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THE BIOSYNTHESIS OF HAEMOGLOBIN*

BY

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We pay tribute, on this occasion, to the memory of Sydney Ringer, whose devotion to the increase of human knowledge and understanding of the mechanism of the living cell is perpetuated not only in the sum total of his discoveries but also by the precise and logical way in which he developed the inspiration of his bold and imaginative mind. I need hardly remind you that in his investigations of the action of salts on the heart, some years before the introduction of the ionic theory by Arrhenius, he was led to the conclusion, to quote his own words, that "the effect, then, in potassium phosphate is due to the potassium and not to the phosphoric acid. Probably any potassium salt is sufficient to obviate the prolonged dilatation occurring when the ventricle is fed with saline solution only." He realized later, of course, that the slow ventricular relaxation was due to an unbalanced trace of calcium salt in the saline, but the concepts of ionic dissociation and of ionic antagonism are here clearly recognizable.

Ringer's work dealt with fundamental aspects of the life of the cell, and I venture to think that he would not be disapproving of the theme of my lecture, since the synthesis of porphyrins, haem, and haemoproteins may well be one of the earliest and most fundamental achievements of living matter. Aerobic respiration depends, in all cells, upon the operation of the cytochromes, which have a haemoprotein structure, and oxygen is transported to the tissues by haemoglobin or a closely related pigment in all the vertebrates and in many lower forms of life as well.

It is the nature of the protein moiety in these haemoproteins which determines their functional character: the haem is in nearly all cases the same—an iron complex of protoporphyrin.

Haemoglobin is therefore globin+haem, and this summarizes what was known about it and its synthesis in the body until only a few years ago. Brilliant x-ray crystallographic work by Bragg, Perutz, and Kendrew, combined with other physiochemical data, has made it very probable that globin consists of five layers of coiled polypeptide chains and that the four haem units present in each molecule of haemoglobin are attached to the surface of the globin structure, rather like flat plates, in a symmetrical distribution and each with one free co-ordination axis of the iron atom projecting outwards from the molecule as a whole. The haem portion is ferroprotoporphyrin 9, and its structure derives largely from the work of H. Fischer, culminating in his synthesis of haemin, in 1929.

That is the qualitative aspect, but now what about the quantitative? There is about 850 g. of haemoglobin in the blood of an adult, and of this some 8 g. is broken down daily into bile pigment and replaced by the activity of the bone marrow. Expressed in another way, each one of you will, during the course of this lecture, destroy about ten thousand million red cells and replace them, for your survival, with an equal number, representing the synthesis of some 300 mg. of globin and 12.5 mg. of haem. This prodigious feat of biosynthesis takes place within the bone marrow, and by examining individual cells of the red series in all stages of development by Caspersson's ultra-violet microspectrographic technique Thorell has demonstrated that the protein moiety appears to be synthesized first and then the haem residues, which attach themselves to the surface of the globin molecule.

In vivo and *in vitro* exposure to x rays has been shown to affect the synthesis of haem and of globin to a different extent, suggesting that the two processes may be largely independent of one another, as indeed do Thorell's own experiments on the incorporation of radioactive glycine into maturing chicken red cells. Some extremely interesting work has also been done by Nizet and collaborators on the biosynthesis of haemoglobin by reticulocytes *in vitro*, but I intend to restrict myself to consideration of the biosynthesis of the haem moiety alone.

Glycine as a Precursor of Haem

Absolutely nothing was known about the chemical precursors of haem in the body until, in 1946, Shemin and Rittenberg made the chance observation that after feeding the amino-acid glycine, labelled with the heavy isotope ^{15}N , to man or animals the concentration of ^{15}N in the circulating haem rose rapidly, remained more or less steady for some 80 days, and then suffered a rapid decline. Of other ^{15}N amino-acids tested, only serine showed a comparable efficiency of utilization.

Certain inferences were immediately possible from this experiment; firstly, that the haemoglobin of the circulating erythrocyte is outside the general metabolic pool of protein interchange; secondly, that glycine is specifically utilized in the synthesis of haem; and, lastly, that the majority of normal red cells have a mean life of about 120 days. Closer scrutiny of such curves has led Evans to suspect that in normal blood there may be a small proportion of cells with a very much shorter life, but this need not concern us now.

