Studies on the Biosynthesis of Blood Pigments

3. HAEM AND PORPHYRIN FORMATION FROM δ-AMINOLAEVULIC ACID AND FROM PORPHOBILINOGEN IN HAEMOLYSED CHICKEN ERYTHROCYTES

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The significance of δ-aminolaevulic acid (ALA) and porphobilinogen (PBG) as specific haem and porphyrin precursors has been outlined in the preceding paper (Dresel & Falk, 1956a); in experiments with intact chicken erythrocytes ALA and PBG behaved very differently, because of the almost complete impermeability of the intact cells to PBG. In the present paper we describe the behaviour of these two precursors in our chicken erythrocyte haemolysate (cf. Dresel & Falk, 1954); in this system the two substrates behave similarly with respect to haem and porphyrin formation, both quantitatively and qualitatively.

Lead has been shown to inhibit the formation of haem from glycine (Dresel & Falk, 1954, 1956a). In this paper we show that lead inhibits porphyrin formation from ALA but not significantly from PBG. This indicates inhibition of ALA dehydrase (Gibson, Neuberger & Scott, 1954), but this enzyme does not appear to be as sensitive to lead as some enzyme involved in the formation of ALA from glycine. In addition to the whole haemolysate we have studied the activity of homogenized preparations and of supernatants obtained from both types of preparation by high-speed centrifuging. These preparations have been compared with similar preparations from duck cells.

Some of the results described in this paper have already been reported briefly (Dresel, 1955; Falk, 1955; Falk, Dresel & Rimington, 1953; Dresel & Falk, 1953).

EXPERIMENTAL

Materials

Radioactive porphobilinogen. Radioactive glycine (200 μc/m-mole; 50 mg.) was given orally each day for three successive days to a rabbit made porphyric by allylisopropylacetamide (cf. Goldberg & Rimington, 1955). The porphobilinogen in the urine was worked up separately each day as described by Cookson & Rimington (1954); we are grateful for the help given us by Dr Goldberg and Professor

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Rimington in this matter. Almost equal and high activities were obtained on the second and third days, and these samples were combined and crystallized to constant activity as the hydrochloride. From a comparison of the counts at infinite thinness (cf. Dresel & Falk, 1956b) of this material with 2:4-dinitrophenylglycine of known radioactivity, it was calculated that the radioactivity of the porphobilinogen so obtained corresponded to 6·26 μc/m-mole of PBG, or 25·04 μc/m-mole of haem formed from PBG. Unlabelled PBG and ALA were obtained as described by Dresel & Falk (1956a). All reagents were of A.R. grade. Glass-distilled water was used throughout.

Radioactive ALA. $^{14}CO_2H.CH_2.CH_2.^{14}CO.CH_2.NH_2$ containing 29·5 μc/m-mole was kindly supplied by Drs Scott and Neuberger. Since, in the formation of protoporphyrin from 8 moles of ALA six of the carboxyl groups are removed, the radioactivity of haem formed from this material should be 147·5 μc/m-mole, that is 5·85 times as high as the haem formed from our radioactive porphobilinogen.

It should be pointed out that, owing to the low radioactivity of the material, the results quoted for haem synthesis from PBG are subject to rather large experimental error. Maximum counts obtained in the isolated copper protoporphyrin ester (after incubation for 4 hr. with non-limiting PBG) were of the order of 10 counts/min. above background counts of about 14 counts/min.

Methods

Tissue preparations. The red cells were washed and haemolysed essentially as described previously (Dresel & Falk, 1954, preparation B), but the haemolysate was brought back to approximate isotonicity with 0·604 M-KCl instead of the phosphate buffer–KCl mixture. 'Homogenates' were prepared from the haemolysates by treatment for 7 min. in a Waring Blendor in a cold room at 2°. 'Supernatant' preparations from these haemolysates and homogenates were obtained at 0° by high-speed centrifuging. By phase-contrast microscopy, kindly carried out by Dr J. Hanson, a few particles of less than 1·0 μ. diameter were found in the crystal-clear supernatants from chicken cells, both when the preparations had been spun at 18 000 g for 20 min. and at 78 000 g for 60 min. Incubations were carried out as described by Dresel & Falk (1954).

