BRITISH MEDICAL JOURNAL

LONDON SATURDAY MAY 3 1958

SOME ASPECTS OF HAEMOGLOBIN BIOSYNTHESIS AND THEIR IMPORTANCE TO MEDICINE*

BY

C. RIMINGTON, Ph.D., D.Sc., F.R.S.

Professor of Chemical Pathology, University College Hospital Medical School, London

Until recently our knowledge of the synthesis of haemoglobin in the body was so scanty that it could be summarized in a few lines. The site of haemoglobin formation in the adult was known to be the red bone-marrow, and haemoglobin could be observed to occur at a certain stage in the development of the cell destined ultimately to become a circulating erythrocyte. In foetal life, or under great stress, other organs, such as the liver, were capable of showing haemopoietic activity. Certain perturbations of haemoglobin biosynthesis were also known and understood to a greater or lesser degree. Thus, simple iron deficiency resulted in an anaemia of microcytic type with poor haemoglobinization of the cell, understandably due to inability to synthesize adequate amounts of haem, the iron-containing pigmented moiety of haemoglobin. In some other forms of anaemia the mechanism was—and to this day still is—little understood. I refer to such states as the anaemias accompanying chronic infections, malignant disease, and so on.

Experimental Work

The first great contribution to the biochemistry of haemoglobin biosynthesis was made by Whipple and his associates, who used dogs kept anaemic by repeated withdrawals of blood and return of the plasma after centrifugation. If animals were required to be both anaemic and deficient in plasma proteins, no restoration was made, and, similarly, by returning the cellular elements but no plasma, a state of plasma-protein deficiency without anaemia could be produced. The basal diet was a poor one—bread and fish-paste—and supplementary proteins or amino-acids could be added at will. Whipple's experiments demonstrated that such dogs had a high power of haemoglobin restoration even when completely starving, that biosynthesis of haemoglobin took precedence over that of plasma proteins, and that different dietary proteins added to the basal diet showed differing degrees of biological efficiency calculated from the number of grammes of haemoglobin synthesized per 100 g. of such protein fed.

It is perhaps not surprising that the protein most efficiently utilized was haemoglobin itself, followed closely by such relatively complete proteins as casein, plasma protein, and liver. The vegetable proteins, so often deficient in one or more essential amino-acids, had a relatively low biological value. Only the globin moiety and

not the haem of administered haemoglobin was used for erythropoiesis, since after intraperitoneal injection of haemoglobin into the anaemic animal its constituent haem was promptly excreted as extra bile pigment while the globin was utilized with 80–90% efficiency.

That the biological value of a protein for haemoglobin synthesis depends upon its amino-acid composition is substantiated by feeding protein hydrolysates or pure amino-acid mixtures and also by experiments in which haemoglobin synthesis is followed by incubating reticulocytes with an appropriate mixture of amino-acids. In the whole animal with exhausted body protein stores, it is the amino-acid in shortest supply in the supplement, as compared with its quantity in the haemoglobin molecule, which will become the limiting factor and determine how much haemoglobin can be produced. Thus Sebrell (1949) found that in rats the most effective supplement for haemoglobin regeneration was histidine, with valine, lysine, phenylalanine, and leucine following closely in that order. No dietary amino-acid plays a convincingly "specific" part.

The work of Aschkenasy and Pariente (1951) on the protective effect of methionine against protein-deficiency anaemia is explicable, it would seem, on the basis of the participation of methionine, together with vitamin B₁₂ and folic acid, in the metabolism of one-carbon fragments required in the biosynthesis of nucleoproteins. The conversion of tryptophan into nicotinic acid is controlled by pyridoxine, and deficiency of any one of these three factors will cause anaemia, but the supplementation of a niacin-deficient diet with tryptophan is protective. A finding rather more difficult to understand is the report by Orten, Bourque, and Orten (1945) that when rats are fed upon beef globin as the sole source of protein the addition of isoleucine promotes haemoglobin formation. This is strange, since the haemoglobins so far analysed are free from isoleucine. The haemoglobin of the rat may be an exception, but it appears more likely that this is a sparing action, the inclusion of isoleucine in the diet reducing breakdown of body proteins and hence rendering available more dietary protein for the synthesis of haemoglobin.