The American workers later found that duck blood, containing nucleated erythrocytes, was capable of carrying out biosynthesis of haem *in vitro* when incubated with suitable precursors, and Shemin's group have done nearly all their subsequent work on this system, whilst, in Great Britain, Professor Neuberger's team have largely used living rabbits

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and my own group have employed chicken blood *in vitro* or a haemolysate made therefrom. I cannot, in what follows, mention by name the investigators responsible for each contribution, but will try, so far as is possible, to indicate the team concerned.

There are four nitrogen atoms in haem, one in each of the pyrrole rings A, B, C, and D, and looking at the formula

FIG. 1.—Degradation of protoporphyrin (after Wittenberg and Shemin, 1950).

of haem or protoporphyrin (Fig. 1) you will notice that A and B are similar with methyl and vinyl β -substituents, whilst C and D form another pair, each carrying methyl and propionic acid β -side-chains.

In all biosynthetic experiments using isotopes to label portions of a complex structure, one's aim is to degrade the molecule step by step so that the location of labelled atoms can be precisely defined. With haem, a start may be made by removing iron and hydrogenating the vinyl groups to ethyl, C₂H₅, after which oxidative scission liberates the four bridge carbon atoms as carbon dioxide and provides two molecules of methylthylmaleinimide from the basic pyrrole rings A and B and two molecules of haematinic acid from the acidic pyrroles C and D.

Although A cannot be distinguished from B, or C from D, it was nevertheless shown almost simultaneously by Neuberger's group and Shemin's group that the ¹⁵N labelling in each pair was similar, so that glycine probably supplies the nitrogen for all four pyrrole rings.

In my own laboratory we excluded both formate and carbon dioxide as precursors of the bridge carbon atoms. From America quickly came the demonstration by ¹⁴C

labelling that the carboxyl group of glycine is not incorporated into haem but that the methylene carbon is, four of these atoms entering into the pyrrole rings and four becoming methyne bridge carbons.

Continuing the stepwise degradation of haematinic acid and methylthylmaleinimide, Wittenberg and Shemin were able, through the scheme shown, to measure the activity of each individual carbon atom of the porphyrin structure. The glycine-methylene groups were thus located as follows (Fig. 2).

The origin of the remaining carbon atoms remained obscure until Shemin's group followed up an earlier observation that acetate was also apparently utilized as a precursor for haem.

The Role of Acetate

Acetic acid is a two-carbon compound, and the experiment may be performed with the ¹⁴C label placed in either the methyl or the carboxyl group. Isolation and step-by-step degradation of the haem synthesized under the two conditions showed that acetate cannot itself be a direct precursor but that the precursor is most likely an unsymmetrical four-carbon compound derived from acetate. The evidence which led to this conclusion is circumstantial. Thus methyl-labelled acetate gave rise to a haem with highest activity in C atoms 6 and 9 (Fig. 3), but considerable activity was also found in positions 4, 8, 5, and 3. Moreover, there was equal labelling, each to each, in the position pairs 6 and 9, 4 and 8, 5 and 3, and corresponding positions were equally labelled in the acid and basic pyrrole rings A+B and C+D respectively. This strongly suggests that the same four-carbon chain is utilized in the formation of both sides of all pyrrole rings, and from the known metabolic fate of acetate the most likely four-carbon compound appeared to be succinate, which is formed from acetate by the operation of Krebs's citric acid cycle.

Powerful evidence in favour of the participation of a succinyl derivative, such as succinyl coenzyme A, was provided by Shemin and Kumin, who degraded the haem synthesized *in vitro* from carboxyl-labelled succinate and obtained the expected qualitative and quantitative distribution of radioactivity in individual atoms of the pyrrole rings. Confirmatory evidence was also obtained by the use of selective inhibitors of the citric acid cycle and has been added to since.

¹⁴C₃H₃COOH Experiment

CH₃C¹⁴OOH Experiment

PROTOPORPHYRIN 9

FIG. 2.—Positions in protoporphyrin derived from the α -carbon atom of glycine (after Wittenberg and Shemin, 1950).

