Studies on the Biosynthesis of Blood Pigments

5. INTERMEDIATES IN HAEM BIOSYNTHESIS*

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In earlier papers (Falk, Dresel & Rimington, 1953; Dresel & Falk, 1953, 1954, 1956*a*, *b*; Falk, Dresel, Benson & Knight, 1956) we have discussed the capacity of chicken-erythrocyte preparations to synthesize haem and porphyrin from substrates such as glycine, δ-aminolaevulic acid (ALA) and porphobilinogen (PBG). Our results, and those of others (Shemin & Russell, 1953; Neuberger & Scott, 1953; Bogorad & Granick, 1953; Shemin, 1955), suggest strongly that both ALA and PBG are highly specific precursors of porphyrins and of haem. It seemed desirable to seek more definite evidence that these substances lie on the normal pathway of haem biosynthesis. In addition, the exact pathway from porphobilinogen to haem has yet to be established. Two alternatives for this pathway have been suggested from indirect evidence obtained with isotopically labelled materials (Shemin & Wittenberg, 1951). In the first, the condensation of four identical monopyrrole precursor molecules leads to the formation of uroporphyrin III, which, by successive decarboxylations and dehydrogenation, leads via coproporphyrin III to protoporphyrin IX and haem. We think that in this series of reactions the incorporation of iron might occur at a step before the formation of protoporphyrin IX. The alternative pathway would involve modifications of the side chains of the monopyrrole precursor molecule before condensation, some of the porphyrins with a high number of carboxyl groups thus being bypassed in the biosynthesis. The fact that porphyrins with eight to two carboxyl groups readily accumulate in systems *in vitro* (Dresel & Falk, 1956*b*; Bogorad & Granick, 1953) as well as *in vivo* in normal and pathological conditions (cf. Rimington, 1954) would appear to favour the first pathway. The present paper describes investigations designed to elucidate the role of some of the free porphyrins known to occur naturally, and of ALA and PBG, in the biosynthesis of haem.

* Part 4: Falk, Dresel, Benson & Knight (1956).

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Much of the work described here has already been presented in outline (Dresel, 1955).

MATERIALS

The samples of PBG and ALA used have been described previously (Dresel & Falk, 1956*a*). The porphyrins used were prepared from the methyl esters by saponification as described by Falk *et al.* (1956). The methyl esters of uroporphyrin I (from a case of congenital porphyria), uroporphyrin III (from turacin) and coproporphyrin III (from *Corynebacterium diphtheriae*) were the reference substances described by Falk & Benson (1953). 'Uroporphyrin III from haemolysate' and *pseudo*uroporphyrin were isolated as a mixture after incubation of a haemolysate preparation with PBG, and, after esterification, were separated on a $CaCO_3$ column (cf. Falk *et al.* 1956). Waldenström porphyrin ester (m.p. 260°, corr.) was a bulked sample from various cases of human acute porphyria; paper chromatography [by the dioxan method of Falk & Benson (1953), and by the lutidine method of Falk *et al.* (1956) on the coproporphyrin isolated after decarboxylation] indicated mainly uroporphyrin III with not more than 5% uroporphyrin I. Haematoporphyrin (Light and Co.) was purified by countercurrent distribution (Falk *et al.* 1956). Protoporphyrin IX ester was prepared from blood by the method of Grinstein (1947) and, after saponification, purified by countercurrent distribution (Falk *et al.* 1956).

METHODS

The tissue preparations, methods of incubation and quantitative determinations of free porphyrins were carried out as described by Dresel & Falk (1956*b*). For haematoporphyrin determinations, the value given by Heilmeyer (1943) for the extinction coefficient in 5% (w/v) HCl was used ($E^{1\%}_{1\,cm.} = 5300$ at 402 mμ.). 1 μmole of 'porphyrin equivalent' denotes 1 μmole of porphyrin, 4 μmoles of PBG and 8 μmoles of ALA (cf. Dresel & Falk, 1956*b*).

Radioactivity determinations. When sufficient material was available, determinations were carried out at 'infinite thickness' as described by Dresel & Falk (1954). Small amounts of highly active material were counted at 'infinite thinness'. A disk of lens paper, a fraction smaller in diameter than the 1 cm.² polythene planchets, was placed on a glass plate, and one drop of concentrated solution of the material blown on to it from a capillary pipette. The solvents used were $CHCl_3$ for porphyrin esters, acetone for 2:4-dinitrophenylglycine and water for PBG hydrochloride. The disk was allowed to dry in air and then placed in the planchet. After the determination of radioactivity, the material

on the disk was dissolved in a known volume of suitable solvent and determined spectrophotometrically (see below). In all cases the radioactivity was directly proportional to the amount of material on the disk with quantities up to at least 50 μg. A similar method has been used by Henriques, Henriques & Neuberger (1955).