Normal Haemoglobin Synthesis

To return to our consideration of the source of material for haemoglobin synthesis in the normal organism, there are clearly three possible sources of protein or protein-breakdown products which could be drawn upon—namely, (1) dietary protein; (2) protein stores in organs, tissues, and

* The Walker Lecture delivered before the Royal Faculty of Physicians and Surgeons of Glasgow on February 12, 1958.

plasma; and (3) the protein, mainly globin, liberated through the continuous normal destruction of red cells—and I shall deal with each of these possibilities in turn. The description which I have already given of Whipple's work and experiments by other investigators makes it quite clear that good dietary protein may provide all that is required for the biosynthesis of haemoglobin, assuming, of course, that iron, vitamins, and so forth are in sufficient supply.

During starvation, body protein is broken down to counteract anaemia caused by deliberate withdrawal of red cells. In the normal well-fed animal we may expect the pool of free amino-acids in the tissues to be drawn upon for haemoglobin restoration, and there are also protein stores present, particularly in the liver. This was clearly recognized by Whipple as a result of his experiments. All these sources can be drawn upon freely and indiscriminately for haemoglobin biosynthesis; we have no convincing evidence for the existence of specific erythropoietic stores.

Utilization of Red-cell Catabolites

Finally, let us consider the possibility of a preferential utilization of red-cell catabolites. It is possible that the proteins of destroyed erythrocytes could be directly and specifically re-utilized for red-cell regeneration. It is even conceivable that such a mechanism could be responsible, in part at least, for the remarkably constant equilibrium that is maintained between regeneration and destruction of red cells. We know for a fact that iron is used again in haemoglobin biosynthesis.

Various types of experiment have been devised in attempts to answer this question. Increased haemolysis provoked by agents such as phenylhydrazine is followed immediately by enhanced haemoglobin synthesis, which might suggest re-utilization of globin after more or less complete degradation, but a more decisive experiment, affording no support for such re-utilization, was performed by Bale, Yuile, DeLaVergne, Miller, and Whipple (1949). They fed the amino-acid lysine, labelled by ^{14}C in its ϵ position, to a dog depleted of both haemoglobin and plasma proteins. The animal was thereafter given a good diet, and determinations of ^{14}C in the washed cells and plasma respectively were made at intervals for the next 234 days. Haemoglobin was not actually separated for determination of radioactivity, but in a parallel experiment on a rat it was shown that the ^{14}C of lysine, when administered under similar conditions, was confined almost exclusively to globin within the red cell. Haemoglobin makes up about 90% of the solid matter of the erythrocyte, so that the intensity of labelling of the whole cell could be taken to indicate that of globin, provided there is no other very metabolically active protein present.

The experimental results showed a rapid labelling of plasma protein, which fell again, roughly exponentially, at a rate indicating a turnover time of about 10 days. Red-cell protein reached its maximum radioactivity rather more slowly, after which the curve exhibited a plateau followed by a fairly sharp fall from the 80th to 150th day. The similarity of this picture to that of haem labelling after the feeding of ^{15}N glycine (Shemin and Rittenberg, 1946) is striking, and the same conclusions are permissible—namely, that the protein fraction investigated (assumed to be globin) is outside the general dynamic exchange of body protein constituents, has an average life of about 115 days, and is *not* preferentially re-utilized for the formation of new erythrocytes.

Order of Synthesis

If we accept that both the globin and haem moieties of haemoglobin are built up *de novo* in the biosynthesis of the pigment in the bone-marrow, we must now inquire whether these processes are simultaneous or whether, for example, globin is synthesized first and haem added later.

The molecule of haemoglobin consists of several layers of coiled polypeptide chains, and x-ray crystallographic measurements show that the four haem residues probably

lie upon the surface of this structure. Prior synthesis of globin would not therefore be an unreasonable supposition. Experiments performed by Thorell (1947) seemed, in fact, to indicate that such was the case. Using a highly sensitive microspectrographic technique, he was able to measure in individual cells, aspirated from the bone-marrow, the content of nucleic acid, total protein, and haem. The absolute amounts of these substances were plotted against the stage of maturation, and curves were obtained which showed clearly that the synthesis of the total protein is largely completed before that of haem takes place, and *if* we assume this new protein to be mainly globin, then globin would appear to be synthesized first and haem added to it later on.