FIG. 3.—Radioactivity of carbon atoms in pyrrole rings of protoporphyrin using either methyl-labelled or carboxyl-labelled acetate as substrate for biosynthesis (after Shemin and Wittenberg, 1951).

Ideas prevalent at this stage of development of the subject were crystallized by Shemin's group in their postulate of a common pyrrolic precursor, carrying acetic acid and propionic acid side-chains on the β and β^1 positions. Union, in some way, of four of these pyrrolic units so as to make a macrocycle could be imagined giving rise to uroporphyrin, and the latter, by loss of CO_2 and further modification, could be visualized as giving rise to coproporphyrin, protoporphyrin and then haem. Uroporphyrin would thus be the first porphyrin appearing in haem biosynthesis, but it would have to be a series III isomer, for all naturally occurring haems belong to the III series, and transformation of one isomer into another is not possible without complete disintegration and rebuilding of the porphin ring. I shall return later to a careful consideration of the exact relation of the porphyrins to the biosynthetic pathway.

Isolation of Porphobilinogen

In 1952 events took a dramatic turn, the description of which necessitates a little preliminary digression. Since 1931 it had been known that sufferers from acute porphyria excrete in their urine a substance giving a red colour with Ehrlich's aldehyde reagent. The isolation of this substance, porphobilinogen, had defied all attempts, although Waldenström had obtained concentrates, the properties of which led him to conclude that porphobilinogen was a dipyrrolic

FIG. 4.—Porphobilinogen.

FIG. 5.—Porphyrins formed from porphobilinogen by red-cell haemolysates (after Falk *et al.*, 1953).

methane—that is, two pyrroles joined by a $-\text{CH}_2$ group—and that on heating it gave rise to uroporphyrin III. I had long regarded the isolation of porphobilinogen as an urgent necessity in the belief that in this metabolic error we might be witnessing the excretion, in great quantity, of a normal intermediate in porphyrin synthesis, just as, for example, homogentisic acid, a product of tyrosine metabolism, is excreted in alcaptonuria. The admission of an acute porphyria patient to our metabolic ward provided abundant raw material, and with great skill Westall achieved the isolation of porphobilinogen, for the first time, from the collected urines.

Cookson and I elucidated its chemical structure (Fig. 4) and showed that it was a monopyrrolic substance with acetic acid and propionic acid side-chains in the $\beta\beta^1$ positions and carrying an α -aminomethyl group which is unusually reactive. I must add that an essential piece of evidence, bearing on the molecular weight of the substance, was derived from Mrs. Kennard's x-ray crystallographic examination, at the National Institute for Medical Research, of the derivatives of porphobilinogen which we prepared and submitted to her. That porphobilinogen gave rise, by chemical manipulation, to uroporphyrin in as high a yield as 75% under the proper conditions was easily demonstrated; and that this was indeed mainly, but not entirely, the series III isomer followed from paper chromatography by a method especially devised in my laboratory. When we incubated porphobilinogen with one of our chicken red-cell haemolysates, Falk, Dresel, and I were highly gratified to find that here, also, efficient transformation took place into uropor-

phyrin, coproporphyrin, and protoporphyrin (Fig. 5). It certainly looked as if porphobilinogen was indeed the "common pyrrolic precursor" postulated by Shemin, but there was much to be done before it could be established as a true intermediate in haem biosynthesis.

Making use of a device current in isotope work, we showed that if a large pool of ordinary porphobilinogen was added to a chicken red-cell haemolysate, synthesizing haem from radioactive glycine, and after about four hours the porphobilinogen of this pool was reisolated and rigorously purified, it had become radioactive, thus conclusively proving that some radioactive porphobilinogen was formed from radioglycine on its way to haem. Although not by itself conclusive, this was substantial additional evidence in favour of porphobilinogen being a true precursor of haem.

There remained the difficulty of understanding how an unsymmetrical series III uroporphyrin could arise chemically or enzymically from the union of four molecules of a single pyrrolic substance. This problem

FIG. 6.—Scheme summarizing biosynthetic steps from glycine + succinate (from the tricarboxylic acid cycle) to porphobilinogen, the simplest pyrrolic substance known to be a precursor of haem and porphyrins (after Rimington, 1955a).

formaldehyde is indeed formed during the transformation of porphobilinogen into uroporphyrin *in vitro*. It is not unthinkable that the enzymically controlled *in vivo* transformation of porphobilinogen into uroporphyrin may take place along similar lines.