In a few cases the yields of Cu protoporphyrin ester from the haemolysate preparation were too small for counting at infinite thickness, and the radioactivity too low for the 'infinite thinness' method. The following procedure, similar to that of Fager (1947), was then adopted. Material (0·5–5 mg.) in concentrated solution in $CHCl_3$ was placed on a nickel planchet (1·77 cm.^{2}) and covered with a lens paper disk to assist the even distribution of the material. After air-drying and determination of radioactivity, the Cu protoporphyrin ester was redissolved in $CHCl_3$ and determined spectrophotometrically. The counts were corrected to 'counts at infinite thickness' by the use of a reference curve.

Samples of the glycine used for the experiments were converted into the 2:4-dinitrophenyl derivative, and the specific activity was determined.

Spectrophotometric determinations

Porphyrin esters were determined by the absorption at the maximum of the Soret band in $CHCl_3$. The positions of the maxima were slightly different in $CHCl_3$ and dioxan, but the extinction coefficients in the two solvents were identical within the limits of experimental error. The molar extinction coefficients in $CHCl_3$ were calculated from the data for the visual bands in dioxan (Stern & Wenderlein, 1934), after the relative absorptions of band IV (about 500 mμ.) and the Soret band in dioxan had been determined. In this way the following millimolar absorption coefficients (ϵ_{mM}) in $CHCl_3$ were derived: protoporphyrin ester, 405–406 mμ., ϵ_{mM} 161; coproporphyrin ester, 400–401 mμ., ϵ_{mM} 169; uroporphyrin ester, 405 mμ., ϵ_{mM} 190. With 'uroporphyrin III from haemolysate' and *pseudo*uroporphyrin, it was assumed that the molar extinction coefficients are identical with those of uroporphyrin. Although exposure to bright daylight was avoided, there was occasional evidence of degradation, particularly of protoporphyrin, on the columns. Samples showing broadening of the Soret band were rejected, and in all instances the observed density readings at the Soret band were corrected by the following formulae, adapted from Rimington & Sveinsson (1950). In these D is the corrected density, and d_{430}, d_{380}, etc., are the observed density readings:

$$\text{Protoporphyrin: } D = \frac{2 \times d_{406} - [d_{380} + d_{430}]}{1{\cdot}33},$$

$$\text{Coproporphyrin: } D = \frac{2 \times d_{400} - [d_{380} + d_{430}]}{1{\cdot}43},$$

$$\text{Uroporphyrin: } D = \frac{2 \times d_{405} - [d_{380} + d_{430}]}{1{\cdot}56}.$$

Porphobilinogen was determined by the method described by Cookson & Rimington (1954).

2:4-*Dinitrophenylglycine* was determined at 360 mμ. in 0·5% (w/v) aqueous $NaHCO_3$ (Dresel & Falk, 1954). ϵ_{mM} at this wavelength was 17·2.

Isolation of materials for radioactivity determinations

Haem. Haem was separated completely from free porphyrins and purified and crystallized as Cu protoporphyrin ester (Dresel & Falk, 1956*a*). Where it was necessary also to isolate PBG, the haemolysate was precipitated with trichloroacetic acid (see below), and haem was extracted from the precipitated protein, first with acetic acid followed by 3 vol. of ethyl acetate, and then with acetone containing 1% HCl. The final yields of Cu protoporphyrin ester were rather low in these cases.

Porphobilinogen. The haemolysate was precipitated with trichloroacetic acid (final concentration 5%, w/v) and centrifuged; the precipitate was washed with 5% (w/v) trichloroacetic acid and recentrifuged until the supernatant gave no colour with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde). The PBG present in the combined supernatants was determined, and then PBG hydrochloride (about 50 mg.) was added in known amount so that the dilution of the radioactive material could be calculated. After several extractions with ether to remove the trichloroacetic acid, the PBG was precipitated with mercuric acetate, and extracted and purified either (Expt. *A*, Table 1) on an alumina column (Prunty, 1945; Westall, 1952) or (Expt. *B*, Table 2) as described by Cookson & Rimington (1954). The PBG hydrochloride was recrystallized to constant radioactivity, three crystallizations being required. In a control experiment, in which the PBG was added after the incubation and immediately before precipitation with trichloroacetic acid, the isolated PBG hydrochloride had no radioactivity.

Porphyrins: Experiment A (*Table* 1). Uro- and coproporphyrins were isolated and determined quantitatively as described by Dresel & Falk (1956*a*). To the acid extracts containing these porphyrins, about 30–40 mg. of the same porphyrin, dissolved as its methyl ester in 25% (w/v) HCl, was added; the total porphyrin was then determined, so that the dilution of the isolated material could be calculated. The acid extracts were then evaporated to dryness *in vacuo*, converted into their methyl esters (Falk *et al.* 1956), passed through a column of Grade III MgO (Nicholas, 1951) and crystallized. Recrystallization did not alter the radioactivity (determined by the method of 'infinite thickness', see above).

