lysine 4, tyrosine 4, histidine 2, methionine 2 and tryptophan 2.

SUMMARY

1. Values are given for nineteen of the common amino-acids in human hair keratin.

2. These account for about 90 % of the expected weight and 86 % of the nitrogen of the keratin;

melanin plus ammonia account for another 3 % of the weight and 7.7 % of the nitrogen.

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Further Isotopic Studies on Haemoglobin Formation in the Rat and Rabbit

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Recent work done *in vivo* with rabbits (Muir & Neuberger, 1950) and *in vitro* with nucleated red cells of birds (Radin, Rittenberg & Shemin, 1950) has shown that the α -carbon atoms of glycine molecules provide eight carbon atoms for the porphyrin moiety of haemoglobin. Earlier investigations (Wittenberg & Shemin, 1949; Muir & Neuberger, 1949) had produced evidence that all four nitrogen atoms of porphyrin are also specifically derived from glycine. The present paper is concerned in the first place with a comparison of the isotope contents of the porphyrin and glycyl residue in rabbit haemoglobin at different time

intervals after administration of methylene-labelled glycine. If glycine is indeed a specific precursor of porphyrin, it would be expected that the ratio of 'molar' radioactivities of porphyrin and globin glycine should approach or be equal to the value of eight, provided that the rate of blood formation is constant and that globin and porphyrin are made in the same cell or cell compartment, during the experiment, i.e. utilize the same glycine 'pool'. It is of course possible that the newly formed porphyrin or globin are diluted to different extents by unlabelled preformed material or that the average times required for the incorporation of the α -carbon atoms of glycine into the porphyrin and globin moieties of haemoglobin differ significantly. In

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either of these cases the shapes of the activity-time curves of these two components will not be identical initially, but the ratio of the molar activities should approach eight, as the curves flatten out, provided the above conditions apply. This investigation was then extended to the rat whose haemoglobin, unlike that of the rabbit, can readily be obtained in a crystalline form (Warburg & Reid, 1931). Preliminary experiments had shown that valyl residues provide the terminal amino groups of the peptide chains of rat haemoglobin, as in the haemoglobins of many other species (Porter & Sanger, 1948). We have therefore injected radioactive glycine and valine in constant proportion to groups of rats and measured the activities of porphyrin, the terminal valine residues, the non-terminal valine residues and the glycine residues at different times after administration of the labelled compound. It was hoped that the data thus obtained would be susceptible to a less ambiguous and more quantitative interpretation than is generally the case with isotope results on protein metabolism, for the following reasons. Haemoglobin isolated from adult mammals, unlike most other intracellular proteins, can be considered to be reasonably homogeneous, as measured by known physical and chemical criteria. Moreover, the haemoglobin of the normal, mature erythrocyte of the mammal is metabolically either completely or almost completely inert and we are justified in interpreting the activity-time curves of haemoglobin obtained for the first weeks after administration of labelled precursors entirely in terms of protein synthesis and in neglecting degradation.

In these experiments protein fractions were obtained which were almost free from haemoglobin and which were derived largely from the red cell membranes. These heterogeneous fractions which were designated 'stromatin', were also hydrolysed and the radioactivities of the valine and the glycine determined. In these and the haemoglobin experiments the rather small amounts of amino-acids available were isolated in the form of 2:4-dinitrophenyl (DNP) derivatives by a method described in detail elsewhere (Perrone, 1951).

EXPERIMENTAL

Treatment of animals and administration of labelled compounds

Rabbit experiments. In the first rabbit experiment a female Hollingsworth half-lopped rabbit (body weight 2.75 kg.) was used. The animal received 57.4 μC . of [α - ^{14}C]glycine having a specific activity of 1 μC ./mg. glycine and 100 μC . of [$\text{carboxy-}^{14}\text{C}$] acetate (8.3 mg. anhydrous sodium acetate) intravenously. At stated intervals blood samples (10 ml. each) were removed from the marginal ear vein. In the second rabbit experiment a Dutch female rabbit

(3.7 kg. body weight) was given, by vein, an isotonic solution containing 20 mg. of [α - ^{14}C]glycine with a total activity of approx. 300 μC . The rabbit was killed 8 hr. after the administration of the glycine. This animal was primarily used for a study of milk protein formation by Dr Campbell and Dr Work, to whom we are grateful for supplying the blood.