Constitution of Intermediate Compounds

Since glycine and succinate, at an early stage, and porphobilinogen, at a later stage, were now fairly well established as raw materials for the synthesis of porphyrins and haem, it became possible to speculate upon the constitution of the compounds intermediate between these stages. Some possible structures were under consideration by Neuberger and Scott when Shemin and Russell announced that δ -aminolaevulinic acid could replace active succinate and glycine for haem synthesis in duck blood. The activity of δ -aminolaevulinic acid in haem and porphyrin production was confirmed by Neuberger's group, while, in my laboratory, we were able to demonstrate its conversion into porphobilinogen as well as porphyrins in the chicken red-cell haemolysate system. The formation of porphobilinogen may therefore be summarized as shown in Fig. 6, Shemin postulating α -amino- β -ketoadipic acid as an intermediate which, being a β -ketonic acid, could readily lose carbon dioxide to form δ -aminolaevulinic acid.

The position of δ -aminolaevulinic acid as a true intermediate in haem biosynthesis has been amply confirmed both by radioactive labelling experiments in Shemin's laboratory and by my group, who have shown that it is entirely equivalent

to porphobilinogen in kinetic studies using the chicken haemolysate system. Neuberger and collaborators have studied its metabolism *in vivo* and have also discovered, in several tissues, including some micro-organisms, an enzyme which brings about the condensation of two molecules of δ -aminolaevulinic acid to form one molecule of porphobilinogen. More recently Scott has shown that this change can also occur chemically. A similar enzyme has been studied by Shemin and by Granick's group in plant tissues and in the alga *Chlorella*, and the latter workers have built up evidence that chlorophyll synthesis follows fundamentally the same pathway as that of haem in animals. I would like also to mention the work of Miss Lascelles, who draws the same conclusion concerning the synthesis of bacteriochlorophyll in the photosynthetic bacterium *Rhodospseudomonas spheroides* and has demonstrated the equivalence in this organism of δ -aminolaevulinic acid to a mixture of glycine and succinate.

A Region of Uncertainty

The development of my theme has now reached the point where we have established glycine and succinate, or a similar tricarboxylic acid cycle intermediate, as the simple precursors of haem, and δ -aminolaevulinic acid and porphobilinogen as almost certain intermediates. On either side of these latter two substances, which stand as a sort of biochemical "half-way house" towards our goal, lie regions of uncertainty and ignorance. I shall try to deal with this *terra incognita* and will lead you first to the region between glycine and δ -aminolaevulinic acid. Shemin has suggested, and has brought forward, some evidence to support his view that the unsymmetrical derivative of succinic acid is, in fact, its compound with coenzyme A, succinyl CoA, in which there is a thioester linkage between the SH of CoA and one of the carboxyl groups of succinic acid. How glycine is made to react with it is unknown, although Lipmann has suggested that the glycine molecule may also be united to CoA and a "head-to-tail" condensation result, as in the formation of acetoacetic acid from two molecules of acetyl CoA. I venture to show you a scheme I have drawn up which, although speculative, is not, I think, at variance with known facts and may provide a working hypothesis for further explorations in this region (Fig. 7).

α -Ketoglutaric acid is visualized as the actual intermediate coming from the tricarboxylic acid cycle. It is a homologue

FIG. 7.—Tentative scheme of biosynthesis of porphobilinogen (after Rimington, 1955b).

of pyruvic acid, and the latter is known to be decarboxylated, in certain systems, whilst in union with a co-factor, lipothiamide. I depict the decarboxylation of α -ketoglutarate to succinate as occurring in the same way, after which a transferring enzyme passes it on to coenzyme A, forming succinyl CoA and reduced lipothiamide, which is then re-oxidized through the diphospho-pyridine nucleotide system so that it becomes available for a further molecule of α -ketoglutarate and the operation of the cycle is preserved. According to Lipmann's suggestion, succinyl CoA and glycyl CoA now condense in the head-to-tail reaction, giving α -amino- β -keto adipic acid, Shemin's postulated precursor of δ -aminolaevulinic acid. One molecule of CoA becomes freed in this process and returns in the cycle to pick up another molecule of succinate. Whether the decarboxylation of the adipic acid derivative to form δ -aminolaevulinic acid occurs before or after removal from CoA is uncertain, but eventually this molecule of CoA will return, carrying into the cycle with it a new glycine molecule, and so the operation becomes continuous as so many biochemical processes are now known to be.