Experiment B (*Table* 1). The isolated free porphyrins were converted into their methyl esters and purified to constant radioactivity (determined by the method of 'infinite thinness', see above) by column chromatography. The adsorbent chosen in each case was one which would hold the porphyrin in pure $CHCl_3$, so that the material, once applied to the top of the column (in $CHCl_3$), could be freely washed with $CHCl_3$ before elution. As expected, radioactive impurities were present in these $CHCl_3$ washings, but they were readily separated from the porphyrin esters in this way. All porphyrins were passed through two successive columns, but in no case was the specific activity changed after the second column, unless there were obvious signs of degradation as with protoporphyrin (see above). Uroporphyrins were purified on Al_2O_3 (grades V–VI) and eluted with $CHCl_3$ containing 0·5% methanol. Copro- and protoporphyrins were purified on MgO (grade III) and eluted with $CHCl_3$ containing 1 and 2% methanol respectively. With small columns of adsorbent (about 1·2 cm. × 6 cm.), as little as 20 μg. of porphyrin ester formed a distinctly fluorescent band, and its passage down the column could be observed with ease under an ultraviolet lamp. In most cases some material remained near the top of the column and was not easily eluted; this was probably partly saponified ester and was rejected.

Calculation of the quantity of newly synthesized material

The method for the calculation of the amount of newly synthesized haem from the radioactivity of the isolated Cu protoporphyrin ester has been described (Dresel & Falk, 1956*a*). Essentially the same method has been used for the calculation of the amounts of newly synthesized material in the PBG and porphyrins isolated after incubation with [α-^{14}C]glycine. It was assumed that glycine is used for the synthesis of all porphyrins and PBG, as one would expect from the evidence for haem (Muir & Neuberger, 1950; Wittenberg & Shemin, 1950) and free protoporphyrin (Dresel & Falk, 1956*a*), that is, that eight α-C atoms of glycine are incorporated per molecule of porphyrin and two per molecule of PBG. From a knowledge of the specific activity of the glycine in the system, the molar specific activity of any newly synthesized material can be calculated. The amount of newly synthesized substance present in the isolated material is equal to $p \times X/Y$, where X is the observed molar specific activity of the isolated material, Y the calculated molar specific activity of the newly synthesized material and p the molar quantity of material isolated. Corrections were made for endogenous glycine, which was assumed to be the same as that determined previously in an exactly similar system (0·0012 M; Dresel & Falk, 1954).

RESULTS

Comparison of the labelling of haem, porphyrins, PBG and ALA by [α-^{14}C]*glycine*

It is established that glycine is an obligatory precursor of haem (Wittenberg & Shemin, 1950; Muir & Neuberger, 1950). A study of the incorporation of [α-^{14}C]glycine into haem in the presence of various unlabelled substances should help to elucidate the role of these in the biosynthesis of haem. Various materials suspected of being intermediates in the biosynthesis were therefore added in known quantity to the chicken-erythrocyte haemolysate (Dresel & Falk, 1954), and this was incubated with radioactive glycine. It was thus possible to study quantitatively, and in the same sample, any reduction in haem synthesis from radioactive glycine due to the added material, and in the event of the latter fraction being radioactive on subsequent re-isolation, the amount of newly synthesized material it then contained. If the radioactivity of the haem was reduced, a comparison of these two measurements should enable one to distinguish between two possibilities: (*a*) that the added material inhibits haem synthesis from glycine, but is not itself a normal intermediate in the synthesis; in this case one would expect very little, if any, radioactivity in the added material re-isolated after incubation; (*b*) that the added material is indeed a true intermediate; in this case one might expect that the loss of radioactivity in the haem should be of the same order as the gain in radioactivity of the added material. Indeed, since most materials were added in quantities greatly in excess of the amount of haem normally synthesized from glycine in this system, one would in the latter case expect most of the radioactivity to be trapped in the added 'pool'.

The results are illustrated in Table 1. There were great differences in the amount of added 'pool' material with the several compounds tested. In view of the very active conversion of PBG into

Table 1. *Comparison of* [α-^{14}C]*glycine incorporation into haem and into various added 'pools'*

Expt. *A*. Haemolysate (21 ml.) was incubated for 2 hr. at 38° in a final volume of 33 ml. with 0·01 M sodium succinate, 0·0036 M-$MgCl_2$ and 0·0065 M [α-^{14}C]glycine (42·7 μc). Porphyrins were added in less than 1 ml. of 0·1 N-$NaHCO_3$. Standard deviations are quoted only where the error of counting was greater than ±5%. All figures are expressed as μm-moles of 'porphyrin equivalents'. Expt. *B*. As Expt. *A* except that the glycine (0·0065 M) contained 107 μc. The porphyrins were added in 1·0 ml. of 0·1 N-$NaHCO_3$, and this was also added to the control flask and the flasks containing PBG and ALA.

