SUMMARY

1. Dried crystalline D-fructose, heated to 120° and cooled suddenly, yielded a product which, it is claimed, contained 51 % of fructofuranose.

2. This product was fermented much more rapidly than fructose at equilibrium in a corresponding solution.

3. This observation supports the theory that fructofuranose is the only form of fructose fermentable by yeast.

One of the authors (M. H.) is indebted to the Department of Scientific and Industrial Research for a grant. This work was recorded in a thesis presented to the University of Birmingham on 1 October 1938.

REFERENCES

Bailey, K. & Hopkins, R. H. (1933). *Biochem. J.* **27**, 1957.
Gottschalk, A. (1943). *Aust. J. exp. Biol. med. Sci.* **21**, 133.
Gottschalk, A. (1945). *Aust. J. exp. Biol. med. Sci.* **23**, 261.
Gottschalk, A. (1947). *Biochem. J.* **41**, 478.
Harding, T. S. (1922). *J. Amer. chem. Soc.* **44**, 1765.
Hopkins, R. H. (1931). *Biochem. J.* **25**, 1783.
Hopkins, R. H. & Roberts, R. H. (1935*a*). *Biochem. J.* **29**, 919.
Hopkins, R. H. & Roberts, R. H. (1935*b*). *Biochem. J.* **29**, 2486.
Suomalainen, H. & Toivonen, T. (1948). *Arch. Biochem.* **18**, 109.

The Biogenesis of Porphyrins

2. THE ORIGIN OF THE METHYNE CARBON ATOMS

BY HELEN M. MUIR AND A. NEUBERGER
The National Institute for Medical Research, Hampstead, London, N.W. 3

(*Received* 23 *December* 1949)

The method which the organism uses to synthesize protoporphyrin has aroused much speculation (Dobriner & Rhoads, 1928, 1940; Rimington, 1938; Turner, 1940). No experimental evidence for the mechanism of porphyrin synthesis was obtained, however, until Bloch & Rittenberg (1945) reported that deuterium, when supplied to rats in the form of acetate, was found in their blood haem. Shemin & Rittenberg (1946*a*, *b*) showed that the blood porphyrin from men and rats who had received ^{15}N-labelled glycine had a relatively high isotope content. Other nitrogen-labelled compounds including ammonia, leucine, proline, glutamic acid, histidine (Tesar & Rittenberg, 1947), aspartic acid (Wu & Rittenberg, 1949), and ethanolamine (Muir & Neuberger, 1949*a*) were relatively ineffective in rats and rabbits respectively.

In a previous paper (Muir & Neuberger, 1949*a*), the partial degradation of mesoporphyrin on a small scale was described. Three products of oxidation were isolated, viz. carbon dioxide, which was derived mainly from the methyne carbon atoms, methylethylmaleic acid imide from the non-acidic rings I and II of the porphyrin, and 1-methyl-2:2′-carboxyethylmaleic acid imide (haematinic acid), from the acidic rings III and IV. It was shown that feeding ^{15}N-labelled glycine to rabbits produced a blood porphyrin in which the isotope content was the same in the acidic and non-acidic fractions. Wittenberg & Shemin (1949) independently carried out an analogous degradation of the haemin obtained from a human subject and from ducks who had received ^{15}N-labelled glycine, and obtained the same result.

In the present investigation the method has been used to find the distribution within the protoporphyrin molecule of labelled carbon atoms derived from likely precursors. These included sodium acetate, glycine, serine and sodium formate labelled with ^{14}C and ^{15}N as indicated by the following formulae:

$^{14}CH_3.COONa$, $CH_3.{}^{14}COONa$,
$NH_2.{}^{14}CH_2.COOH$, $^{15}NH_2.{}^{14}CH_2.COOH$,
$^{14}CH_2OH.CH(NH_2).COOH$ and $H^{14}COONa$.

For preliminary communication see Muir & Neuberger (1949*b*).

EXPERIMENTAL

Treatment of animals and administration of compounds

Rabbits. Male and female Hollingsworth half-lopped rabbits were used (2·5–3 kg. body weight). They were given the Institute stock diet, except in the experiment in which glycine was administered. In this experiment the animal was kept on a low protein diet of bran, hay and cabbage during the experiment, and for 1 month prior to it. Except in the experiment with $^{14}CH_3.COONa$, to increase the amount of isotope incorporated into the haem, an anaemia was produced by withdrawing a total of 50–70 ml. of blood over 2–4 days. The labelled compound was injected sub-

cutaneously on the day following the last bleeding. The animals were killed from 3–9 days after the injection. They were anaesthetized with ether, and the blood was collected from the aorta, while heparin-containing saline was perfused through the vena cava.

Rats. Albino rats of the Institute stock (250–300 g. body weight) were used for two experiments in which serine and formate were given. These animals were also being used for other experiments. No anaemia was produced. In the serine experiment the rats received Na benzoate and γ-phenyl-α-aminobutyric acid as well as serine, and in the formate experiment the animal was given glycine and Na benzoate as well as Na formate. The labelled compounds were mixed with the food, and were not injected.