A third rabbit (3.3 kg. body weight female half-lopped Hollingsworth) was given a low protein diet for 48 hr. and then fasted for 24 hr. It was then given by mouth 7 μC . of [α - ^{14}C]lysine monohydrochloride (460 mg.) admixed with a small amount of pelleted diet. The lysine which was mainly in the L-form, but contained approx. 15% of the D-isomer and which had been synthesized in this laboratory (Arnstein, Hunter, Muir & Neuberger, 1952). Blood samples (10 ml. each) were taken from the marginal ear vein at stated intervals. We are grateful to Dr A. S. McFarlane for supplying us with the washed corpuscles for this experiment.

Rat experiments. Male and female rats of the Institute albino strain (200–270 g. body weight) were used. Each rat was injected intraperitoneally with an amount of a solution containing 5 μC . of [α - ^{14}C]glycine and 3.5 μC . of [$\text{gem-methyl-}^{14}\text{C}$]DL-valine/100 g. body weight. The glycine had a specific activity of 1 μC ./mg. and the valine a specific activity of 1 μC ./0.7 mg. Pairs of rats were killed by ether at various stated intervals of time and the blood removed by heart puncture. The labelled compounds excepting lysine were obtained from the Radiochemical Centre, Amersham.

Isolation of substances

Haemoglobin. In the first rabbit experiment the washed corpuscles were laked with distilled water and the haemoglobin precipitated by pouring the haemolysate into 10 vol. of acetone. The precipitate was further extracted successively with boiling acetone and CHCl_3 . The extracts were used for the preparation of cholesterol (see Muir, Perrone & Popják, 1951). In the second and third rabbit experiments the washed red cells, after removal of the 'buffy coat' containing the leucocytes, were lysed by addition of distilled water and the 'stromatin' precipitated by passage of CO_2 into the mixture. The solution was centrifuged and the precipitate discarded. Rabbit haemoglobin could not be crystallized, but the preparations obtained by the second method appeared to be uniform on electrophoresis.

In the rat experiments the washed red cells, after removal of the white cell layer, were lysed by addition of 4 vol. of water saturated with CO_2 . The precipitate containing the 'stromatin' was removed on the centrifuge. The haemoglobin was crystallized by addition of acetone (0.3 vol.) and leaving the mixture at 0° for 18 hr. The haemoglobin was recrystallized by dissolving the crystals in CO_2 -saturated water followed by addition of acetone (0.3 vol.) and cooling to 0° . The 'stromatin' was washed repeatedly with CO_2 -saturated water, until no more haemoglobin could be detected in the supernatant fluid.

Porphyrin and globin. Protoporphyrin methyl ester was obtained from the rabbit haemoglobin by the method of Grinstein (1947); the residue insoluble in acidic methanol was used for the preparation of amino-acid derivatives. In the rat experiments the globin was prepared by the method of Anson & Mirsky (1930). The supernatant solution from the acetone-HCl treatment was evaporated to dryness and the protoporphyrin methyl ester obtained from the residue according to Grinstein (1947). In the one experiment in which a degradation was carried out, the protoporphyrin

ester was reduced to mesoporphyrin ester and the latter oxidized with CrO_3 as described by Muir & Neuberger (1950).

Amino-acids. The proteins were hydrolysed and DNP-glycine prepared as described by Perrone (1951). In the rat experiments the globin was treated with 1:2:4 fluorodinitrobenzene (FDNB) (Sanger, 1945; Porter & Sanger, 1948) and the DNP-globin was hydrolysed. Extraction with ether and chromatographic separation (Perrone, 1951) gave crystalline DNP-L-valine derived from 'end-group' valine. The remaining free amino-acids were again treated with

about 40 days. This figure is lower than the values obtained in earlier experiments with ^{15}N (Neuberger & Niven, 1951). The difference may be explained as follows: the blood samples taken in the present investigation were rather large (10 ml.) and by the twenty-second day approximately 30% of the initial labelled haemoglobin had been removed and this was presumably replaced by protein of negligible isotope content. Moreover, the particular

Table 1. *Radioactivities of samples of protoporphyrin methyl ester and of DNP-glycine obtained at various times from haemoglobin specimens of a rabbit which had received 57.4 μc . of [$\alpha\text{-}^{14}\text{C}$]glycine*

(The values for molar radioactivity were obtained by multiplying the observed counts/min. by the mol.wt. and dividing by 1000.)