Determination of PBG. PBG was determined spectrophotometrically (Cookson & Rimington, 1954), in filtrates obtained after precipitation of the tissue preparations with trichloroacetic acid to a final concentration of 5 % (w/v).
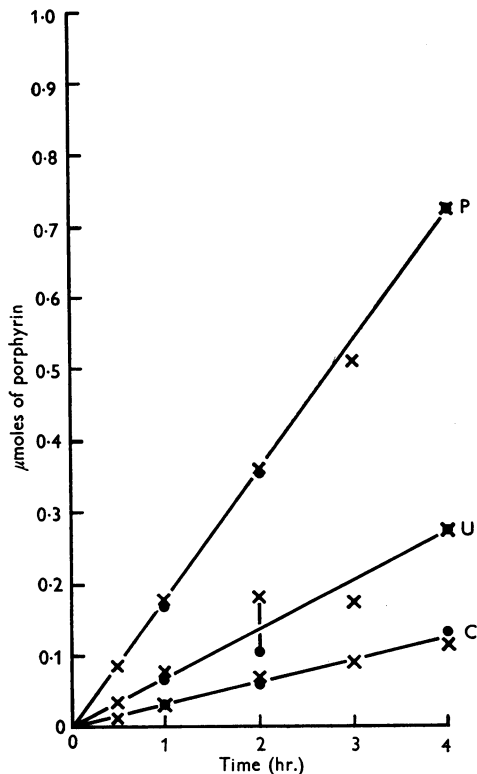
Fig. 1. Effect of time on synthesis of porphyrins from non-limiting amounts of PBG (×) (2·12 μmoles 'porphyrin equivalents') and ALA (●) (2·09 μmoles 'porphyrin equivalents') in the haemolysate. P, protoporphyrin; C, coproporphyrin; U, uroporphyrin. Other conditions as in Table 1.

Fig. 2. Effect of time on synthesis of porphyrins from a limiting amount of PBG (0·52 μmole 'porphyrin equivalents') in the haemolysate. P, protoporphyrin; C, coproporphyrin; U, uroporphyrin. Other conditions as in Table 1.

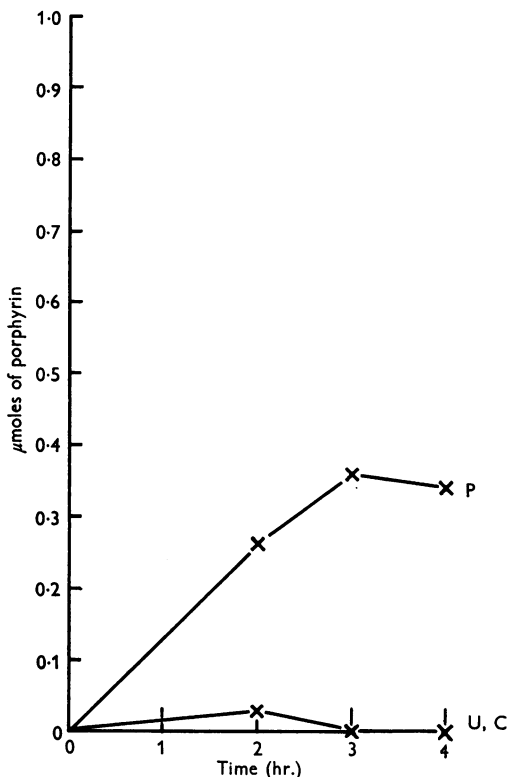There was considerable fading of the colour of the PBG–Ehrlich reagent complex in these extracts; when readings were taken as quickly as possible (about 30 sec.) recoveries of the order of 70–80 % were obtained. By determining the rate of fading and extrapolating to zero time, recoveries were increased to 90–100 %.

Determinations of haem and porphyrins. These were carried out as described by Dresel & Falk (1956a).

RESULTS

The quantitative equivalence of PBG and ALA as substrates for haem and porphyrin formation is indicated in Figs. 1, 3 and 4. The concentrations of these two substrates are usually expressed as 'porphyrin equivalents', to allow for the fact that eight molecules of ALA, but only four of PBG, are required to synthesize one molecule of porphyrin; i.e. the concentrations quoted have to be multiplied by 8 and 4 respectively to give the actual molarities of ALA and PBG.

Effect of time of incubation. The time curves for porphyrin formation from ALA and PBG at non-limiting substrate concentrations are shown in Fig. 1. The system was obviously stable for at least 4 hr. Protoporphyrin, coproporphyrin and uroporphyrin were formed in molar ratios of approximately 6:1:2. The constitution of these porphyrins, as revealed by chromatographic studies, is discussed below. When the substrate concentration was rate-limiting (Fig. 2), the small amounts of uroporphyrins and coproporphyrins present at 2 hr. disappeared after a further hour of incubation, and protoporphyrin increased during this hour. This might suggest conversion of uroporphyrins and coproporphyrins into protoporphyrin. The amounts of porphyrin were, however, rather small, and evidence from some recovery experiments at similar levels suggests that part, at least, of the disappearance of uroporphyrins and coproporphyrins is due to degradation during incubation.