Isotopic compounds

^{14}C used in these experiments was obtained from the U.S. Atomic Energy Commission. The two ^{14}C-labelled acetates were synthesized by the Radiochemical Centre, Amersham, Buckinghamshire. $NH_2.{}^{14}CH_2COOH$ and $H^{14}.COOH$ were supplied by Dr H. R. V. Arnstein, who also synthesized the $^{14}CH_2OH.CH(NH_2).COOH$ (Arnstein, 1949). The ^{15}N-labelled glycine was made by the method of Schoenheimer & Ratner (1939), from isotopic NH_4NO_3.

Isolation of mesoporphyrin

The haemin from washed erythrocytes was crystallized, converted to protoporphyrin methyl ester and then reduced to mesoporphyrin as described by Muir & Neuberger (1949*a*). To obtain larger quantities for degradation, the haemin was generally diluted with an appropriate quantity of inactive haemin, before being converted to mesoporphyrin.

Degradation of mesoporphyrin with CrO_3

Apparatus. The oxidation was carried out in a Kjeldahl apparatus. N_2, washed free of CO_2, was introduced through the inlet tube, the side arm of which carried a short burette attached by a rubber connexion to a right-angle tube leading to an absorption train. Thus the flask could be shaken during the reaction. The absorption train contained three units, each of which consisted of a tapered pyrex centrifuge tube (11 cm. long, 1·2 cm. internal diameter) previously weighed, containing 3·905 N-NaOH free of CO_2. Each tube had a B 19 cone joint at the top, which fitted into a socket carrying an inlet tube reaching to within 1 cm. of the bottom of the tube. The socket also carried an outlet tube which connected with the next absorption unit through a B 10 joint. Both inlet and outlet tubes were fitted with taps, so that each unit could be shut off in turn. The whole apparatus could be dismantled for cleaning.

Oxidation. The mesoporphyrin methyl ester was first dissolved in 50% (v/v) H_2SO_4, then cooled to 0° while the CrO_3 was added from the burette in small quantities over 1 hr., with occasional shaking. An excess of 10–12% of available O was used. After 5–6 hr. the unused O was estimated iodometrically, and the imides extracted from the reaction mixture with ethyl acetate. The separation and purification of the imides was performed as described by Muir & Neuberger (1949*a*).

Isolation and estimation of CO_2. The CO_2 was carried over in a stream of N_2. At the end of the reaction each absorption unit was disconnected in turn, the tip of the inlet tube washed with CO_2-free water, and an excess of saturated $BaCl_2$ added. The tube was capped at once, and centrifuged. No $BaCO_3$ was ever obtained in the third tube. The $BaCO_3$ was resuspended in CO_2-free water, and washed on the centrifuge until free of Na^+ and Cl^-. It was finally washed with ethanol and ether, dried *in vacuo* at 100° and the centrifuge tube weighed again. The original supernatant and the washings from the $BaCO_3$ precipitation were combined, made up to volume, and the unused NaOH estimated by titration. Thus the CO_2 was estimated both volumetrically and gravimetrically. The values agreed to within 3%. An experiment in which CO_2 was liberated from 250·7 mg. of pure $NaHCO_3$ with H_2SO_4 showed that CO_2 could be recovered quantitatively by this method.

Isotope determinations

^{15}N was estimated with the mass spectrometer, and ^{14}C was determined with a bell-shaped He-filled Geiger-Müller counter, having a thin mica window. This counter has an unusually low background count of 10–11 counts/min. The radioactivity measurements were carried out on solid samples placed on disks of identical geometry. The material was spread evenly by the pellet technique (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949; Popják & Beeckmans, 1950). The layer of material was 'infinitely thick', i.e. not less than 25 mg./sq. cm. Under such conditions the number of counts is linearly proportional to the specific activity. No correction for self-absorption was made. When $BaCO_3$ samples were compared with samples of organic material, a back-scattering correction of 4% was made. This was determined experimentally as recommended by Calvin *et al.* (1949).

In order to compare the radioactivities of compounds of different molecular weight, the counts/min./mg. sample were multiplied by the mol.wt. $\times 10^{-3}$ of the substance. These values are designated counts/min./mmol. Standard errors were calculated as described by Calvin *et al.* (1949). Some disk samples were counted on a windowless proportional counter, which had greater sensitivity.

Conversion of haemin to deuterohaemin

Haemin (70 mg.) was converted to deuterohaemin by heating with resorcinol (Fischer & Hummel, 1929). (Found: Cl 5·9. Calc. for $C_{30}H_{28}O_4N_4FeCl$: Cl 5·9%.)

RESULTS

Particulars of conditions used in the various experiments are summarized in Table 1.