	Times of collection after administration of labelled compound (days)					
	0.25	1.0	3.0	7.0	22.0	52.0
Porphyrin ester:						
Counts/min.	49.9	224.9	391.6*	476.6	386.8	178.0
Molar radioactivity	29.45	132.6	240.5*	281.2	228.5	105.0
DNP-glycine from globin:						
Counts/min.	23.4	80.46	145.2	127.4	112.6	54.3
Molar radioactivity	5.64	19.64	35.0	30.7	21.7	13.1

* This sample amounted to 11.0 mg. only; correction to infinite thickness is given for molar radioactivity only, whilst counts/min. represents the value observed.

FDNB and the crystalline DNP-glycine and DNP-L-valine derived from non-terminal valine were obtained from this fraction. Lysine was obtained from the phosphotungstic acid-insoluble fraction of the globin hydrolysate by precipitation as copper-benzylidene complex (Turba, 1948); this was decomposed with H_2S and the lysine isolated as dihydrochloride after removal of the benzaldehyde with ether.

Isotope determinations

Radioactivities were measured as described (Muir & Neuberger, 1950), using an experimentally determined correction curve, if the samples were smaller than 20 mg./sq.cm. The results are given as counts/min. for samples of 'Infinite' thickness and 1 sq.cm. area. In an earlier paper (Muir & Neuberger, 1950) activities were expressed in an identical manner but erroneously defined as counts/min./mg. sample. In order to facilitate comparisons between different molecular species these values are multiplied by the molecular weight and divided by 1000. Such corrected figures are termed 'molecular activities'.

RESULTS

Comparison between the radioactivities of the porphyrin and the glycine residue in globin

In the rabbit experiment (Table 1) appreciable radioactivity was found both in the porphyrin and glycine of the globin as early as 6 hr. after the administration of labelled glycine. The maximum activity was reached after approximately 3 days; after the seventh day activity decreased and had fallen to about 40% of its highest value by the fifty-second day of the experiment, suggesting an average half-life span for the rabbit erythrocyte of

rabbit used increased its body weight during the experiment and probably also its blood volume by about 12%. These two factors thus account for the apparently short life-span, but they will not affect the ratio of activity between porphyrin and globin glycine in which we were primarily interested.

The relatively small amount of carboxyl-labelled acetate which was given to the rabbit, together with the labelled glycine, will produce a very slight activity in the carboxyl groups of protoporphyrin. However, the dilution factor for acetate is very large compared with that of glycine and we have therefore assumed in our calculations that the whole activity of the porphyrin is derived from the α -carbon atom of glycine.

In the rat experiment (Table 2) two animals were used for each time interval. Although the animals were kept under identical conditions and doses of radioactive amino-acids were exactly proportional to body weight, individual differences were not entirely eliminated. Such differences, which will affect the absolute values, should not influence to the same extent the ratios of radioactivities of different components of haemoglobin and it is only the ratios which are being considered.

Table 3 gives the ratios of molar activities of porphyrin and globin glycine from both rat and rabbit experiments for different time intervals. If we consider in the first place only those values which were obtained 24 hr. or more after administration of labelled glycine, we find that the mean molar activity of porphyrin is about 7.7 times that of the glycine isolated from the same haemoglobin sample.

This mean figure is reasonably close to the value of 8 which is expected on the basis of earlier work based on degradation of porphyrin (Muir & Neuberger, 1950; Wittenberg & Shemin, 1950). However, in these experiments the assumption was made that the radioactivity or the ^{15}N content of the

1950) and the α -carbon atom of the glycine residue in globin have identical activities within ± 10 or 15 %.

In haemoglobin samples taken 6 or 12 hr. after administration of radioactive glycine, the activity ratio was significantly less than 8, the values varying

Table 2. *Radioactivities of samples of protoporphyrin methyl ester and of DNP-glycine obtained from crystalline haemoglobin specimens of rats which had received 5 μC . [α - ^{14}C] glycine and [gem-methyl- ^{14}C] DL-valine/100 g. body weight*

	Time of collection after administration of labelled compounds (days)					
	0.5	1.0	3.0	7.0	14	21
Porphyrin ester:						
Counts/min.	162.5	N.d.	419.9	297.8	375.6	326.5
Molar radioactivity	95.9	N.d.	247.7	175.8	221.6	192.3
DNP-glycine from globin:						
Counts/min.	73.5	70.5	125.0	104.8	136.0	95.71
Molar radioactivity	17.7	17.0	30.1	25.3	32.8	23.1

N.d., not determined.