Porphyrin precursors of the leucoporphyrin type (cf. Falk, 1954) were sought on the basis of their conversion into porphyrins by treatment with iodine (Schwartz, Zieve & Watson, 1951), but no increase in free porphyrin was ever found by this method.

Effect of substrate concentration. At low substrate concentration protoporphyrin alone is formed (Fig. 3); uroporphyrin and coproporphyrin appear only when a substrate concentration is reached at which protoporphyrin formation is reaching a maximum. This could be due either to a stepwise conversion of uroporphyrin through coproporphyrin into protoporphyrin, or, on the other hand, to parallel independent syntheses with differing enzyme–substrate affinities. The curve (Fig. 3) does not reflect initial rates over the whole range of substrate concentrations. The yields of porphyrins were those found after 4 hr., but at low substrate concentrations the formation of porphyrins would already have been complete before this time (cf. Fig. 2).

At rate-limiting substrate concentrations (e.g. 2.4×10^{-5} M PBG or 0.6×10^{-5} M 'porphyrin equivalents') as much as 90% of the added material could be accounted for as haem and protoporphyrin. Under optimum conditions of substrate concentration (20×10^{-5} M PBG, or 5×10^{-5} M 'porphyrin equivalents'), the total yield of porphyrins accounted for some 55% of added PBG. In addition, about 10% of added PBG was recovered. It will be shown below that the capacity of the system to convert PBG into haem is relatively slight. In the present case the haem formed would have accounted for only about 2% of

the added PBG. The fate of the remaining 33% was not discovered (cf. Discussion).

PBG was recovered almost quantitatively, and only a trace, if any, of uroporphyrin was formed, after incubation under the usual conditions (approx. 2.5×10^{-4} M at 37° for 4 hr.) with boiled haemolysate or with 0·01 M phosphate buffer pH 7·3. This was important because of the ready non-enzymic conversion of PBG into uroporphyrins under certain conditions (cf. Cookson & Rimington, 1954). The yields of porphyrins from optimum concentrations of ALA or PBG were unchanged when succinate (0·01 M) and glycine (0·01 M) were also added to the system.

Haem synthesis. Maximum haem synthesis in 4 hr. was obtained from 1.5×10^{-5} M ALA or PBG 'porphyrin equivalents' Fig. 4. Some three to four times as much substrate is required for maximum synthesis of porphyrin (Fig. 3), the amount of the latter being about thirty times greater than the amount of haem synthesized.

Haem synthesis was probably not quite linear for 4 hr. (Table 1). The synthesis after 2 hr. amounted to about 60% of that observed after 4 hr., although we have insufficient data to establish this point accurately. The time curve up to 4 hr. is not dissimilar to that obtained for the synthesis of haem from glycine (Dresel & Falk, 1954), so that a comparison of the formation of haem from glycine and from ALA and PBG at 4 hr. is perhaps permissible. Under optimum conditions, haem synthesis from PBG was apparently only some 50% higher than haem synthesis from glycine (Expt. no. 164, Table 1). This is in strong contrast to the relative amounts of free protoporphyrin. From PBG and ALA the maximum protoporphyrin formed was about fifty times as much as from glycine. The inequality of the yields from PBG and ALA in the two different experiments of Table 1 is due to the variation in activity of
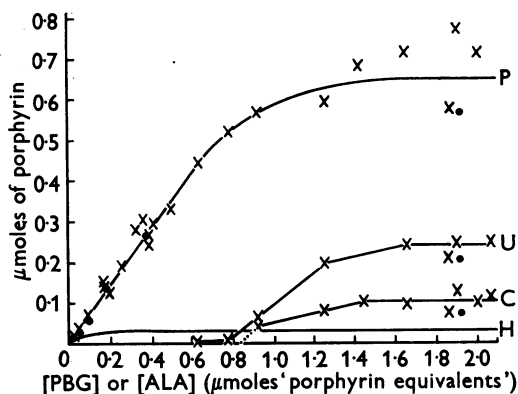