There is another way by which this point may be tested. It is known that eight molecules of glycine are utilized in the synthesis of one molecule of haem, and glycine is also required, of course, for incorporation into globin. By giving an animal 2- ^{14}C labelled glycine, any pool of this amino-acid in the erythropoietic tissues should be uniformly labelled within a short time, and were new molecules of globin and of haem to be synthesized *simultaneously* from this pool one would expect to find a constant ratio of eight between the radioactivity of one mol of haem and that of one mol of glycine isolated from the globin portion of the same sample of haemoglobin. Ratios greater or less than eight would indicate prior synthesis of haem or of globin respectively. In such an experiment carried out on rabbits by Muir, Neuberger, and Perrone (1952), the figures obtained 24 hours and more after the administration of the radioactive glycine were consistent with simultaneous synthesis of globin and haem. At earlier intervals a lower ratio suggested some priority in globin synthesis, but the interpretation demands caution.

Yet another way of approaching this problem is by following the biosynthesis of haemoglobin by reticulocytes under suitable conditions *in vitro*. Kruh and Borsook (1956), and more particularly Nizet (1953, 1955, 1957), have used this method. In Nizet's scrupulously careful work, reticulocyte-rich dog blood is first incubated with the marker. The haem and globin fractions are then carefully purified, glycine is isolated as the pure dinitrophenyl derivative after hydrolysis of the globin, and the radioactivity of the two fractions is determined in the form of barium carbonate. His results point to a simultaneous synthesis of globin and of haem. Even more interesting is the fact that if he adds to the system agents which stimulate or depress *either* globin or haem formation, the other component rises

FIG. 1.—Parallel stimulation of haem and globin synthesis in dog's reticulocytes *in vitro* by an ultrafilterable plasma factor.

or falls in a parallel manner. We know that violent treatment such as haemolysis will destroy protein-synthesizing ability whilst leaving that of haem intact; but in the complete, normal cell Nizet concludes that the synthesis of haem regulates and controls that of globin. The parallelism is well exemplified in a series of unpublished experiments he and I performed together and in which we added a stimulating factor derived from plasma. This material considerably enhances haem production by red-cell haemolysates, but when added to the surviving reticulocyte preparation both globin and haem production are stimulated, and each to an approximately equal extent (Fig. 1).

Relation of Globin and Haem Synthesis

The mechanism by which globin synthesis is so precisely related to that of haem is at present obscure. The implications of this finding for the pathologist are, however, obvious. Although disability to synthesize haem is infrequent we have one clear example in iron-deficiency anaemia, and another perhaps in copper-deficiency anaemia, and the first authentic case in man of anaemia due to deficiency of pyridoxine in the diet has been recently described. Pyridoxine is known to be concerned in the elaboration of porphyrins and haem, and, according to the work of Dr. Goldberg, in Glasgow, so also is ascorbic acid, which apparently plays an important part in the final incorporation of the iron atom into protoporphyrin. The clinical association of some anaemias with ascorbic acid deficiency is well known.

I may pause here to recall an interesting finding of the Whipple school. It is that in a well-nourished dog the production of a sterile inflammatory lesion causes anaemia, lowering of plasma iron, and marked delay in the uptake of ^{59}Fe . A similar lesion in an already anaemic and iron-deficient dog does not, however, have these effects. It was concluded that anaemia following inflammation is relatively mild and easily overcome if the bone-marrow is already sufficiently stimulated. My own impression is that we have much more to learn about the interplay of the various factors stimulating or inhibiting biosynthesis of the two fragments of haemoglobin in pathological conditions such as this.

I purposely propose to avoid any review of the so-called haemopoietins. Enough has been said to indicate that plasma factors, at least, do exist but that more work has to be done upon isolated systems before the physiological tangle caused by crude stimulation, as by reduced oxygen tension, can be unravelled. Just how complex the situation may be is revealed by the demonstration given by Jacobson and co-workers (1957) of the essential part played by the kidney in response to such stimuli. It must either activate a precursor of a haemopoietin or elaborate and secrete such a precursor, which is then transformed into the physiologically active material at another site—a story reminiscent of hypertension production from hypertensinogen under the influence of renin.

Gene-controlled Defects in Globin Production

An absolute inability to biosynthesize the globin moiety of haemoglobin would of course prove fatal, but there are certain examples of gene-controlled defects or abnormalities in globin production. The best known of these is sickle-cell anaemia, or trait, in which a certain proportion of the haemoglobin produced differs in its protein part from normal haemoglobin. Skilled biochemical work over the past few years has culminated in the discovery that the difference here lies in the substitution of a molecule of valine for a molecule of glutamic acid in one of the polypeptide chains or of globin (Ingram, 1956). Such a relatively slight abnormality has nevertheless serious consequences. As is well known, the reduced form of sickle-cell haemoglobin is so sparingly soluble that it can separate out in liquid crystal form in the affected erythrocyte.