Table 3. *Ratio of molar radioactivities in rats and rabbits of protoporphyrin and glycine obtained from haemoglobin samples obtained at different times after administration of labelled glycine*

(The molar activities are given in Tables 1 and 2.)

Rabbits		Rats	
Time after administration of [α - ^{14}C]glycine (days)	Molar activity of porphyrin/ molar activity of globin glycine	Time after administration of [α - ^{14}C]glycine (days)	Molar activity of porphyrin/ molar activity of globin glycine
0.25	5.2	0.5	5.4
0.25	5.6	3.0	8.2
1.0	6.8	7.0	7.0
3.0	6.9	14.0	6.8
7.0	9.1	21.0	8.3
22.0	8.4	—	—
52.0	8.0	—	—

Table 4. *Comparison of radioactivities of products of oxidation of mesoporphyrin**

(The distribution of radioactivities was obtained by multiplying the molar activities shown in the second line by 2 in the case of haematinic acid and methylethylmaleic acid, and by 6 in the case of barium carbonate.)

	Mesoporphyrin methyl ester	Haematinic acid	Methylethylmaleic acid imide	Barium carbonate
Counts/min.	295.2 $\pm$ 4.3	108.7 $\pm$ 1.2	145.5 $\pm$ 2.4	71.4 $\pm$ 0.7
Counts/min. $\times$ mol.wt. 1000	175.2 $\pm$ 2.6	19.9 $\pm$ 0.2	19.94 $\pm$ 0.3	14.09 $\pm$ 0.13
Percentage distribution of radioactivity	(100)	22.72	22.77	48.27

* Mesoporphyrin isolated from second rabbit diluted with inactive mesoporphyrin.

maleimide was derived to an equal extent from rings *A* and *B* of protoporphyrin and the activity of haematinic acid from rings *C* and *D*. The unlikely possibility that only two rings, one of each type, and two methene carbon atoms were labelled was not completely excluded. The present results clearly justify the assumption made in the earlier interpretation and suggest that all four methene carbon atoms, all carbon atoms in the 2-positions of the pyrrolic rings (notation of Wittenberg & Shemin,

between 5.2 and 5.6. These values are well outside the limits of variation found with the later samples. The possibility was considered that this was due to the complexity, and hence possible slowness of the reactions leading from glycine to either the methene groups or to the formation of the pyrrole rings. If one of these reactions were slow in relation to the other, a degradation of a porphyrin specimen obtained a few hours after administration of glycine, should reveal considerable differences in the relative

activities of the degradation products. Such an experiment (Table 4) carried out with a haemoglobin sample obtained 6 hr. after administration of labelled glycine gave results which were more or less identical with those obtained with a haemoglobin specimen obtained several days after administration of labelled glycine (Muir & Neuberger, 1950). In fact, agreement with theory was better than in the earlier experiments. It can therefore be concluded that the rates of conversion of the α -carbon atom of glycine to the methene groups and its incorporation into the pyrrole rings must be of similar magnitude.

The incorporation of labelled glycine and valine into stromatin

The quantities of stromatin available were rather small and thus only about 2–3 mg. of DNP-glycine and DNP-valine were obtained in most cases; however, the samples were chromatographically pure and had correct melting points. The DNP-derivatives were diluted as described in Table 6 and the accuracy of the activity measurements is somewhat lower than with the globin samples. Comparison between the activity-time curves of the two amino-

Table 5. *Radioactivities of DNP-valine isolated from terminal and non-terminal positions of rat globin samples obtained at various intervals after administration of labelled valine and glycine*

(Values for molar activity of globin glycine were taken from Table 2.)

Time after administration of labelled amino-acids (days)	Radioactivity of terminal valine		Radioactivity of non-terminal valine		Ratio of molar activity of terminal over non-terminal valine	Molar activity of glycine from globin	Ratio of molar activity of glycine over non-terminal valine
	(Counts/min.)	Molar activity	(Counts/min.)	Molar activity			
0.5	S.L.	S.L.	29.15	8.3	S.L.	17.7	2.13
1.0	33.1	9.36	33.1	9.36	1.00	17.0	1.82
3.0	74.2	21.1	76.1	21.5	0.98	30.15	1.40
7.0	58.1	16.5	59.5	16.8	0.98	25.2	1.50
14.0	102.7	29.2	102.0	29.1	1.00	32.8	1.13

S.L., sample lost.