Degradation of mesoporphyrin

In a previous paper (Muir & Neuberger, 1949*a*) it was stressed that a knowledge of the yields in the oxidation is important for the interpretation of degradation experiments involving tracers, and the belief was expressed that 80–90% of the carbon dioxide evolved comes from the four methyne carbon atoms, and the two carbon atoms of the methyl ester groups. In agreement with earlier results the yields of the two imides (Table 2) varied between 63 and 78%. The oxygen consumption varied from 93 to 102% of the theoretical, and the carbon dioxide

Table 1. *Details of biological experiments*

Exp. no.	Species	Body wt. (kg.)	Blood withdrawn (ml.)	Labelled compound injected: Formula	Wt. injected (mg.)	Isotopic dose ($\mu c.$)	Radioactivity of haemin (counts/min./mmol.)
1	Rabbit	3·9	0	$^{14}CH_3.COONa$	34·2	416	121·9±4·2
2	Rabbit	3·0	73	$CH_3.^{14}COONa$	29·8	363	83·4±2·6
3*a*	Rabbit	2·7	45	$^{15}NH_2.^{14}CH_2.COOH$	566·1	54	733·0±8·1
3*b*	Rat	0·302	0	$NH_2.^{14}CH_2.COOH$	9·6	9·6	138·8±4·2
4	Rats (2)	0·55	0	$^{14}CH_2.OH.CH(NH_2).COOH$	122	10	3·5±1·5
5	Rat	0·387	0	$H.^{14}COOH$	41·7	22·25	3·54±0·5*

* Estimated as protoporphyrin methyl ester.

production from 98 to 99 %. These findings indicate that the formation of the two imides is not quantitative, but that the side reactions which occur do not consist of extensive further oxidation of the two maleic imides.

Table 2. *Results of oxidation experiments*

(Particulars of animals used and compounds administered are given in Table 1.)

Exp. no.	Wt. of mesoporphyrin methyl ester (mg.)	Percentage of theoretical: O consumed	Percentage of theoretical: CO_2 produced	Wt. of crude imides obtained
1	269·7	93·0	68	67
2	246	100·1	99	78
3*a*	495·2	102	98·7	65
3*b*	100·1	99·5	97	63·5
4	33	98·5	70	—

Comparison of activities of haemin samples

Of all the substances tested, glycine produces by far the most active haemin (Table 1). Both serine and formate are relatively inefficient precursors. Carboxyl-labelled acetate is less efficient than methyl-labelled acetate, even though the rabbit used in the latter experiment was not anaemic. In a preliminary experiment with $CH_3.^{14}COOH$, the haemin obtained from a rabbit that was not anaemic had a very low activity. Radin, Rittenberg & Shemin (1949) have stated that the utilization of $^{14}CH_3.COOH$ is about six times more efficient, but it is not clear whether the experiments were carried out *in vivo* or *in vitro*.

Relative activities of degradation products

The experimental results are shown in Table 3. The proportions of the total activity of the original mesoporphyrin accounted for by the products are

Table 3. *Radioactivity of products of oxidation*

(Particulars of animals used and compounds administered are given in Table 1 and yields of products in Table 2. Results expressed as counts/min./mmol. —, means 'not determined'.)

Exp. no.	Compound injected	Mesoporphyrin methyl ester (*a*)	Haematinic acid (*b*)	Methylethylmaleic acid imide (*c*)	$BaCO_3$ (*d*)
1	$^{14}CH_3.COONa$	27·9 ±0·67	7·55±0·25	6·5 ±0·22	0·08
2	$CH_3.^{14}COONa$	32·35±1·09	12·66±0·31	2·97±0·15	0·44±0·3
3*a*	$^{15}NH_2.^{14}CH_2.COOH$	131·2 ±1·09	20·4 ±0·25	19·8 ±0·198	11·4 ±0·27
3*b*	$NH_2.^{14}CH_2.COOH$	30·45±0·65	3·88±0·12	—	—
		187·0 ±3·68*	—	—	14·73±0·79*
4	$^{14}CHOH.CH(NH_2).COOH$	11·07±3·3*	—	—	0

* Values obtained on proportional counter.

Table 4. *Comparison of activities of haematinic acid and methylethylmaleic acid imide*

(Letters *a*, *b*, *c* refer to molecular activities of mesoporphyrin, haematinic acid, and methylethylmaleic acid imide respectively. Results expressed as counts/min./mmol.)

Compound injected	$\frac{2b}{a}\times 100$	$\frac{2c}{a}\times 100$	$\frac{2(b+c)}{a}\times 100$	$\frac{b}{c}$
$^{14}CH_3.COONa$	54·1±4·36	46·6 ±4·0	100·7 ±6·3	1·16
$CH_3.^{14}COONa$	78·3±2·7	18·36±1·75	96·66±4·65	4·26
$^{15}NH_2\,^{14}CH_2.COOH$	31·1±2·06	30·2 ±1·74	61·3 ±2·69	1·03
			$\frac{4c}{a}\times 100$	
$NH_2\,^{14}CH_2.COOH$	—	25·5 ±4·2	51·0 ±8·4	—

given in Table 4. The assumption is made that the methylethylmaleic acid imide isolated represents equally both rings I and II, and the haematinic acid rings III and IV. Therefore the proportion of the activity which they represent is obtained by multiplying their molecular activities by two.