Fig. 3. Effect of PBG and ALA concentration on porphyrin synthesis during 4 hr. incubation with the haemolysate. P, protoporphyrin; C, coproporphyrin; U, uroporphyrin; H, haem (for more detailed comparison see Fig. 4). ×, [PBG]; ●, [ALA]. Other conditions as in Table 1.
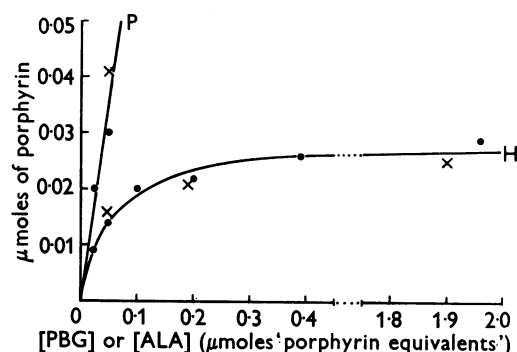

Fig. 4. Effect of PBG and ALA concentration on haem synthesis in the haemolysate. Conditions as in Fig. 3. P, protoporphyrin; H, haem; ×, [PBG]; ●, [ALA].

Table 1. *Time relationships of haem and protoporphyrin formation from ALA and PBG*

Haemolysate (21 ml.) was incubated in a final volume of 33 ml. (see Methods) containing $0.0036\,M\text{-}MgCl_2$.

Expt. no.	Addition (μmoles 'porphyrin equivalents')	Time (hr.)	Haem (μmoles $\pm$ s.d.)	Proto-porphyrin (μmoles)
164	PBG, 1·94	2	0·021±0·0017	0·379
	PBG, 1·94	4	0·037±0·0019	0·818
	Glycine, 53·4*	4	0·025±0·0010	0·015
186	ALA, 1·96	2	0·017±0·0008	0·289
	ALA, 1·96	4	0·029±0·0009	0·576
	PBG, 1·90	4	0·025±0·0018	0·587

* Glycine concentration corresponds to $0.013\,M$ (cf. Dresel & Falk, 1954).

Table 2. *Effect of $FeSO_4$ on haem and porphyrin formation from PBG*

Flasks were incubated for 4 hr. under conditions as described in Table 1. All figures are expressed as μmoles 'porphyrin equivalents'.

Additions	Haem ($\pm$s.d.)	Proto-porphyrin	Copro-porphyrin	Uro-porphyrin
PBG, 1·94	0·037±0·0019	0·818	0·112	0·255
PBG, 1·94 + $FeSO_4$ (0·001 M)	0·330±0·012	0·377	0·051	0·164

different haemolysate preparations, which was exceptionally large in this particular case.

The low yield of haem from PBG and ALA may be due to a lack of available iron. Indeed, addition of $10^{-3}\,M\text{-}FeSO_4$ to the system led to a tenfold increase of haem which was paralleled by a fall in the free protoporphyrin, approximately equivalent to the amount of extra haem formed (Table 2). The coproporphyrin and uroporphyrin fractions were also reduced. This could be due to a formation of urohaem and coprohaem, which would have been lost during fractionation. Similarly, the raised protohaem formation in the presence of iron in this system is probably the result of the non-specific formation of a complex of iron with the available protoporphyrin.

It was of some interest to know whether, at low concentrations, PBG is preferentially converted into haem. As shown in Fig. 4, it was not found to be so, the free protoporphyrin amounting to about twice the haem formed at the lowest concentration of PBG which could be studied.

Porphyrin and haem formation from ALA and PBG in soluble preparations from chick and duck haemolysates and homogenates

The supernatant fraction obtained after spinning the chick haemolysate at high speed (see Methods) will convert PBG and ALA into coproporphyrin and, at high substrate concentrations, also into 'uroporphyrins', but not significantly into protoporphyrin or haem (Falk *et al.* 1953; Dresel, 1955). This is further illustrated in Table 3. The small amounts of porphyrin which appeared in the 'protoporphyrin' fraction of the supernatants always had Soret maxima at slightly shorter wavelength than true protoporphyrin; in addition, a sample