The rate of incorporation of valine into globin

Table 5 shows that the rates of incorporation of radioactive valine in terminal and non-terminal positions of the peptide chains of the globin molecule are identical. The very small differences found were well within the limits of error of counting. A comparison between the activities of glycine and of valine obtained from the same globin samples indicates that the ratio of the two activities decreases somewhat with time. A very similar change was found in a comparison of a change of ratio of the activities with time in the case of stromatin (Table 7).

The incorporation of L-[α - 14 C]lysine into globin

This experiment was carried out with material of relatively low specific activity; moreover, the total activity injected was small. The counting errors in the early samples were therefore large. The following values (counts/min.) on disks of 2 sq.cm. were obtained on samples of L-lysine-dihydrochloride (infinite thickness): 2 hr., 0; 6 hr., 3.3; 10 hr., 4.5; 14 hr., 15.2; 19 hr., 31.5; 24 hr., 38.4; and 29.5 hr., 52.6. (The times in hours indicate the intervals between administration of lysine and collection of samples.) The protoporphyrin methyl ester isolated from the haemoglobin sample which had been collected 29.5 hr. after administration of labelled lysine was completely inactive.

acids in globin and stromatin shows that the activities of both the DNP-glycine and the DNP-valine obtained from the 12 hr. stromatin specimens

Table 6. *Radioactivities of DNP-glycine isolated from stromatin samples obtained at various intervals after simultaneous administration of labelled glycine and valine*

(The amounts of DNP-glycine obtained from stromatin were generally too small to be counted directly under conditions of 'infinite thickness'. The specimens after recrystallization were therefore dissolved in ether; a known amount of unlabelled DNP-glycine was added to a solution of the labelled DNP-glycine which had also been carefully weighed. The mixture was then evaporated to dryness and the crystalline residue counted. The dilution was generally threefold. The molar activities given have been corrected for this dilution effect. Values for molar activity of globin glycine were taken from Table 3.)

Time after administration of labelled amino-acids (days)	Molar activity of stromatin glycine	Molar activity of globin glycine	Ratio of molar activity of globin glycine over that of stromatin glycine
0.5	6.15	17.7	2.9
3.0	40.1	30.15	0.75
7.0	23.2	25.2	1.09
14.0	25.0	32.8	1.31

were very low compared with the corresponding values of globin (Tables 6 and 7). In the case of 3-day specimens the activities of the stromatin samples are

higher, to fall again relative to globin in the 1-week and 2-week samples. It is interesting that the ratios of the molar activities of valine and of glycine in stromatin and globin respectively were so similar for identical time intervals.

DISCUSSION

The interrelationship between the formation of porphyrin and that of globin

It is generally agreed that both components of the haemoglobin molecule, i.e. haem and globin are synthesized in the maturing erythrocyte. From microscopic observation it had been known for some time that the appearance of pigment occurs relatively late in the development of the red cell, and recently Thorell (1947) has shown by the use of spectroscopic methods that formation of the bulk of the protein precedes the synthesis of porphyrin. The methods used by Thorell, which depend on selective ultraviolet absorption, did not enable him to demonstrate that this protein is in fact globin or chemically related to globin and the sequence of the two processes, formation of globin and of porphyrin, cannot be deduced from these observations. Experiments done *in vitro* with homogenates of spleen and bone marrow of rabbits and rats (Richmond, Altman & Salomon, 1951) and *in vivo*, with rats (Salomon, Altman, Casarett & Noonan, 1949), have shown that exposure of the living animal to X-rays affects the synthesis of haem and of globin to a different extent. Thus it appears that the two processes are largely independent of one another, although they occur in the same cell and lead to a common end product, haemoglobin.