Addition	'Pool' size: Before incubation	'Pool' size: After incubation	Haem synthesized from [α-^{14}C]glycine	Newly synthesized 'pool' substance remaining
Expt. *A*				
None	—	—	20	—
PBG	2610	1300	0·05±0·07	6·7±0·46
Uroporphyrin (Waldenström)	297	241	9	0·6±0·08
Coproporphyrin III	378	314	14	0·5±0·04
Expt. *B*				
None	—	—	19	—
PBG	2380	1200	0·5±0·07	7·9±0·54
ALA	2240	—	0·3	—
Uroporphyrin III (Turacin)	292	183	3·8	1·1±0·08
Uroporphyrin III (haemolysate)	119	68	12	0·7±0·06
*pseudo*Uroporphyrin	51	40	14	0·4
Coproporphyrin III	387	321	11	1·0
Haematoporphyrin IX	408	351	16	—
Protoporphyrin IX	215	116	18	9·8

porphyrins (Dresel & Falk, 1956*b*), this substance was added at a relatively high concentration to assure sufficient material for re-isolation and radioactivity determination after incubation. This consideration did not apply to the porphyrins; these were added at considerably lower concentrations to avoid, as far as possible, simple inhibition of normal haem biosynthesis by the added materials. Shortage of purified material sometimes necessitated very low concentrations; even then, the amount of added material recovered at the end was such that more radioactivity should have appeared in this fraction than in the haem if the added porphyrin had behaved as a true intermediate.

Porphobilinogen 'pool'. The two experiments with added PBG gave very similar results. In both the radioactivity of the haem was negligible. Only some 50% of the added PBG was recovered after incubation, a considerable part of the loss being due to the conversion into porphyrins (cf. Dresel & Falk, 1956*b*). Even so, the radioactivity of the re-isolated PBG corresponded to some 35–40% of that found in the haem in the control experiments.

Aminolaevulic acid 'pool'. ALA was added at the same molar concentration (expressed as porphyrin equivalents) as PBG. As with the PBG 'pool', haem synthesis from glycine was almost completely suppressed by the ALA 'pool'. At the time of the experiment no technique for the re-isolation of ALA was available. Instead, the porphyrins formed from ALA were isolated and their radioactivities compared with those derived from the PBG 'pool' (Table 2). The specific activities of the porphyrins from ALA were double those from PBG. The total amounts of porphyrins synthesized from PBG were not determined in this experiment, but we have good evidence that the yields of porphyrins from ALA and PBG are of the same order under these conditions (Dresel & Falk, 1956*b*). The synthesis of porphyrins from glycine was therefore twice as high in the presence of ALA than of PBG. In spite of this difference, which can be interpreted in several ways, the results would appear to indicate a certain similarity in the behaviour of ALA and PBG in this system.

Uroporphyrin 'pools'. The several uroporphyrins tested were added at different concentrations and direct comparisons are difficult. Haem synthesis from glycine was reduced in all cases, but it was still considerably greater than the corresponding synthesis of uroporphyrin from glycine. The figures quoted for newly synthesized uroporphyrin were calculated from the specific activity of the porphyrin recovered in the ethyl acetate fraction after precipitation of the haemolysate (Dresel & Falk, 1956*a*); only about half of the total porphyrin is recovered in this fraction, the remainder being precipitated with the protein (Dresel & Falk, 1956*a*). It was conceivable that the newly synthesized uroporphyrin might preferentially follow the protein precipitate, and that the calculated amounts might therefore have been artificially low. To test this, the protein precipitates from two samples were treated with methanol–5% H_2SO_4 and the specific activities of the extracted porphyrin esters determined. In the one case the specific activities found for the two fractions were not significantly different, and in the other the specific activity of the uroporphyrin from the precipitate was only about 50% of that in the ethyl acetate. Although this difference is difficult to understand, the results indicate that the newly synthesized porphyrin does not preferentially follow the protein precipitate.

In the control flask (Expt. *B*) carrier uroporphyrin was added after incubation, to determine the newly synthesized uroporphyrin present at the end. This was, approximately, 0·05 μm-mole, which was only about one-tenth or less of that trapped when uroporphyrin was added before incubation.

Table 2. *Radioactivities of porphyrins formed in presence of ALA and PBG in Expt. B (Table 1)*

For conditions of incubation see Table 1.