Other gene-controlled abnormalities of globin synthesis leading to the appearance of haemoglobins differing from haemoglobin A in their electrophoretic mobility are en-

countered, and at least nine non-sickling haemoglobin variants are known. In haemoglobin C, lysine appears instead of glutamic acid in the same position in the peptide chain as was incriminated in the case of sickle-cell haemoglobin, but one of the most interesting of these abnormal haemoglobins is haemoglobin M, so named because it occurred as methaemoglobin in the red cells of a family with an unusual form of familial methaemoglobinemia discovered by Hörlein and Weber in 1948. The defect is usually due to lack of a flavoprotein co-factor forming a link in the oxidation-reduction chain which keeps the haemoprotein in the erythrocyte in the ferrous form. In this family, however, the abnormality was in the structure of the globin itself, for, when this was exchanged for normal globin, reduction to ferrous haemoglobin offered no difficulty.

In Cooley's anaemia, Mediterranean anaemia, or thalassaemia, as it has been variously called, there is a genetic abnormality which shows itself in an inability to synthesize ordinary haemoglobin A. The homozygous condition results, therefore, in a severe and often fatal anaemia, whilst the heterozygous condition, thalassaemia minor, may also result in severe disability.

Biochemistry of Porphyrin Synthesis

Do genetically determined errors occur also in the biosynthesis of the porphyrins or haem part of haemoglobin? The answer is, "Yes, so far as we know, in one instance; in congenital porphyria." Drug-induced methaemoglobinemia and sulphaemoglobinemia arise through alterations of normal pigment, and I do not include them, therefore, under this heading.

In congenital porphyria, however, many immature and circulating red cells are loaded with porphyrins belonging to the abnormal isomeric series I. The haem isolated from the haemoglobin of these cells is the normal series III pigment; the reason why none of the series I type haem is found is because there is an absolute specificity in the oxidative decarboxylation of coproporphyrin III to the corresponding protoporphyrin. This enzyme system is unable to utilize coproporphyrin I added to it in *in vitro* experiments, so we

FIG. 2.—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.

have the picture of normal haem—that is, iron protoporphyrin—together with masses of uroporphyrin I and coproporphyrin I, the end stages of the abnormal synthesis proceeding in some erythrocytes.

To understand this situation in more detail it is necessary to recall what is now known about the biochemistry of porphyrin synthesis, and this I have summarized in Fig. 2. Glycine and succinic acid are the two simple materials from which the complex ring structure of porphyrin is built up. An initial condensation, for which pyridoxine and coenzyme A appear to be essential, results in the formation of δ -aminolaevulinic acid. Two molecules of this substance are then combined in such a way that a pyrrolic substance is formed. This is porphobilinogen, the urinary excretion of large quantities of which, it is interesting to note, is the biochemical abnormality characterizing acute intermittent porphyria, a disease involving the liver rather than the haemopoietic system. Porphobilinogen can be transformed either chemically or enzymatically into uroporphyrin, and in each case it is the unsymmetrical series III isomer which is the principal product.

Many schemes have been put forward to explain the way in which combination of four identical porphobilinogen units could result in a porphyrin of this configuration, and I shall not discuss the merits and demerits of each hypothesis here. It is sufficient to note that this is the normal course of events. We (Booij and Rimington, 1957) have found, however, that if our enzyme system, extracted from red blood cells, is heated for some time at about 60° its character is altered so that when incubated with porphobilinogen it now forms exclusively uroporphyrin I, the symmetrical uroporphyrin isomer, which, as I have already said, is excreted in large quantities by congenital porphyria. Not only this, but if we prepare an enzyme system from the erythrocytes of animals or human patients with congenital porphyria and incubate it with porphobilinogen *in vitro*, we find that both uroporphyrins I and III are formed and the series I isomer may even predominate (Rimington and Booij, 1957).

It is thus clear that by mild heat treatment of the enzyme system from normal cells we have made it resemble that of the congenitally abnormal cells of this rare familial disease. Just what happens as a result of heating is under investigation at the present moment in my department. When we know, we shall, I feel convinced, understand also the inborn error responsible for congenital porphyria.