The relative rates of incorporation of radioactive carbon derived from α -labelled glycine into the porphyrin and globin, respectively, have already been measured in the rat by Altman, Casarett, Masters, Noonan & Salomon (1948) who followed the isotope contents of the two components over a considerable period of time. However, their results are not easy to interpret quantitatively, since only the activity of the globin and not that of the glycine was determined. The glycine originally administered will give rise to activity in other amino-acids and the fraction of the radioactivity of the whole protein not due to glycine is likely to increase with time. In the present experiments a crystalline glycine derivative was isolated and its activity compared with that of porphyrin. It was found both in the rat and rabbit (at least with samples obtained 24 hr. or more after administration of the labelled glycine), that the ratios of the molar activities of glycylic residue in globin and of the porphyrin were within $\pm 15\%$ of a constant value. This result is to be expected on the basis of the concept of the steady state, provided that the two components are formed from the same

glycine 'pool'. The postulate of the steady state requires that both the amounts of the substances under consideration and their rates of synthesis from the common precursor should be constant during the period of the experiment. As already pointed out, the fact that this ratio of molar activities is approximately 8 agrees well with current theories which assume that eight carbon atoms of protoporphyrin are exclusively derived from the α -carbon atom of glycine.

With haemoglobin samples obtained 6 or 12 hr. after administration of labelled glycine, the molar activity of the porphyrin was only about 5.0 to 5.5 times higher than that of the DNP-glycine. This high isotope content of the globin glycine relative to that of the porphyrin found with the early samples could be explained by assuming that the newly formed labelled globin combines with preformed unlabelled haem. However, this deduction, which implies that porphyrin formation precedes globin synthesis, is only valid if the times required for the incorporation of the labelled α -carbon atom of glycine into the various positions of the porphyrin moiety and of the glycylic residue of the globin are the same. It is possible that since the reactions which lead from glycine to protoporphyrin or haematin are more complex than the incorporation of the glycine residue into globin, the former reactions will take longer. Hence the first cells which appear in the circulation after administration of glycine, will contain a higher proportion of isotope in the globin than in the haem. The time relationships in relative activities will also be affected by the size of the 'pools' of the glycine, the protoporphyrin and the various intermediates, and in the absence of any knowledge on these points it would be hazardous to draw conclusions from the differences observed. In any case the relatively high labelling found in these early samples suggests that both components are synthesized at a relatively late stage in the development of the red cell.

The rate of incorporation of labelled valine and glycine into globin

The rate at which labelled amino-acid residues appear in the haemoglobin of circulating red cells is determined by a number of factors. The time required for the synthesis of the protein from the amino-acids is probably quite short, but the interval between the synthesis of haemoglobin in the erythroblast and the release of the reticulocyte from the bone marrow may vary between species and may not be constant for different red cells of the same animal. Moreover, haemoglobin formation occurs with varying intensity during different morphological stages in the development of the cell and thus the activity-time curves of the amino-acids obtained from haemoglobin of circulating erythrocytes do not

accurately reflect the rate of incorporation of the labelled amino-acids into globin. But apart from these factors which are concerned with the physiology of the cell, we must also consider the changes in activity of the free intracellular amino-acids which are presumably the direct precursors of the protein. The isotope content of the free amino-acid will rise very quickly, on parenteral administration, to a maximum, at least in actively metabolizing and highly vascularized tissue such as bone-marrow. This will be followed by a sharp decrease in isotope content owing to the occurrence of fast metabolic reactions and distribution of the amino-acid over

5 $\mu\text{c.}/100\text{ g.}$ as compared with 3.5 $\mu\text{c.}/100\text{ g.}$ for DL-valine. A difficulty in interpreting the observed relative dilution factors arises from the fact that the injected valine was racemic and the exact proportion of the D-isomer which is inverted and becomes available for protein synthesis is unknown. An inversion in the case of leucine was demonstrated by Ratner, Schoenheimer & Rittenberg (1940), and the same probably applies to valine. However, the nutritional experiments of Rose (1938) suggest that such an inversion, if it takes place, is inconsiderable in the rat. Allowing for the difference in dosage and assuming that D-valine is not inverted to a signifi-

Table 7. *Radioactivities of DNP-valine isolated from rat stromatin samples which had been collected at various intervals after administration of labelled valine and glycine*

(Dilution of samples was done as described in Table 6. The molar activities are corrected for dilution. Values for molar activities of globin valine and stromatin glycine are given in Tables 5 and 6 respectively.)