I must emphasize that this scheme, leading up to δ -aminolaevulinic acid formation from small molecules, is tentative and must be at best an over-simplification, but it does offer some chance of experimental verification. It also shows how many stages in haem synthesis could be vulnerable to disorganization by metabolic upset in pathological conditions even in the earliest stages of haem formation. It is here, I am sure, that the key to at least two forms of human porphyria will ultimately be found.

On the other side of our halfway-house, represented by porphobilinogen, considerable exploration has already been carried out. The various porphyrins which one encounters normally and in excessive amounts in some diseases are, clearly, closely related to haem. It was tempting, in fact, to assume that one might write the sequence of events from porphobilinogen onwards as uroporphyrin $\rightarrow$ coproporphyrin $\rightarrow$ protoporphyrin, since all have been demonstrated at one time or another in bone marrow or red blood cells. There remained the difficulty that, prior to work with isotopes, no biological transformation of one porphyrin into another had ever been indubitably established.

If any substance is a true intermediate in a process it should be able to replace its precursors both qualitatively and quantitatively under suitable experimental conditions. Falk, Dresel, and I found that on incubation of pure uroporphyrin III with chicken red-cell haemolysate, 53% was converted into protoporphyrin, but added coproporphyrin III was largely recovered unchanged and no significant transformation into protoporphyrin could be demonstrated, notwithstanding the fact, already mentioned, that porphobilinogen in this same system gives rise to all these porphyrins. The failure to achieve a positive result could conceivably have been due to impermeability of the mitochondrial or other subcellular particles to the added substrate. This explanation was rendered less likely by the finding that the supernatant of an ultra-centrifuged haemolysate could bring about conversion of uroporphyrin I to coproporphyrin I, though not of uroporphyrin III to coproporphyrin III.

Another explanation of the lack of quantitative utilization of added porphyrins could be that they do not, in fact, lie on the direct line of haem biosynthesis but arise as by-products from the true precursors. To help decide between these possibilities, experiments were carried out in which haemolysate preparations were allowed to synthesize haem from radioactive glycine in the absence and in the presence of an unlabelled "pool" of the suspected intermediate, and the radioactivity of the resulting haem was carefully measured and also that of the reisolated pool substance. A true intermediate would act as a diluent of the small radioactively labelled amount travelling along the pathway, and would thus drastically reduce labelling in the haem while the pool itself acquired activity.

Experimental Results

I can summarize the results of my group by saying that porphobilinogen and δ -aminolaevulinic acid behaved as expected of true intermediates. At the other extreme, added protoporphyrin scarcely affected the radioactivity of the biosynthesized haem at all and had little effect upon the normal protoporphyrin production. Uroporphyrin III and coproporphyrin III occupied intermediate positions, somewhat depressing the haem labelling and significantly diverting radioactivity into their pool. In other words, none of these three porphyrins fulfils the requirement of a true intermediate; simple enzyme inhibition was excluded, but I must repeat the warning that permeability barriers may be playing a part here which it is difficult to assess.

From chemical considerations, it would seem plausible that the first porphyrin formed from porphobilinogen is uroporphyrin, which, by stepwise decarboxylation and, finally, dehydrogenation and iron incorporation, would lead to protoporphyrin and haem, and the enzyme catalysis of this last step has recently been demonstrated by Goldberg. In support of such a mechanism are the observations of porphyrins with from eight to two carboxyl groups in many biological situations. If these porphyrins are not themselves true intermediates they are almost certainly closely related structurally to them. My group has put forward the following scheme (Fig. 8), showing this hypothetical relationship, where X represents the true intermediate and the number

FIG. 8.—Suggested scheme for mechanism of haem biosynthesis from porphobilinogen.

denotes the number of carboxyl groups it carries. We suggested that the X substances might be the porphyrinogens or reduced forms of their respective porphyrins, and within the last few weeks a paper has appeared from Aldrich's group in Portland claiming that reduced uroporphyrin III does in fact behave as a true intermediate. The pathway for handling the series I isomers may differ from the other in that the free porphyrins *do* themselves lie on it.