The molecular activity of the two imides was almost equal after injecting $^{14}CH_3.COONa$ and $^{15}NH_2.^{14}CH_2.COOH$. But after injecting $CH_3.^{14}COOH$, the molecular activity of the haematinic acid was more than four times that of the other imide.

Origin of methyne carbon atoms

The activity of the methyne carbon atoms has been determined in two ways. The activity of the carbon dioxide isolated in the oxidation is a direct measure of the activity of the methyne carbon atoms, but the accuracy is reduced, owing to side reactions discussed above. Moreover, the carbonate fraction contains carbon dioxide derived from the methyl ester groups, and it is assumed that these are quantitatively oxidized. Since six molecules of carbon dioxide are produced from one molecule of mesoporphyrin ester, the molecular activity of the barium carbonate is multiplied by six. The figures given in Table 5 show clearly that in the two glycine experiments, the barium carbonate fraction accounts for approximately 50 % of the total activity of the mesoporphyrin, but after giving either of the acetates or serine, the activity was negligible.

calculated value for the relative activity of the methyne carbon atoms was $38{\cdot}7 \pm 2{\cdot}8$ % of the total. In the second experiment only one imide was isolated, and it was assumed that the molecular activities of the two imides were equal (Table 4). On this basis, the relative calculated activity of the methyne carbon atoms was found to be 49 %. The relative molecular activity of the barium carbonate fraction in the two experiments were $50{\cdot}0 \pm 2{\cdot}1$ and $45{\cdot}9 \pm 5{\cdot}9$ % respectively.

Although the agreement between the values obtained in the various experiments is only moderately good, nevertheless it seems justified to conclude that (*a*) of all the compounds tested methylene-labelled glycine alone contributes significantly to the formation of the methyne bridges; (*b*) that the methyne bridges account for 40–50 %, and the pyrrole rings 50–60 % of the total activity of the porphyrin obtained after glycine administration.

Comparison of isotope ratios of doubly labelled glycine and haemin

From the results given in Table 6, it can be calculated that the dilution of ^{15}N, i.e. the ratio of ^{15}N (atom % excess) of the glycine administered to that of the haemin is 82·5.

To compare the two dilution factors, the ^{14}C results are given in counts/min./100 mg. N. The ^{14}C dilution factor, as calculated from the results of Table 6, is 38·02. The ratio of the two dilution

Table 5. *Comparison of activities of methyne carbon atoms as calculated by difference and as found in carbonate fraction*

(Letters *a*, *b*, *c*, *d* refer to molecular activities of mesoporphyrin, haematinic acid, and methylethylmaleic acid imide and barium carbonate respectively. Results expressed as counts/min./mmol.)

Compound injected	$\frac{a-2(b+c)}{a} \times 100$	$\frac{a-4c}{a} \times 100$	$\frac{6d}{a} \times 100$
$^{14}CH_3.COONa$	$-0{\cdot}3 \pm 6{\cdot}3$	—	0
$CH_3.^{14}COONa$	$3{\cdot}34 \pm 4{\cdot}65$	—	0
$^{15}NH_2.^{14}CH_2.COOH$	$38{\cdot}7 \pm 2{\cdot}82$	$39{\cdot}6 \pm 3{\cdot}58$	$50{\cdot}02 \pm 2{\cdot}13$
$NH_2.^{14}CH_2.COOH$	—	$49{\cdot}0 \pm 8{\cdot}7$	$45{\cdot}9 \pm 5{\cdot}9$

The relative activity of the methyne carbon atoms has also been calculated by deducting the proportion of the activity due to the two imides (Table 4, column 4), from the activity of the original mesoporphyrin. These values are given in Table 5. This calculation shows that with both acetates the imides account for the whole of the activity of the mesoporphyrin. In agreement with the barium carbonate results this shows that neither of the two carbon atoms of acetate contributes to any appreciable extent to the formation of the methyne bridges. On the other hand, with glycine, the imides appear to account for only 50–60 % of the activity of the porphyrin. Thus, in the first glycine experiment, the factors will give the relative proportions of labelled carbon and nitrogen atoms entering the porphyrin molecule. This ratio is 2·17, i.e. 2·17 methylene carbon atoms are used for porphyrin synthesis for each nitrogen atom derived from the same amino-acid.

Table 6. *Comparison of* ^{15}N *and* ^{14}C *contents of glycine and haemin*

	^{15}N (atom % excess)	^{14}C (counts/min./100 mg. N)
Glycine	28·7	$50{\cdot}06 \times 10^6$
Haemin	0·348	$1{\cdot}317 \times 10^{6*}$

* No correction for back scattering.