Time after administration of labelled amino-acids (days)	Molar activity of DNP-valine from stromatin	Molar activity of DNP-valine from globin	Ratio of molar activity of valine from globin over that of stromatin	Ratio of molar activity of stromatin glycine over stromatin valine
0.5	3.7	8.3	2.2	1.66
3.0	28.8	21.5	0.73	1.39
7.0	18.8	16.8	0.88	1.23
14.0	22.3	29.1	1.31	1.08

tissues with relatively low permeability. During the third period the isotope content will probably decrease relatively slowly to an asymptotic value of zero (for discussion, see Arnstein & Neuberger, 1951a). Thus some of the factors which will determine the change of isotope content of one particular amino-acid in a given cell are the following: permeability characteristics of the tissues for the compound under consideration, the rate of conversion to other substances, the rates of protein formation and of synthesis of the amino-acid from unlabelled precursors. It can be expected that these factors will operate in a quantitatively different manner with different amino-acids. Valine, for example, cannot be synthesized by the animal and dilution will therefore be largely due to dietary intake and liberation of the amino-acid by hydrolysis of proteins. Glycine, on the other hand, is synthesized at the rate of about 2 m-moles/100 g. of rat (Arnstein & Neuberger, 1951b) and it can be predicted that the isotope content of the free amino-acid will decrease more rapidly with time than that of valine. It is expected, therefore, that the specific activity of the valine incorporated into protein per unit time should decrease more slowly than that of glycine. This prediction is borne out by the data presented in Tables 5 and 7 which show that the ratios of the activities of DNP-glycine to DNP-valine tend to decrease with time both in globin and stromatin.

The dose of radioactive glycine injected was

cant extent, the data of Tables 5 and 7 suggest that radioactive glycine is diluted between two or three times as much as valine, the ratio changing with time for reasons discussed above.

It was found that the ratio of the activities of the terminal valine to that of the non-terminal valine was unity and this suggests that both types of residue are incorporated into globin at approximately the same time. We do not know how far the two types of valine are separated from one another in the peptide chains of the globin molecule, but unless all or most of the valine groups are situated near the end of the chain the results suggest that the average time interval between the synthesis of a peptide involving a valine residue in terminal position and that of a peptide involving a valine residue in a non-terminal position is very short compared with the rate of decrease of isotope content in the free valine of the cell. These findings are compatible with either a mechanism of globin synthesis involving simultaneous condensation of amino-acids or, more probably, a process of rapid successive condensations involving intermediates of relatively short life. Anfinsen & Steinberg (1951) have recently found a considerable difference in isotope content between aspartic acid residues present in a peptide which is liberated from egg albumin during the conversion of the latter to plakalbumin and the aspartic acid residues present in the rest of the albumin molecule. It is possible that this system represents a special case or that different

mechanisms of synthesis operate with different proteins. Further work with homogeneous proteins is obviously required before any more general conclusions can be drawn.

The findings reported here with respect to the isotope contents of the protein and porphyrin after administration of labelled lysine indicate definitely that lysine is not a specific precursor of any part of the porphyrin molecule. This conclusion is in complete agreement with the theory based on degradation experiments that glycine and glutamic acid, or their derivatives, are the only specific precursors of protoporphyrin (see Neuberger, Muir & Gray, 1950; Shemin & Wittenberg, 1951). The finding of Altman, Miller & Richmond (1950) that labelled lysine gives rise to labelling in protoporphyrin may be explained, as already suggested by Altman *et al.* (1950), by conversion to glutamic acid which has been shown to occur (Miller & Bale, 1949).

The metabolic behaviour of stromatin

The experiments with stromatin were done in order to find out whether the proteins of the cell membrane are as inert metabolically as haemoglobin. The results obtained are not conclusive. The protein mixture termed stromatin is obviously very heterogeneous and may not comprise all the proteins of the membrane and may also contain proteins derived from the interior of the cell. The low isotope contents in the first glycine (Table 6) and valine sample (Table 7) compared with the corresponding values for globin indicate that the bulk of the proteins of this fraction are formed earlier than the haemoglobin. The values found in the other experiments are not greatly different from those observed for globin and this suggests that the bulk of the proteins comprising this fraction are, once formed, metabolically as inert as haemoglobin. If stromatin contained a large fraction with a turnover rate similar, for example, to that of plasma proteins, we should have found initial isotope contents very much higher than those of globin. The apparent

decrease in activity relative to that of globin which was observed with both glycine and valine, may indicate that stromatin contains a fraction which is in the dynamic state even in the mature cell. However, the present data are not sufficient for definite conclusions to be drawn.

Most of the results given here have already been presented by one of us at an isotope conference (Neuberger, 1951). The present data, however, are more extensive and the interpretations have been slightly modified.

SUMMARY

1. The incorporation of [α - 14 C]glycine into the protoporphyrin and globin moieties of the haemoglobin of rats and of a rabbit has been determined at various intervals of time after administration of glycine. Labelled valine was also administered to the rats.