Conclusion

In this survey I have tried to view haemoglobin biosynthesis as a whole and then to indicate how experimental findings over a wide field are slowly but steadily being related to various steps in this vital process. Nearly 100 years have elapsed since Körber (1866) first noticed the difference between foetal and adult haemoglobins, and we cannot flatter ourselves on the speed of our progress; nevertheless the record of the past few years holds out some promise that before another century dawns we may have achieved understanding and control of some, at least, of the medical problems associated with haemoglobin biosynthesis.

REFERENCES

- Aschkenasy, A., and Pariente, P. (1951). *Rev. Hémat.*, **6**, 166.
 Bale, W. F., Yuile, C. L., DeLaVergne, L., Miller, L. L., and Whipple, G. H. (1949). *J. exp. Med.*, **90**, 3.15.
 Booij, H. L., and Rimington, C. (1957). *Biochem. J.*, **65**, 4P.
 Hörlein, H., and Weber, G. (1948). *Dtsch. med. Wschr.*, **73**, 476.
 Ingram, V. M. (1956). *Nature (Lond.)*, **178**, 792.
 Jacobson, L. O., Goldwasser, E., Fried, W., and Plzak, L. (1957). *Ibid.*, **179**, 633.
 Körber, E. (1866). *Inaug. Dissertation*. Dorpat.
 Kruh, J., and Borsook, H. (1956). *J. biol. Chem.*, **220**, 905.
 Muir, H. M., Neuberger, A., and Perrone, J. C. (1952). *Biochem. J.*, **52**, 87.
 Nizet, A. (1953). *Rev. méd. Liège*, **8**, 506.
 — (1955). *Ibid.*, **10**, 392.
 — (1957). *Bull. Soc. Chim. biol. (Paris)*, **39**, 265.
 Orten, J. M., Borque, J. E., and Orten, A. U. (1945). *J. biol. Chem.*, **160**, 435.
 Rimington, C., and Booij, H. L. (1957). *Biochem. J.*, **65**, 3P.
 Sebrell, W. H. (1949). *Fed. Proc.*, **8**, 568.
 Shemin, D., and Rittenberg, D. (1946). *J. biol. Chem.*, **166**, 621.
 Thorell, B. (1947). *Studies on the Formation of Cellular Substances During Blood Cell Production*. Kimpton, London.

THE PRODUCTION RATE OF CORTISOL IN MAN

BY

C. L. COPE, D.M., F.R.C.P.

AND

E. BLACK, B.Sc.

From the Postgraduate Medical School of London, W.12

Hitherto the great majority of proposed measures of adrenal cortical activity have involved the estimation of a particular chemical group of metabolites in the urine. These have all suffered from the disadvantage that it has not been possible to check their validity by comparison with any more direct measure of adrenal activity, and the relation between the amount of excreted compounds and the actual production rate by the adrenal cortex has been a matter of considerable conjecture. Indeed, some tests have relied for their justification on little more than the observation that they give low readings in clinical hypoadrenalism and high readings in Cushing's syndrome.

One of the major products of the adrenal cortex is now proved to be cortisol (hydrocortisone), and measurement of the production rate of this hormone must therefore be regarded as one of the major indices of adrenal activity.

The recent introduction of radioactive cortisol, labelled with ^{14}C in ring A, has made possible a much more direct estimation of the rate of production of endogenous cortisol than could be achieved before. Peterson and Wyngaarden (1956) have pioneered its use for this purpose. By injecting trace amounts of ^{14}C -labelled cortisol into human subjects and by making serial estimations of the changing ^{14}C content (specific activity—S.A.) of the circulating cortisol they were able to calculate turnover rates by methods conventional in such isotope studies. In nine normal subjects they reported a mean daily cortisol production of 21 mg.

The estimates of these authors were based on events occurring during an observation period of two to four hours, and daily output was calculated on the assumption that the observed production rate persisted throughout the 24 hours, an assumption which is probably not justified. While such an estimate of production over a short period is valuable for many purposes, a simpler method capable of indicating the daily output without recourse to difficult serial analyses would also be of very great clinical value.

We suggest here an alternative method of using ^{14}C -labelled cortisol which is believed to give a useful estimate of the daily production of cortisol by the subject, which is relatively easy to apply clinically even to ill patients, and which does not make excessive demands on the time and skill of laboratory staff. Results in a variety of clinical conditions using this method are presented.

Principle

The principle of the measurement is the well-established one of isotope dilution. If a small amount of ^{14}C -labelled cortisol is introduced into the body it may be expected to be metabolized in precisely the same manner as the endogenous cortisol. It will thus enter equally into all the metabolites, known and unknown, of cortisol and will appear in these in the urine.