Comparison of activity of haemin and deuterohaemin

When haemin is converted to deuterohaemin, two vinyl groups of rings I and II are lost. The relative molecular activity of the two compounds will give a measure of the radioactivity of the four carbon atoms removed. Table 7 shows that, after injecting $CH_3.{}^{14}COONa$, 7% of the activity is lost with the vinyl groups. This value is probably statistically significant and suggests that the carboxyl carbon atom of acetate is utilized to some extent for the formation of the vinyl groups of protoporphyrin. After injecting $^{14}CH_3.COONa$ the loss of activity was 23·2%. It is concluded that the methyl carbon atom of acetate is used to a greater extent than the carboxyl carbon atom, for the formation of the vinyl groups.

Table 7. *Comparison of radioactivity of haemin*

(Results expressed as counts/min./mmol.)

Compound injected	Haemin (A)	Deuterohaemin (B)	$B/A \times 100$
$^{14}CH_3.COONa$	121·9±4·2	93·6±3·6	76·8±5·15
$CH_3.{}^{14}COONa$	83·4±2·6	77·5±2·4	93·0±4·4

DISCUSSION

The origin of the methyne carbon atoms

Previous work has already shown that glycine is a biological precursor of protoporphyrin; the nitrogen atom (Shemin & Rittenberg, 1946*a*, *b*), and the methylene carbon atom (Altman, Casarett, Masters, Noonan & Salomon, 1948), but not the carboxyl carbon atom (Grinstein, Kamen, & Moore 1948) are used for the synthesis of the porphyrin. The experiments described in this paper demonstrate that the methylene carbon atom is the source of at least some of the methyne bridge atoms of protoporphyrin. The specificity of glycine in this respect is indicated by the observation that neither of the two acetates, nor formate nor serine produces significant activity in the methyne carbon fraction. Bufton, Bentley & Rimington (1949) have also found that formate is not utilized for the synthesis of haem by fowl erythrocytes *in vitro*. The hydroxymethyl group of serine is probably a precursor of the metabolic 'one-carbon' fragment (Sakami, 1949*b*) and the negative results obtained with serine and formate suggest that the methyne carbon atoms are not derived directly from such a 'one-carbon' fragment. These results also refute the hypothesis that, because glycine can be converted to serine (Sakami, 1948, 1949*a*; Siekevitz & Greenberg, 1949; Goldsworthy, Winnick & Greenberg, 1949), serine and not glycine is the immediate porphyrin precursor.

As already shown (Muir & Neuberger, 1949*a*; Wittenberg & Shemin, 1949), glycine must provide at least two of the four nitrogen atoms of protoporphyrin. However, as no other nitrogenous precursor has yet been found, amongst numerous compounds tested, it is likely that all four nitrogen atoms of the porphyrin are derived from glycine. This is supported by the observation that the newly formed porphyrins of a congenital porphyric have a higher ^{15}N content even than the glycine excreted as hippuric acid (Gray & Neuberger, 1950).

By using $^{15}NH_2.{}^{14}CH_2.COOH$, it was found that at least twice as many labelled carbon as nitrogen atoms appear in protoporphyrin. This ratio of 2 : 1 cannot be explained by loss of ^{15}N from doubly labelled glycine followed by reamination, because such a reversible deamination does not occur to a significant extent (Shemin, 1946, Elliott & Neuberger, 1950; Arnstein & Neuberger, unpublished observations). It would therefore appear that for each glycine molecule which supplies both nitrogen and methylene carbon atoms, there is approximately one additional molecule, which provides only the methylene carbon atom for the synthesis of protoporphyrin. As all four nitrogen atoms of the porphyrin appear to be derived from glycine, it must follow that at least eight carbon atoms are also derived from this source. The actual number of labelled carbon atoms calculated on this basis from the isotope ratio of 2·17, is 8·68. If all the four methyne carbon atoms are derived from glycine, it would follow that there are 4·68 labelled atoms in the pyrrole rings. In support of this deduction, the relative activity of the rings, as determined by degradation experiments, was found to be greater than that of the methyne bridges. The ratio was 61·3/38·7 in the first glycine experiment in rabbits. However, by calculation from the $^{14}C/^{15}N$ ratio, the ratio of activity of the pyrrole rings to that of the methyne bridges should be 54/46. It might therefore be argued that only three methyne carbon atoms are derived from glycine, in which case there should be 5·68 labelled atoms in the rings, resulting in a ratio of activities of 65·5/34·5. The accuracy of the results does not justify a decision being made. Nevertheless, in the second glycine experiment in rats, the ratio of activity of the ring to that of the methyne carbon atoms, was found to be 51/49, which would support the supposition that there are an equal number of labelled atoms in the rings and methyne bridges.