2. After intervals of time greater than 24 hr. the molecular radioactivity of protoporphyrin was approximately 8 times greater than that of the glycine isolated from the same sample of haemoglobin, in agreement with current theories of haem synthesis. After shorter time intervals smaller values were obtained. Possible explanations are discussed.

3. Valine was also isolated from the 'end-groups' and remainder of the rat haemoglobin molecule. The radioactivities of both fractions were identical at all intervals of time. The implications of this finding are discussed.

4. The relative radioactivity of glycine to that of valine isolated from haemoglobin and from the heterogeneous protein 'stromatin' decreased as time went on. This is attributed to increasing dilution of radioactive glycine by endogenous synthesis.

5. The isotope contents of glycine and valine isolated from 'stromatin' and haemoglobin were compared. It is concluded that stroma proteins are formed earlier and are for the most part as metabolically inert as haemoglobin.

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Lignin in Young Plants

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In a previous communication (Bondi & Meyer, 1948) we showed that lignins extracted by alkali ('alkali lignin') from annual plants yield a number of aromatic degradation products similar to those obtained from wood lignins. The preparation of lignin from faeces collected from sheep fed one plant exclusively was used to investigate the changes occurring in lignin during digestion in the ruminant body. It was found that the lignin molecule is not attacked to any appreciable degree during its passage through the animal body. It was found, however, that the alkali-extractable lignins differed considerably both in yield and composition from those obtained by the usual methods using acid (cf. Bondi & Meyer, 1943). The aim of the experiments described in this paper was, therefore, to investigate the influence of various hydrolytic treatments of the starting materials used for the preparation of alkali-extractable lignins and of the isolated lignins on their composition. Furthermore, an attempt was made, by studying methylation of the lignins with diazomethane and dimethyl sulphate, to draw conclusions with regard to the structure of the lignin building unit.

EXPERIMENTAL

Materials

Barley. Barley was harvested at the age of 2.5 months and dried in air. The hay was used for lignin extraction, and part of it was fed to sheep as their only food. Their faeces were collected and lignin was extracted from them by the same methods as from barley.

De-linted cotton-seed hulls. In this case, lignin could be prepared only from the undigested material, since it proved impossible to maintain sheep on rations containing only cotton-seed hulls.

Methods

The extraction and purification of lignins extracted by alkali, their methylation with diazomethane and dimethyl sulphate and the method of methoxyl determination have been described previously (Bondi & Meyer, 1948). Acid-processed lignins were prepared by the method of Kalb (1932) by treating the starting materials with a mixture of HCl and H₂SO₄. Samples of 1 g. were used throughout, since lignin prepared by the acid method in larger batches often showed marked inhomogeneity and the batches differed widely in composition.

Hydrolytic treatment of the starting materials

(a) *With dilute H₂SO₄.* 300 g. plant or faecal material (finely ground and passing a 0.5 mm. sieve) were suspended in 200 ml. of 0.5N-H₂SO₄ and heated under reflux, with stirring for 6 hr. The mixture was then filtered through cheesecloth and washed free from acid with hot water. The residue was dried in air and used for the preparation of alkali-extractable lignin.

(b) *By heating with water under pressure.* Finely ground plant or faecal material (300 g.) was suspended in 2 l. of water and autoclaved at 210° for 2 hr. The mixture was filtered through cheesecloth, washed with 6 l. of hot water on the filter and the residue was dried and used for the preparation of alkali-extractable lignin. (Attempts to hydrolyse plant and faecal material with N- or 2N-acid at 210° failed owing to extensive decomposition and charring of the materials together with the formation of ether-soluble degradation products, mostly phenols, presumably from the lignin.)

Hydrolytic treatment of the isolated lignins

(a) *By heating with dilute H₂SO₄.* Isolated lignins remain unchanged when refluxed with 0.5N-acid; they were therefore treated with the acid at higher temperatures in the following manner: lignin (15 g.) was suspended in 200 ml. of 0.5N-H₂SO₄ and heated for 1 hr. at 150° in a glass-lined autoclave. After cooling the lignin residue was filtered off, washed free from acid and dried in air.

(b) *By heating with water.* Lignin (20 g.) was suspended in 200 ml. of water and autoclaved at 210° for 2 hr. The lignin residue was filtered off, washed free from acid and dried.

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