Addition	Porphyrins formed: Protoporphyrin	Coproporphyrin	Uroporphyrin + *pseudo*uroporphyrin
ALA			
Specific activity (counts/min./μm-mole)	3·83	4·03	5·80
Amount recovered (μm-moles)	350	44	246
Amount synthesized from [α-^{14}C]glycine (μm-moles)	2·2	0·3	2·5
PBG			
Specific activity (counts/min./μm-mole)	1·94	1·97	—

Coproporphyrin 'pool'. The two experiments with added coproporphyrin III gave similar results. The reduction in the radioactivity of the haem was considerably less than in the flasks to which uroporphyrin had been added at approximately similar levels. At the same time, the amount of newly synthesized porphyrin trapped in the coproporphyrin 'pool', although significant, was of the same low order as that trapped by the uroporphyrin 'pools'. These results appear to suggest inhibition rather than dilution of haem synthesis by the added coproporphyrin.

Haematoporphyrin 'pool'. Added haematoporphyrin caused even less reduction in the radioactivity of the haem than did added coproporphyrin III. The haematoporphyrin re-isolated after incubation was degraded before its radioactivity could be determined. We do not know, therefore, whether any haematoporphyrin was formed from glycine in this system.

Protoporphyrin 'pool'. There was scarcely any inhibition of haem synthesis from glycine when protoporphyrin was added to the system during incubation. Furthermore, the amount of newly synthesized protoporphyrin in the protoporphyrin isolated after incubation (9·8 μm-moles) was of the same order as the additional protoporphyrin found by quantitative determination in the control flask (12 μm-moles). Thus the synthesis from glycine of both haem and protoporphyrin apparently was unaffected by the added protoporphyrin 'pool'.

Incubation of haemolysate with added porphyrins

Uroporphyrins. The conversion of uroporphyrins into other porphyrins on incubation with the haemolysate or the supernatant preparation is illustrated by the results given in Table 3. Considerable amounts of protoporphyrin, but no traces of coproporphyrin, were formed from uroporphyrin III in the whole haemolysate preparation. The identity of the protoporphyrin was established as described by Falk *et al.* (1956). At low concentrations the yield amounted to some 50% of the added uroporphyrin (Expt. 110, Table 3); however, the quantity of protoporphyrin formed was not raised by increasing the concentration of uroporphyrin, so that the maximum amount of protoporphyrin formed from uroporphyrin III was only some 10% of that formed from PBG under similar conditions of incubation (cf. Dresel, 1955).

There was no appreciable conversion of uroporphyrin III into either protoporphyrin or coproporphyrin by the supernatant preparation. The amount of porphyrin isolated as 'protoporphyrin' was too small to identify, and the position of maximum absorption differed from that of pure protoporphyrin.

Uroporphyrin I, added to the haemolysate, gave rise only to traces of 'protoporphyrin'; the amounts were again too small to study the nature of this porphyrin. On the other hand, there was an appreciable amount of porphyrin in the coproporphyrin fraction. Lutidine paper chromatography showed that this was mainly coproporphyrin I with traces of coproporphyrin III and a substance behaving like a 'pentacarboxyl' porphyrin (Falk *et al.* 1956). The system responsible for the conversion of uroporphyrin I into coproporphyrin I was present also in the supernatant preparation (Table 3).

Table 3. *Incubation of haemolysate with added porphyrins*

Preparations (21 ml.) were incubated at 38° in a final volume of 33 ml. with 0·0036 M-$MgCl_2$ and, in Expts. no. 108, 110 and 118, 0·01 M sodium succinate. Figures with asterisks imply that the porphyrin was of uncertain identity since the Soret peak was at 406–408 mμ., whereas that of pure protoporphyrin was at 409 mμ.

Expt. no.	Addition (μm-moles)	Time (hr.)	Porphyrins formed (μm-moles): Whole haemolysate — Coproporphyrin	Whole haemolysate — Protoporphyrin	Haemolysate supernatant — Coproporphyrin	Haemolysate supernatant — Protoporphyrin
110	Uroporphyrin III (120)	4	0	62	—	—
	Uroporphyrin III (313)	4	0	63	—	—
192	Uroporphyrin III (282)	4	0	50	0	7*
202	Uroporphyrin III (142)	2	0	32	—	—
	Uroporphyrin III (142)	4	0	39	—	—
118	Uroporphyrin I (241)	2	19	3	—	—
170	Uroporphyrin I (246)	4	18	1	—	—
192	Uroporphyrin I (190)	4	10	3	13	3*
108	Waldenström porphyrin (119)	4	15	34	—	—
118	Waldenström porphyrin (241)	2	9	37	—	—
192	Waldenström porphyrin (272)	4	4	13	5	3*
108	Coproporphyrin III (152)	4	—	10*	—	—
	Coproporphyrin III (455)	4	—	11*	—	—

The results with Waldenström porphyrin were somewhat variable, but indicate that this porphyrin behaves like a mixture of uroporphyrin III and uroporphyrin I in the sense that both protoporphyrin and coproporphyrin are formed from it.