The $^{14}C/^{15}N$ ratio is not a whole number and leads to a calculated value of 8·68 labelled carbon atoms

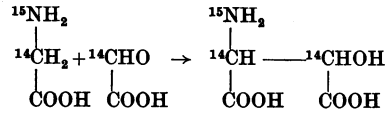
for the molecule. If eight are derived directly from glycine, the remaining 0·68 might represent the aggregate non-specific labelling of the molecule. Sprinson (1949) has shown that the methylene group of glycine is converted to an appreciable extent to both the methyl and carboxyl carbon atoms of acetate. If this conversion occurred to any extent in the bone marrow, then the acetate immediately available for porphyrin synthesis would be appreciably radioactive, because it would not be diluted by the total acetate of the body. Furthermore, if the non-specific labelling is due to the secondary conversion of glycine to acetate, the additional activity should appear in the pyrrole rings of the porphyrin, and not in the methyne carbon atoms, in agreement with the experimental results. The difference between the ratios of activities of the rings/methyne bridges, in the two glycine experiments with rabbits and rats could be explained by a variation in the non-specific labelling in the two species. Taking into account the effect of non-specific labelling, it would appear that the carbon atoms directly derived from glycine are equally distributed over the rings and methyne bridges. If it is assumed that all four nitrogen atoms are derived from glycine, it would then follow that all four methyne carbon atoms are also formed from glycine.

Distribution of labelled carbon atoms in the pyrrole rings

After administration of glycine and methyl-labelled acetate, the two imides were found to have almost equal molecular activities. It follows that the activity which derives from these two precursors is equally distributed between rings I + II and rings III + IV. On the other hand, the haematinic acid obtained from the experiment with carboxyl-labelled acetate, is more than four times as active as the methylethylmaleic acid imide. Presumably the radioactivity is due largely to the carboxyl group, which is what distinguishes the haematinic acid from the other imide. Radin *et al.* (1949) have also stated that the carboxyl group of acetate gives rise to the carboxyl groups of protoporphyrin. The haemin obtained after injecting methyl-labelled acetate lost almost 25 % of its activity when it was converted to deuterohaemin. At least one of the two carbon atoms of the vinyl groups must therefore be formed from the methyl group of acetate. It has recently been shown, with the use of deuterium-labelled acetate, that about one-third of the hydrogen atoms of haemin are derived from the hydrogen atoms of acetate (Ponticorvo, Rittenberg & Bloch, 1949). Some of the carbon atoms of other side chains besides the vinyl groups must therefore also be formed from the methyl group of acetate. The haemin from carboxyl-labelled acetate lost only 7 % of its activity when it was converted to deuterohaemin, which suggests that the carboxyl group of acetate is only used to a slight extent for the formation of the vinyl side chains.

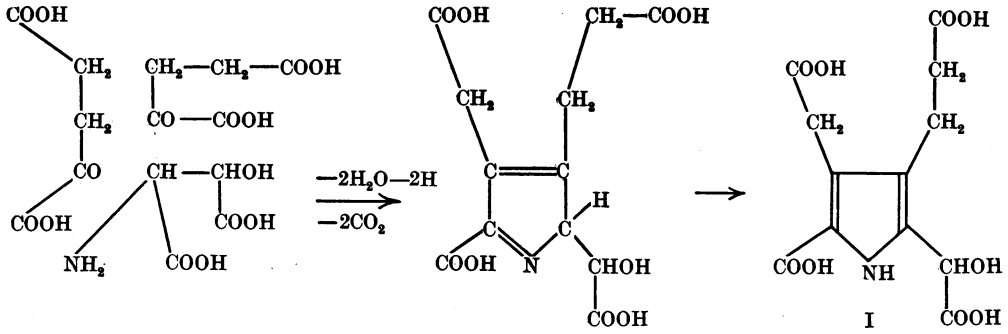
A suggested mechanism of porphyrin synthesis

The experimental results have shown that the methyne carbon atoms of protoporphyrin are largely, if not exclusively, derived from the methylene carbon atom of glycine, and that the amino-acid provides probably eight methylene carbon atoms and four nitrogen atoms for the synthesis. There is no reason to suppose that the N—C bond of the glycine molecule supplying the nitrogen is ever broken, so that the methylene carbon atom of glycine provides, presumably, one of the two carbon atoms of each pyrrole ring in the porphyrin molecule, in addition to the adjacent bridge carbon atoms. On the basis of these results, it is postulated that in the process of porphyrin synthesis, two molecules of glycine condense with loss of one nitrogen atom to a four-carbon compound. The four-carbon compound must subsequently lose its two carboxyl groups, because the carboxyl groups of glycine are not utilized (Grinstein *et al.* 1948). The nitrogen of glycine is utilized very efficiently, and therefore in any postulated intermediate the C—N link should have a metabolic stability similar to that in glycine. The four-carbon fragment is therefore probably not aspartic acid, because Wu & Rittenberg (1949) showed that ^{15}N-labelled aspartic acid loses its label too rapidly to be demonstrated as a nitrogenous precursor of protoporphyrin. However, Ratner, Nocito & Green (1944) have isolated a glycine oxidase from various species of animals, and have shown that this enzyme produces glyoxylic acid from glycine. Glyoxylic acid might therefore be involved in the formation of the four-carbon compound, which could be either hydroxyaspartic acid, or the related dehydration compound aminofumaric acid.