subjected to 'lutidine' paper chromatography (Falk, Dresel, Benson & Knight, 1956) revealed only a small proportion of 'dicarboxylic' porphyrin, together with porphyrins in the tricarboxylic and tetracarboxylic positions. The relatively small amount of the dicarboxylic porphyrin had probably originated from endogenous sources rather than from PBG. It seems, then, that the enzyme system responsible for the formation of protoporphyrin is absent from the supernatant preparation, a finding contrary to that of Shemin, Abramsky & Russell (1954), who have made similar studies with duck-erythrocyte homogenates. For this reason we have performed a few experiments with duck haemolysate and homogenate preparations (Table 3). Certain differences between the two species will be noted. Under optimum conditions for porphyrin synthesis (Expt. 206, Table 3) almost twice as much protoporphyrin was formed in the duck whole haemolysate as in the chick whole haemolysate, but the accumulation of coproporphyrin and uroporphyrin, though still appreciable, was considerably less in the duck preparation. The total porphyrin formed was greater in the duck whole haemolysate than in the chick whole haemolysate, but in the corresponding supernatant preparations the situation was reversed. The smaller total yield of porphyrins in the duck-preparation supernatant was due largely to a much smaller coproporphyrin fraction; in fact the protoporphyrin fraction was rather higher in this preparation than in the corresponding chick preparation; in this case the Soret maximum corresponded closely to that of true protoporphyrin. Even so, less than 10 % of the system responsible for protoporphyrin formation in the duck-blood haemolysate was present in its supernatant.

Table 3. *Haem and porphyrin formation from ALA and PBG in various tissue preparations*

21 ml. preparations were incubated for 4 hr., except in Expt. no. 206, (5·25 ml. incubated for 2 hr.). For easier comparisons, figures in this experiment were multiplied by 8; this treatment implies somewhat greater experimental inaccuracies since the actual determinations were on smaller amounts. The 'solids' preparation (Expt. no. 112) was obtained after centrifuging 21 ml. of haemolysate at 18 000 g, washing the spun-down fraction twice with 0·9 % (w/v) KCl at 18 000 g and finally making up to 21 ml. with 0·9 % KCl. Supernatant fractions were obtained after centrifuging for 20 min. at 18 000 g (Expts. no. 112, 162 and 186) or 60 min. at 78 000 g (Expts. nos. 206 and 214). All preparations were used fresh with the exception of those in Expt. no. 214, which had been stored at $-10°$ for 4 weeks. Quantities of ALA and PBG are expressed as μmoles 'porphyrin equivalents'.

Expt. no.	Tissue preparation	Addition	Porphyrins formed (μmoles)			
			Proto-porphyrin	Copro-porphyrin	Uro-porphyrin	Haem $\pm$ S.D.
112	Chicken haemolysate	PBG, 0·266	0·177	0·0	0·0	—
	Chicken haemolysate 'solids'	PBG, 0·266	0·0	0·0	0·0	—
	Chicken haemolysate supernatant	PBG, 0·266	0·012*	0·068	0·0	—
	Chicken haemolysate supernatant + solids	PBG, 0·266	0·174	0·0	0·0	—
162	Chicken haemolysate	PBG, 0·521	0·362	0·0	0·0	0·024 $\pm$ 0·0017
	Chicken haemolysate supernatant	PBG, 0·521	0·012*	0·167	0·012	0·0
186	Chicken haemolysate	ALA, 0·49	0·347	0·0	0·0	0·026 $\pm$ 0·0009
	Chicken haemolysate supernatant	ALA, 0·49	0·012*	0·147	0·028	0·0
206	Chicken haemolysate	PBG, 3·472	0·640	0·120	0·162	—
	Chicken haemolysate supernatant	PBG, 3·472	0·040	0·320	0·424	—
	Chicken homogenate	PBG, 3·472	0·216	0·0	0·008	—
	Chicken homogenate supernatant	PBG, 3·472	0·056	0·024	0·016	—
	Duck haemolysate	PBG, 3·472	1·168	0·048	0·080	—
	Duck haemolysate supernatant	PBG, 3·472	0·112	0·088	0·344	—
	Duck homogenate	PBG, 3·472	0·160	0·0	0·0	—
	Duck homogenate supernatant	PBG, 3·472	0·112	0·0	0·008	—
214	Chicken haemolysate	PBG, 0·382	0·149	0·025	0·0	0·024 $\pm$ 0·0026
	Chicken homogenate	PBG, 0·382	0·081	0·007	0·0	0·039 $\pm$ 0·0029
	Chicken homogenate supernatant	PBG, 0·382	0·020	0·012	0·0	0·0
	Duck haemolysate	PBG, 0·382	0·155	0·025	0·0	0·034 $\pm$ 0·0031
	Duck haemolysate supernatant	PBG, 0·382	0·021	0·014	0·0	0·0
	Duck homogenate	PBG, 0·382	0·098	0·021	0·0	0·021 $\pm$ 0·0029
	Duck homogenate supernatant	PBG, 0·382	0·051	0·010	0·0	0·0

* The Soret peak of these fractions was at 406–408 mμ., whereas that of pure protoporphyrin is at 409 under similar conditions.