Coproporphyrin. Added coproporphyrin III gave rise to only traces of 'protoporphyrin' (Table 3). The amounts were again too small for rigid identification, and the absorption maximum was again anomalous.

DISCUSSION

Interpretation of the experiments in which 'pool' substance and radioactive glycine were both added is complicated by the fact that the various 'pool' compounds tested for their role in haem biosynthesis were added at different concentrations. Furthermore, these concentrations decreased to differing extents during the incubation. Even so, a very marked difference between the results with PBG on the one hand, and with the porphyrins on the other, becomes apparent from an inspection of Table 1. With all the porphyrins, there was considerably greater incorporation of radioactive glycine into the haem than into the added porphyrin. In all cases the size of the added 'pool', even at the end of incubation, was considerably in excess of the haem and porphyrin synthesized in the control flask, which amounted to 20 μm-moles of haem and 12 μm-moles of protoporphyrin. The results therefore indicate that haem synthesis from glycine was able to bypass the added porphyrin pools, and that the various porphyrins tested were not behaving as true intermediates of haem biosynthesis in this system. In the experiment with added PBG the distribution of the radioactivity was reversed. Haem synthesis from radioactive glycine was almost totally suppressed, but a considerable amount of newly synthesized PBG was trapped by the 'pool'. The amount recovered corresponded to 35–40 % of the haem synthesis, or some 25 % of haem plus protoporphyrin synthesis in the control flask. The latter comparison would seem the more valid, since it is probable that the same monopyrrole is the precursor of both haem and free protoporphyrin. The appearance of 25 % of the porphyrin synthesized in the control in the form of newly synthesized and trapped PBG seems quite impressive, particularly when it is remembered that only half the added PBG was recovered. Furthermore, it is conceivable that the artificially increased concentration of a reaction product might have caused an appreciable reduction in the normal rate of synthesis of PBG from glycine. We conclude, therefore, that the results with PBG indicate that this compound is a normal intermediate in the synthesis of haem from glycine. The results with ALA show a close similarity to those with PBG, and are therefore in concordance with the present belief that ALA is a normal intermediate in the synthesis. It could be argued that this similarity in results was simply due to the rapid conversion of ALA into PBG, known to occur in this system, and that ALA does not itself lie on the direct pathway. This possibility could not be tested, since there was no method for the recovery of ALA for radioactivity determination. Similarly, the results of Shemin & Russell (1953) are not entirely conclusive in this respect. These authors showed that, in their duck-erythrocyte system, labelled ALA is incorporated into haemin more efficiently than labelled glycine or succinate, and also, that the radioactivity of haemin after incubation with labelled glycine or succinate is much reduced by the addition of unlabelled ALA. Such results could also be obtained if ALA were not itself the normal intermediate but were actively converted into an intermediate by the tissue system.

This treatment of our results may be invalid if permeability barriers to the added materials exist in the haemolysate preparation. It is conceivable that the apparent difference in the behaviour of ALA and PBG from that of the porphyrins is due to such factors. Further, these could also be the cause of the inefficient utilization of added uro- and copro-porphyrins for protoporphyrin synthesis. However, the inability of the supernatant preparation to decarboxylate uroporphyrin III is against this possibility. Uro- and copro-porphyrins are freely formed in the supernatant preparation from added PBG (Dresel & Falk, 1956*b*). If the reaction sequence is PBG → uroporphyrin III → coproporphyrin III, one would expect some coproporphyrin III to be formed from added uroporphyrin III in this system, where the existence of permeability barriers seems highly improbable, and in which indeed, uroporphyrin I is converted into coproporphyrin I. All these findings would appear to favour the hypothesis that uroporphyrin III is not a normal intermediate of haem and protoporphyrin synthesis, but that it is relatively inefficiently converted into such an intermediate by a factor present in the whole haemolysate, but not in its supernatant preparation. It appears either that there is no similar factor capable of converting coproporphyrin III into an intermediate of haem biosynthesis, or that if such a factor exists, it must be very much less efficient.