It has generally been assumed that the presence of a nucleus, as in avian erythrocytes, is essential for porphyrin and haem biosynthesis, since the mature human red cell is unable to synthesize any haem from added glycine. Recent work, still in progress in my laboratory, indicates that this is only partly true. Although mature human red cells cannot utilize glycine, they will form copious amounts of uroporphyrin III and coproporphyrin III from added δ -aminolaevulinic acid. Interestingly enough, however, they cannot alone bring about the further oxidative decarboxylation of coproporphyrin (or 4X) to protoporphyrin, but will do so if a suspension of rat liver mitochondria is also added. High-speed centrifugation of a nucleated red-cell haemolysate—for example, of chicken blood—yields a supernatant similarly requiring a particulate system for the 4X to protoporphyrin conversion, as do also extracts of *Chlorella*.

Conclusion

This is where our knowledge of the normal biosynthesis of haem rests at the present day. I must confess to an oversimplification in what I have told you of some parts of the story, and I have omitted nearly all references to the synthesis of the globin moiety of haemoglobin and to the action of stimulating or inhibiting factors on these processes at the cellular or physiological levels.

Many investigators have contributed to our present knowledge, and work is actively proceeding in the belief that when we know every detail of the normal course of events, pathological deviations and the aberrations of disease will also become intelligible and perhaps amenable to correction.

In the biosynthesis of haem the body uses the simplest of building-stones, glycine and acetate, and yet the discoveries I have traced will have made it clear how many stages, beautifully co-ordinated, are gone through to reach the final product—and may I remind you again that the synthesis of haem is almost certainly one of the earliest achievements of living matter.

Whether chlorophyll preceded haem is a matter for debate, but each is essential, in its setting, for the provision of the energy without which life is impossible: chlorophyll harnesses the sun's rays for the reductive splitting of water; the haem enzymes achieve the release of energy by a recombination to water of metabolically produced hydrogen and the oxygen of the air!

It is not unusual to conclude a memorial lecture such as this with some quotation. Goethe says,

"Was kann der Mensch im Leben mehr gewinnen,
Als dass sich Gott-Natur ihm offenbare,
Wie sie das Feste lässt zu Geist verinnen,
Wie sie das Geisterzeugte fest bewahre!"

which can, I think, be translated,

"What greater end can Man in life attain
Than that, to him, 'God-Nature' should Himself reveal;
Material Substance into Spirit changing,
The Spirit-born to Substance She transforms!"

Mighty music! But for the majesty of the full close we must turn, I suggest, to the unknown Hebrew author of Ecclesiastes: "What profit hath he that worketh in that wherein he laboureth? I have seen the travail, which God hath given to the sons of men to be exercised in it. He hath made every thing beautiful in his time; also he hath set the world in their heart, so that no man can find out the work that God maketh from the beginning to the end."

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DIAGNOSTIC USE OF ADRENAL INHIBITION IN CUSHING'S SYNDROME

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In 1955 Cope and Harrison reported in detail the case of a patient with severe Cushing's syndrome due to adrenal cortical hyperplasia in whom it was shown that a temporary reduction in adrenal activity could be produced by the administration of the recently introduced halogen-substituted steroid 9 α -fluorohydrocortisone. They suggested that the response to this drug might prove of diagnostic value in Cushing's syndrome as a means of distinguishing cases with hyperplasia from those due to tumour. That such a distinction can be made in the case of the adrenogenital syndrome by observing the inhibitory effect of cortisone has already been well shown by Lawson Wilkins and his colleagues (1952) and has been more recently confirmed by Jailer, Gold, and Wallace (1954).

In the adrenogenital syndrome the main product of the adrenal gland is an androgen, and the urinary excretion of 17-ketosteroids is increased. Cortisone given orally or intramuscularly causes inhibition of a hyperplastic gland but not of a tumour. In the former the inhibition is demonstrated by a prompt fall in the