Table 4. *Effect of lead acetate on haem and porphyrin synthesis from glycine, ALA and PBG*

A phosphate buffer–KCl–haemolysate (cf. Dresel & Falk, 1954, preparation B) was used in Expt. no. 92, but the usual KCl–haemolysate (see Methods) was used in Expts. nos. 216 and 218. Other conditions as in Table 1; 0·01 M sodium succinate was added in Expt. no. 92. Incubation for 2 hr. in Expt. no. 92 and for 4 hr. in Expts. nos. 216 and 218. — denotes not determined.

Expt. no.	Substrate	Pb acetate (M)	Synthesis as percentage of control				
			Haem	Proto-porphyrin	Copro-porphyrin*	Uro-porphyrin*	Total free porphyrins
92	Glycine (0·0065 M, 12·7 μc)	10^{-6}	86 $\pm$ 4·0	—	—	—	—
		10^{-5}	45 $\pm$ 1·9	—	—	—	—
		3×10^{-5}	16 $\pm$ 1·1	—	—	—	—
		10^{-4}	11 $\pm$ 0·7	—	—	—	—
218	Glycine (0·013 M, 14·1 μc)	10^{-5}	54 $\pm$ 1·8	44	—	—	—
		10^{-4}	23 $\pm$ 1·0	17	—	—	—
216	ALA (0·33 $\times$ 10^{-4} M†)	10^{-6}	—	102	96	88	92
		10^{-5}	—	100	88	106	95
		10^{-4}	—	50	0	<2	33
	PBG (0·3 $\times$ 10^{-4} M†)	10^{-4}	80 $\pm$ 8·0	98	75	85	92

* No entries where porphyrins were absent from control flasks.　　　　　† Expressed as 'porphyrin equivalents'.

It seemed conceivable that homogenization might alter the distribution of the enzymes, but in our hands the main effect of this was a very considerable inactivation of all the enzymes. Those enzymes responsible for the formation of uroporphyrins and coproporphyrins appeared to be more sensitive in this respect than those concerned with protoporphyrin formation. There was possibly slightly more protoporphyrin formed by the homogenate supernatant than by the haemolysate supernatant, but the difference was small.

Constitution of the porphyrins formed

The details of the methods of analysis of these porphyrins are presented separately in the next paper (Falk *et al.* 1956). The 'protoporphyrin' fraction consisted mainly of protoporphyrin, accompanied by trace amounts of a material which corresponded on paper chromatograms to a tricarboxylic porphyrin. Chromatographically, the 'coproporphyrin' fraction corresponded mainly to coproporphyrin III. There was occasionally a suggestion of 'tailing' to the coproporphyrin I position on the paper chromatograms, but the presence of this isomer could not be confirmed. It was present, if at all, in very small amounts compared with the coproporphyrin III. This fraction also contained trace amounts of materials, the behaviour of which on the chromatograms suggested that they might be tri-, penta-, hexa- and hepta-carboxylic porphyrins. The 'uroporphyrin' formed on incubation of the haemolysate with either ALA or PBG always corresponded chromatographically to uroporphyrin III and *pseudo*uroporphyrin (Falk *et al.* 1956; Dresel & Falk, 1956*a*) in about equal amounts.

The 'coproporphyrin' and 'uroporphyrin' fractions behaved identically on chromatograms, whether they arose from whole haemolysate or from supernatant preparations.

Effect of lead

Haem synthesis from radioactive glycine, studied as described by Dresel & Falk (1954), was almost totally inhibited by 10^{-4}M lead acetate (Table 4). At the same concentration haem and porphyrin formation from PBG was inhibited only slightly, if at all. In contrast to the results with PBG, 10^{-4}M lead acetate caused a very marked inhibition of porphyrin synthesis from ALA (Table 4). Uroporphyrin and coproporphyrin did not accumulate at all, and protoporphyrin formation was reduced to 50%. This differential effect on the synthesis of the porphyrins is almost certainly due to the reduced accumulation of porphobilinogen (cf. the effect of substrate concentration on porphyrin formation, Fig. 3), resulting from the inhibition of ALA dehydrase. An inhibition of this

enzyme is not unexpected, since Gibson *et al.* (1954) have demonstrated its sensitivity to thiol reagents. It would seem probable, therefore, that one of the enzymic steps of haemopoiesis affected in lead poisoning is the conversion of ALA into PBG. A comparison of the extent of inhibition of haem synthesis from glycine and porphyrin synthesis from ALA by different concentrations of lead would, however, suggest that some early step, leading to the formation of ALA, is even more sensitive to the action of lead than ALA dehydrase itself. Thus with 10^{-5}M lead acetate there was a 50% inhibition of haem and porphyrin formation from glycine, but no significant inhibition of porphyrin formation from ALA (Table 4). These findings are surprising in view of the excessive formation of porphyrins in lead intoxication, and possible causes of this will be discussed elsewhere.