It is reasonable to suppose that the pyrrole rings are built up by the condensation of the four-carbon fragment with one of the intermediates of the Krebs cycle. It has been suggested by Lemberg & Legge (1949) and Lemberg (private communication), that α-ketoglutarate might be the compound involved. On this basis, the utilization of acetate must occur by its entry into the tricarboxylic acid cycle. Slightly modifying Lemberg's suggestion it is proposed that the four-carbon compound derived from glycine condenses with two molecules of α-keto-

glutarate to form a substituted pyrrole-2-carboxylic acid (I).



The participation of ketoglutarate in this way is consistent with most of the results of this paper. The greater use of the methyl than the carboxyl group of acetate for porphyrin synthesis is explained, since entry of carboxyl-labelled acetate into the cycle by any of the mechanisms believed to occur in the animal organism (for reviews see Krebs, 1943; Wood, 1946; Buchanan & Hastings, 1946; Bloch, 1947) would produce α-ketoglutarate labelled only on the carboxyl groups. The suggested mechanism for the formation of the pyrrole and the subsequent synthesis of porphyrin must eliminate most of the labelled carboxyl groups. On the other hand, the entry of methyl-labelled acetate into the cycle would at first lead to labelling of the ketoglutarate on one of the methylene carbon atoms, to be followed by a general redistribution over all the other carbon atoms as the cycle is repeated. In the synthetic reactions discussed above, only a small proportion of the activity due to the methyl group of acetate would thus be lost. This hypothesis would also explain why the haematinic acid fraction obtained after administration of carboxyl-labelled acetate is considerably more active than the methylethylmaleic acid imide. However, the fact that the latter has any activity at all suggests that there is a mechanism, as yet undiscovered, by which the carboxyl carbon atom of acetate is transformed to the methylene or carbonyl atoms of ketoglutarate. Radin *et al.* (1949) have also reported that in the haem obtained from bird red cells incubated with carboxyl-labelled acetate, only half of the total activity is due to the carboxyl groups. The formation of the pyrrole (I) by the proposed mechanism would explain the finding that at least one of the vinyl carbon atoms is derived from the methyl-carbon atom of acetate, and it would also explain the observation of Ponticorvo *et al.* (1949) that deuterium supplied as acetate is found in haem. Since acetate does not form the methyne groups, the methyne hydrogen atoms cannot come from this source, and therefore the deuterium must be situated in the side-chains. This agrees with the suggested mechanism, which implies that at least one carbon atom in each of the eight side chains of protoporphyrin is derived from the methyl carbon atom of acetate.

Rimington (1949) has pointed out that an attractive analogy for the biological synthesis of porphyrins exists in a chemical synthesis from substituted 5-hydroxymethylpyrrole-2-carboxylic acids, which readily undergo self-condensation to form porphyrins by decarboxylation and oxidation (Siedel & Winkler, 1943). Self-condensation of the pyrrole (I) by the Siedel-Winkler reaction would produce a uroporphyrin, which would finally lose all but two of its carboxyl groups when it is converted to protoporphyrin. This reaction would explain the fact that none of the carboxyl groups of the glycine molecules, and only a small number of the carboxyl groups of the acetate molecules which are used for the synthesis of the porphyrin survive in the final product. In the conversion of the pyrrole (I) to protoporphyrin there must also be a dehydrogenation, involving loss of four hydrogen atoms/protoporphyrin molecule. At what stage this occurs remains obscure.

This mechanism fully explains the formation of porphyrins of series I. In the formation of porphyrins of series III, however, it would have to be assumed that one of the rings, probably ring IV is formed by a somewhat different mechanism leading to an inverted arrangement of the side chains.

SUMMARY

1. Porphyrin synthesis in rabbits and rats was investigated after administration of sodium acetate, glycine, serine and formic acid labelled as indicated in the following formulae:

$^{14}CH_3.COONa$, $CH_3.{}^{14}COONa$,
$NH_2.{}^{14}CH_2.COOH$, $^{15}NH_2.{}^{14}CH_2.COOH$,
$^{14}CH_2OH.CH(NH_2).COOH$, $H.{}^{14}COOH$.

The porphyrin was degraded by oxidation to carbon dioxide, methylethylmaleic acid imide and haematinic acid.

2. The results showed that just over twice as many carbon atoms derived from the methylene group of glycine were incorporated as nitrogen atoms from the same amino-acid. About half the labelled carbon atoms were found in the methyne carbon fraction, whilst approximately one-quarter was found in each of the two imides isolated.