Demonstration of a system capable of converting uroporphyrin I into coproporphyrin I is of considerable interest in view of the normal and pathological excretion of coproporphyrin I (cf. Weatherall, 1954). The detection of uroporphyrin I in the Waldenström porphyrin by its conversion into coproporphyrin I suggests that the haemolysate

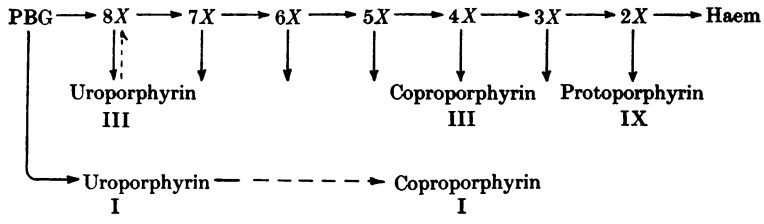

Fig. 1. Suggested scheme for mechanism of haem biosynthesis from PBG.

preparation might offer a sensitive and quantitative method for the detection of traces of uroporphyrin I in mixtures of uroporphyrin isomers.

The experiments described give no final answer to the question of the role of porphyrins as such in the intermediate pathway of haem biosynthesis. Our results show, however, that the free porphyrins of the III isomer series are not readily utilized by the haemolysate, and, although they are synthesized *de novo* from glycine, the capacity for this is slight when compared with the synthesis of PBG and haem; it might be supposed, therefore, that they arise as side products during the synthesis. A consideration of all our findings has led us to believe that the intermediate pathway of haem biosynthesis might follow a scheme as outlined in Fig. 1 (cf. Dresel, 1955). The number in each intermediate indicates the number of side chains possessing a carboxyl group, and *X* denotes a porphyrin-like structure common to all the intermediates which are not, however, true free porphyrins. A slight reversibility of the side reaction leading to uroporphyrin III is indicated. Further, a direct pathway for handling the I series porphyrins is suggested in view of our findings with the supernatant preparation.

It is of interest that Bogorad (1955), using extracts of spinach leaf and *Chlorella*, has recently come to the conclusion that a colourless precursor of uroporphyrin, rather than true uroporphyrin, is the intermediate between PBG and porphyrins with seven to three carboxyl groups. Thus there is independent evidence for a non-porphyrin intermediate corresponding to 8*X* in Fig. 1. The origin of his porphyrins with seven to three carboxyl groups was not studied, but it is conceivable that they also arose as the result of side reactions as in Fig. 1.

SUMMARY

1. The intermediate pathway of haem biosynthesis from glycine has been investigated with a chicken-erythrocyte haemolysate preparation.

2. The incorporation of [α-^{14}C]glycine into haem and simultaneously into various added porphyrins and porphyrin precursors was studied. The results indicated that porphobilinogen, and probably also δ-aminolaevulic acid, are true intermediates in the synthesis. None of the porphyrins tested, including uroporphyrin III, *pseudo*uroporphyrin, coproporphyrin III, haematoporphyrin IX and protoporphyrin IX, behaved as true intermediates in the haemolysate preparation.

3. Added uroporphyrin III gave rise to some free protoporphyrin in the whole haemolysate, but to neither proto- nor copro-porphyrin in the supernatant preparation. Some coproporphyrin I was formed from added uroporphyrin I in both preparations.

4. The results are discussed, and it is suggested that some compounds related to the porphyrins, rather than the porphyrins themselves, are the true intermediates in haem biosynthesis.

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REFERENCES

Bogorad, L. (1955). *Science*, **121**, 878.
Bogorad, L. & Granick, S. (1953). *Proc. nat. Acad. Sci., Wash.*, **39**, 1176.
Cookson, G. H. & Rimington, C. (1954). *Biochem. J.* **57**, 476.
Dresel, E. I. B. (1955). In *The Biosynthesis of Porphyrins and Porphyrin Metabolism, Ciba Foundation Conf.* p. 72. London: Churchill.
Dresel, E. I. B. & Falk, J. E. (1953). *Nature, Lond.*, **172**, 1185.
Dresel, E. I. B. & Falk, J. E. (1954). *Biochem. J.* **56**, 156.
Dresel, E. I. B. & Falk, J. E. (1956*a*). *Biochem. J.* **63**, 72.
Dresel, E. I. B. & Falk, J. E. (1956*b*). *Biochem. J.* **63**, 80.
Fager, E. W. (1947). *Report of a Symposium on the use of Isotopes in Biological Research*, Chicago (3–4 March), p. 13.
Falk, J. E. & Benson, A. (1953). *Biochem. J.* **55**, 101.
Falk, J. E., Dresel, E. I. B., Benson, A. & Knight, B. C. (1956). *Biochem. J.* **63**, 87.
Falk, J. E., Dresel, E. I. B. & Rimington, C. (1953). *Nature, Lond.*, **172**, 292.
Grinstein, M. (1947). *J. biol. Chem.* **167**, 515.
Heilmeyer, L. (1943). *Spectrophotometry in Medicine*. London: Hilger.