DISCUSSION

The results given in this paper do not necessarily support the hypothesis that protoporphyrin formation proceeds through uroporphyrin and coproporphyrin. Evidence, obtained by isotope-dilution techniques and other methods, which throws doubt on this hypothesis, is presented in a later paper (Dresel & Falk, 1956*b*; cf. also Dresel, 1955).

The relation between haem and porphyrin formation in the haemolysate from the substrates ALA and PBG, and their formation from glycine, is interesting. The maximum amount of haem formed from glycine, without added iron, is of the same low order as that formed from ALA or PBG. While porphyrins are formed in greater quantity than haem from ALA and PBG, protoporphyrin is the only detectable porphyrin from glycine, and is formed in smaller amounts even than haem. Thus when much free protoporphyrin is present (when the substrate is ALA or PBG), haem formation is limited to about the same level as when relatively very little free protoporphyrin is present (when glycine is the substrate). The possibility that this is due to lack of iron has been discussed above. In these tissue preparations it is difficult to determine whether haem is formed by a mechanism similar to that occurring *in vivo*, or simply by a chemical artifact.

Porphyrin formation proceeds more efficiently from ALA or PBG than from glycine, and this may well be due to relatively low activity in this system of the enzymes of the tricarboxylic acid cycle, which must be involved in the synthesis of ALA from glycine (Shemin & Wittenberg, 1951). That some cofactors are limiting seems clear, since boiled yeast juice caused a marked stimulation of haem formation (Dresel & Falk, 1954). This was not due to iron added with the yeast juice, because,

with glycine as substrate, added $FeSO_4$ alone caused no stimulation. The stimulation occurred apparently at a stage between glycine and PBG, since we have found that yeast juice causes no increase in porphyrin formation from PBG.

The supernatant fractions possess only a small proportion of the original capacity for protoporphyrin formation of the whole haemolysate, although uroporphyrin and coproporphyrin are freely formed. Shemin *et al.* (1954), using duck erythrocytes, found no difference in the amount of haem formed from ALA in haemolysed or homogenized preparations, and they observed that supernatants from their homogenate contained almost the whole of this activity. Shemin *et al.* (1954) appear to have avoided the inactivation that we have found on homogenization of either chicken- or duck-erythrocyte haemolysates; unfortunately the exact details of their method are not available.

The factors required for the formation of protoporphyrin can also be supplied to the haemolysate supernatant by a preparation of rat-liver mitochondria (Dresel, 1955). Granick (1955) has found a similar effect on addition of liver mitochondria to supernatant preparations of *Chlorella* extracts; indeed, it appears that the free porphyrins formed from PBG in his systems are not dissimilar to those we find with chicken-haemolysate preparations.

The efficiency of the utilization of PBG for porphyrin formation is remarkable. In an experiment where PBG concentration was limiting (2.4×10^{-5} M) and no PBG remained at the end of incubation, 90 % was accounted for as protoporphyrin and haem. The conversion was probably even higher, because we have repeatedly found that porphyrins cannot be recovered quantitatively after incubation with the haemolysate. In another experiment, where PBG concentration was not rate-limiting (see Results), some 67 % of the PBG was accounted for at the end as porphyrins or unchanged PBG. Like the porphyrin recoveries, PBG recoveries from tissue preparations are low, because of the fading of the colour of the Ehrlich-reagent complex. These low recoveries could account for at least 20 % of the added PBG, and it seems that very little PBG can have been converted into products other than porphyrins.

The substrate-affinity of the rate-limiting enzyme in the conversion of PBG into porphyrin must be remarkably high. Although we have not carried out detailed rate studies at very low substrate concentrations, our data show that the maximum initial rate of formation of porphyrins from PBG is reached at less than 10^{-4} M.

The complete equivalence of haem and porphyrin formation from ALA and PBG indicates that ALA dehydrase was never rate-limiting under our conditions, with the exception of the experiments with lead, when it was inhibited.

SUMMARY

1. The quantitative and qualitative equivalence of haem and porphyrin formation from ALA and PBG in the fowl haemolysate preparation is demonstrated.

2. At low substrate concentrations, only haem and protoporphyrin were formed, but, with higher substrate concentrations, porphyrins with more than two carboxyl side-chains also accumulated. The main porphyrins other than protoporphyrin were coproporphyrin III, uroporphyrin III and a new porphyrin which we have called *pseudo*-uroporphyrin.