3. The methyl carbon atom of acetate was utilized more efficiently than the carboxyl carbon atom, and the activity of the former was found to an equal extent in both the imides, but not in the methyne carbon fraction. The activity of the carboxyl carbon atom of acetate was found chiefly in the haematinic acid. One or both of the vinyl carbon atoms were derived from the methyl carbon atom of acetate. Serine and formate were not utilized to any significant extent.

4. It is suggested that one molecule of glycine is oxidized to glyoxylic acid and then condenses with another molecule of glycine to give either hydroxyaspartic or aminofumaric acid. This four-carbon compound then condenses with two molecules of α-ketoglutarate to form a highly carboxylated pyrrole, which by further condensation with loss of carbon dioxide could give rise to porphyrins.

The authors wish to thank Dr R. Bentley for mass spectrometric analyses, Dr H. R. V. Arnstein for supplying some of the l abelled compounds, Dr G. Popják and Dr F. C. MacIntosh for help in animal experiments, and Miss B. Wiltshire and Mr R. Colin for technical assistance.

REFERENCES

Altman, K. I., Casarett, G. W., Masters, R. E., Noonan, T. R. & Salomon, K. (1948). *J. biol. Chem.* **176**, 319.
Arnstein, H. R. V. (1949). *Nature, Lond.*, **164**, 361.
Bloch, K. (1947). *Physiol. Rev.* **27**, 574.
Bloch, K. & Rittenberg, D. (1945). *J. biol. Chem.* **159**, 45.
Buchanan, J. M. & Hastings, A. B. (1946). *Physiol. Rev.* **26**, 120.
Bufton, A. W. P., Bentley, R. & Rimington, C. (1949). *Biochem. J.* **44**, xlix.
Calvin, M., Heidelberger, C., Ried, J. C., Tolbert, B. M. & Yankwich, P. F. (1949). *Isotopic Carbon.* New York: Wiley.
Dobriner, K. & Rhoads, C. P. (1928). *J. clin. Invest.* **17**, 95.
Dobriner, K. & Rhoads, C. P. (1940). *Physiol. Rev.* **20**, 416.
Elliott, D. F. & Neuberger, A. (1950). *Biochem. J.* **46** (in the Press).
Fischer, H. & Hummel, G. (1929). *Hoppe-Seyl. Z.* **181**, 107.
Gray, C. H. & Neuberger, A. (1950). *Biochem. J.* **46** (in the Press).
Goldsworthy, P. D., Winnick, T. & Greenberg, D. M. (1949). *J. biol. Chem.* **180**, 341.
Grinstein, M., Kamen, M. D. & Moore, C. V. (1948). *J. biol. Chem.* **179**, 359.
Krebs, H. A. (1943). *Advanc. Enzymol.* **3**, 191.
Lemberg, R. & Legge, J. W. (1949). *Haematin Compounds and Bile Pigments*, p. 637. New York: Interscience.
Muir, H. M. & Neuberger, A. (1949*a*). *Biochem. J.* **45**, 163.
Muir, H. M. & Neuberger, A. (1949*b*). *Biochem. J.* **45**, xxv.
Ponticorvo, L., Rittenberg, D. & Bloch, K. (1949). *J. biol. Chem.* **179**, 839.
Popják, G. & Beeckmans, M.-L. (1950). *Biochem. J.* **46**, 99.
Radin, N., Rittenberg, D. & Shemin, D. (1949). *Fed. Proc.* **8**, 240.
Ratner, S., Nocito, V. & Green, D. E. (1944). *J. biol. Chem.* **152**, 119.
Rimington, C. (1938). *C.R. Lab. Carlsberg*, **22**, 454.
Rimington, C. (1949). *Haemoglobin.* London: Butterworth's Scientific Publications.
Sakami, W. (1948). *J. biol. Chem.* **176**, 995.
Sakami, W. (1949*a*). *J. biol. Chem.* **178**, 519.
Sakami, W. (1949*b*). *J. biol. Chem.* **179**, 495.
Schoenheimer, R. & Ratner, S. (1939). *J. biol. Chem.* **127**, 301.
Shemin, D. (1946). *J. biol. Chem.* **162**, 297.
Shemin, D. & Rittenberg, D. (1946*a*). *J. biol. Chem.* **166**, 621.
Shemin, D. & Rittenberg, D. (1946*b*). *J. biol. Chem.* **166**, 627.
Siedel, W. & Winkler, F. (1943). *Liebigs Ann.* **554**, 162.
Siekevitz, P. & Greenberg, D. M. (1949). *J. biol. Chem.* **180**, 845.
Sprinson, D. B. (1949). *J. biol. Chem.* **178**, 529.
Tesar, C. & Rittenberg, D. (1947). *J. biol. Chem.* **170**, 35.
Turner, W. J. (1940). *J. Lab. clin. Med.* **26**, 323.
Wittenberg, J. & Shemin, D. (1949). *J. biol. Chem.* **178**, 47.
Wood, H. G. (1946). *Physiol. Rev.* **26**, 198.
Wu, H. & Rittenberg, D. (1949). *J. biol. Chem.* **179**, 847.