, Tenn., J. cell. comp. Physiol.* **54**, Suppl. 1, 109.
- Ichihara, A. & Greenberg, D. M. (1955). *Proc. nat. Acad. Sci., Wash.*, **41**, 605.
- Ichihara, A. & Greenberg, D. M. (1957). *J. biol. Chem.* **225**, 949.
- Johnson, D. & Fisher, H. (1956). *J. Nutr.* **60**, 275.
- Kikuchi, G., Kumar, A. & Shemin, D. (1959). *Fed. Proc.* **18**, 259.
- Kikuchi, G., Kumar, A., Talmage, P. & Shemin, D. (1958). *J. biol. Chem.* **233**, 1214.
- Kisliuk, R. L. & Sakami, W. (1954). *J. biol. Chem.* **214**, 47.
- Krasna, A. I., Rosenblum, C. & Sprinson, D. B. (1957). *J. biol. Chem.* **225**, 745.
- Lascelles, J. (1956). *Biochem. J.* **62**, 78.
- Laver, W. G., Neuberger, A. & Scott, J. J. (1959a). *J. chem. Soc.* p. 1474.
- Laver, W. G., Neuberger, A. & Scott, J. J. (1959b). *J. chem. Soc.* p. 1483.
- Laver, W. G., Neuberger, A. & Udenfriend, S. (1958). *Biochem. J.* **70**, 4.
- Lynen, F. (1959). *Symposium on Enzyme Reaction Mechanisms, Oak Ridge, Tenn., J. cell. comp. Physiol.* **54**, Suppl. 1, 33.
- Mauzerall, D. & Granick, S. (1956). *J. biol. Chem.* **219**, 435.
- Metzler, D. E. (1957). *J. Amer. chem. Soc.* **79**, 485.
- Mijović, M. P. V. & Walker, J. (1960). *J. chem. Soc.* p. 909.
- Nakada, H. I. & Weinhouse, A. (1953). *J. biol. Chem.* **204**, 831.
- Neilands, J. B., Neuberger, A. & Scott, J. J. (1959). *J. Amer. chem. Soc.* **82**, 214.
- Nemeth, A. M., Russell, C. S. & Shemin, D. (1957). *J. biol. Chem.* **229**, 415.
- Neuberger, A., Perrone, J. C. & Slack, H. G. B. (1951). *Biochem. J.* **49**, 199.
- Osborn, M. J., Vercamer, E. N., Talbert, P. T. & Heunneken, F. M. (1957). *J. Amer. chem. Soc.* **79**, 6565.
- Reiner, J. M. (1953). *Arch. Biochem. Biophys.* **46**, 53, 80.
- Rothberg, S. & Steinberg, D. (1957). *J. Amer. chem. Soc.* **79**, 3274.
- Sakami, W. (1948). *J. biol. Chem.* **176**, 995.
- Sakami, W. (1949). *J. biol. Chem.* **178**, 519.
- Sanadi, D. R. & Bennett, M. J. (1960). *Biochim. biophys. Acta*, **39**, 367.
- Shemin, D. (1946). *J. biol. Chem.* **162**, 297.
- Shemin, D. (1955). *Ciba Foundation Symp., The Biosynthesis of Porphyrins and Porphyrin Metabolism*, p. 4.
- Weinhouse, S. & Friedmann, B. (1951). *J. biol. Chem.* **191**, 707.
- White, K. (1958). *Arch. Biochem. Biophys.* **75**, 215.
- Wiley, R. (1950). *Science*, **111**, 259.