3. The conversion of ALA and PBG into porphyrins was very efficient and approached 90 % under certain conditions.

4. Haem synthesis was relatively inefficient in this preparation, but was much increased, with a corresponding decrease of free protoporphyrin, by the addition of 10^{-3} M ferrous sulphate.

5. Supernatant fractions obtained from the haemolysate after high-speed centrifuging converted ALA and PBG into 'coproporphyrins' and 'uroporphyrins' only, no significant amounts of protoporphyrin or haem being observed. Some comparisons of these preparations with similar preparations from duck cells, and also with homogenized preparations, are described.

6. Addition of 10^{-4} M lead acetate resulted in an almost total inhibition of haem synthesis from glycine, scarcely any inhibition of haem and porphyrin formation from PBG, and a considerable inhibition of porphyrin formation from ALA. It is suggested that, if lead specifically inhibits haem synthesis *in vivo*, the step most sensitive to this reagent must be among the early steps leading to the synthesis of ALA.

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Studies on the Biosynthesis of Blood Pigments

4. THE NATURE OF THE PORPHYRINS FORMED ON INCUBATION OF CHICKEN ERYTHROCYTE PREPARATIONS WITH GLYCINE, δ-AMINOLAEVULIC ACID OR PORPHOBILINOGEN

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In the preceding papers of this series (Dresel & Falk, 1954, 1956*a*, 1956*b*), the incubation with certain substrates of chicken erythrocytes or of preparations obtained by haemolysis has been shown to result in the formation of various porphyrins. The substrates used have been glycine, δ-aminolaevulic acid (ALA) or porphobilinogen (PBG). The constitution of these porphyrins was defined as closely as possible. Some paper-chromatographic techniques that have proved very useful for studies of uroporphyrin and coproporphyrin are now described in detail. As well as making possible some definition of the isomeric types of the uroporphyrin and coproporphyrin formed, these methods have led to the recognition of a material which we have called *pseudo*uroporphyrin and which may have significance in the biosynthesis of protoporphyrin. The 'protoporphyrin' formed has been studied by a variety of techniques, including countercurrent distribution. The instability of protoporphyrin in aqueous HCl under certain conditions is well known, and some aspects of the acid-catalysed hydration of its vinyl groups have also been investigated, with particular reference to methods for the hydrolysis of protoporphyrin ester.

EXPERIMENTAL

Materials

The samples of uroporphyrins I and III and coproporphyrins I and III used for markers in the paper-chromatographic studies have been specified previously (Falk &

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Benson, 1953). Coproporphyrins II and IV were synthetic materials from the collection of the late Professor Hans Fischer, kindly made available to us by Professor S. Goldschmidt. Three samples of synthetic uroporphyrins were kindly supplied by Dr S. F. MacDonald. Samples *a* and *c* were uroporphyrins II and IV respectively, prepared by rational syntheses (MacDonald & Michl, 1955); sample *b* was uroporphyrin II, separated from a synthetic mixture of uroporphyrins (MacDonald, 1952).

The 2:6-lutidine (Hopkin and Williams, London) was used as received. The purification of the other solvents has been described by Falk & Benson (1953). The CaCO₃ used for column chromatography was very kindly given to us by Professor C. J. Watson of Minnesota; we have not found a satisfactory sample in England. All other reagents were of A.R. grade.

Methods

The methods of extraction of the 'uroporphyrin', 'coproporphyrin' and 'protoporphyrin' fractions from the tissue preparations have been described (Dresel & Falk, 1956*a*). These materials were prepared for chromatography by the following method: The HCl solutions in which the 'copro' and 'uro' fractions were determined spectrophotometrically, were evaporated to dryness *in vacuo*. The 'proto' fraction was transferred from the HCl solution to ether, the ether solution was washed with water and evaporated to dryness *in vacuo*. All samples were then esterified by standing for 20 hr. in HCl–methanol (cf. Rimington & Miles, 1951) or in 5% (v/v) H_2SO_4–methanol at 20°, except protoporphyrin samples, which were esterified at −10°. When uroporphyrin samples were esterified by standing in HCl–methanol for 20 hr. at −10°, some material remained on and near the base line when the product was chromatographed by the method of Falk & Benson (1953). When, however, the esterification was carried out for 20 hr. at 20° in either HCl–methanol or H_2SO_4–methanol, little or no material remained near the base line. The slow-running material was apparently un-esterified porphyrin; esterification of the molecule was