Henriques, O. B., Henriques, S. B. & Neuberger, A. (1955). *Biochem. J.* **60**, 409.
Muir, H. M. & Neuberger, A. (1950). *Biochem. J.* **47**, 97.
Neuberger, A. & Scott, J. J. (1953). *Nature, Lond.*, **172**, 1093.
Nicholas, R. E. H. (1951). *Biochem. J.* **48**, 309.
Prunty, F. T. G. (1945). *Biochem. J.* **39**, 446.
Rimington, C. (1954). *Rep. Progr. Chem.* **51**, 311.
Rimington, C. & Sveinsson, S. L. (1950). *Scand. J. clin. Lab. Invest.* **2**, 209.
Shemin, D. (1955). *Rep. 3rd Int. Congr. Biochem., Brussels*, p. 170.
Shemin, D. & Russell, C. S. (1953). *J. Amer. chem. Soc.* **75**, 4873.
Shemin, D. & Wittenberg, J. (1951). *J. biol. Chem.* **192**, 315.
Stern, A. & Wenderlein, H. (1934). *Z. phys. Chem.* A, **170**, 345.
Weatherall, M. (1954). *Pharmacol. Rev.* **6**, 133.
Westall, R. G. (1952). *Nature, Lond.*, **170**, 614.
Wittenberg, J. & Shemin, D. (1950). *J. biol. Chem.* **185**, 103.

Studies in the Biochemistry of Micro-organisms

97. FLAVIPIN, A CRYSTALLINE METABOLITE OF *ASPERGILLUS FLAVIPES* (BAINIER & SARTORY) THOM & CHURCH AND *ASPERGILLUS TERREUS* THOM*

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The Aspergilli are divided by Thom & Raper (1945), mainly on morphological characters, into fourteen groups, two of which are of interest in the present communication. The *Aspergillus flavipes* group consists of a single species, *A. flavipes* (Bainier & Sartory) Thom & Church. The closely related *A. terreus* group includes three species, of which the commonest and most important is *A. terreus* Thom and its three varieties, *boedijni* (Bloch.) n.var., *floccosus* Shih and *aureus* n.var. It is interesting to note that old cultures of *A. flavipes* often resemble *A. terreus*, the two species being readily distinguished, however, when young cultures are examined.

We have found that culture filtrates of one strain each of *A. flavipes* grown on Raulin–Thom solution and of *A. terreus* grown on Czapek–Dox solution give an intense blue–black ferric reaction and an immediate heavy red precipitate with Brady's reagent (2:4-dinitrophenylhydrazine in aqueous 2N hydrochloric acid). We have isolated in a state of purity the metabolic product responsible for these reactions and propose for it the trivial name flavipin, since it does not appear to have been described previously.

There appears to be no published record of the isolation of any metabolic product of *A. flavipes*, although there are a number of references in the literature to the formation by this species of culture filtrates having an antibacterial spectrum similar to that of penicillin and containing a substance which is destroyed by penicillinase. In marked contrast, different strains of *A. terreus* have been shown to produce a large variety of metabolites, among which are: itaconic acid (Calam, Oxford & Raistrick, 1939); itatartaric acid $HO_2C.C(OH)(CH_2.OH).CH_2.CO_2H$ (Stodola, Friedkin, Moyer & Coghill, 1945); expansine, $C_7H_8O_4$ (Kent & Heatley, 1945); terrein, $C_8H_{10}O_3$, and citrinin, $C_{13}H_{14}O_5$ (Raistrick & Smith, 1935); geodin, $C_{17}H_{12}O_7Cl_2$, and erdin, $C_{16}H_{10}O_7Cl_2$ (Raistrick & Smith, 1936); and terreic acid, $C_7H_6O_4$, from *A. terreus* var. *aureus* n.var. (Pansacker, Philpot, Jennings & Florey, 1947).

* Part 96: Galarraga, Neill & Raistrick (1955).

Production of flavipin

The influence of cultural conditions, and particularly of the composition of the culture medium used, on the course of mould metabolism is well illustrated by a comparison of the metabolism of *A. flavipes* with that of *A. terreus*. Thus we have shown that, although good yields of flavipin are produced by *A. flavipes* when grown on Raulin–Thom solution, no flavipin could be detected when this species was grown on Czapek–Dox solution. *A. terreus*, on the contrary, failed to produce any flavipin on Raulin–Thom solution, though it gave moderate yields on Czapek–Dox solution. It was further shown that, whereas the optimum incubation period at 24° for maximum production of flavipin is 12–14 days with *A. flavipes* grown on Raulin–Thom medium, the corresponding time is 7–9 days for *A. terreus* grown on Czapek–Dox medium. The results obtained showed clearly that flavipin cannot be regarded as an 'end' product of the metabolism of either species, since, after the concentration of flavipin in the culture fluid had reached a maximum, flavipin was decomposed at about the same rate as the glucose in the medium