Studies on the Biosynthesis of Blood Pigments

2. HAEM AND PORPHYRIN FORMATION IN INTACT CHICKEN ERYTHROCYTES*

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In Part I of this series (Dresel & Falk, 1954), we reported studies of haem and porphyrin formation from glycine by haemolysed chicken erythrocytes. During the past three years, important additions have been made to knowledge of some of the earlier steps in porphyrin biosynthesis. It has been established (Shemin & Russell, 1953; Neuberger & Scott, 1953; Shemin, 1955) that δ-aminolaevulic acid (ALA), arising from glycine and a derivative of succinic acid, is a specific precursor of haem and porphyrins. Porphobilinogen (PBG), a substance excreted in the urine of patients with acute porphyria, has been isolated and crystallized (Westall, 1952), and its structure has been determined (Cookson & Rimington, 1954). PBG has also been shown to be a specific precursor of porphyrins (Falk, Dresel & Rimington, 1953; Bogorad & Granick, 1953). Falk *et al.* (1953) also demonstrated the biochemical formation of PBG from glycine. The conversion of ALA into PBG by a tissue system has been established (Dresel & Falk, 1953), and the enzyme catalysing this conversion, ALA-dehydrase, has been studied and considerably purified (Gibson, Neuberger & Scott, 1954; Gibson, unpublished observations; Schmid & Shemin, 1955; Granick, 1954; Schulman, 1955). In this paper, we report studies of porphyrin and haem formation in intact chicken erythrocytes. The substrates ALA and PBG were studied as well as glycine. Certain cell-permeability phenomena have been encountered. The intact cell system offered an opportunity to study the effect of certain factors on the utilization of iron for haem formation. Among such factors, lead has a particular interest in relation to the mechanism of lead poisoning and some experiments bearing on this problem are described.

EXPERIMENTAL

Materials

*cyclo*Hexanone (British Drug Houses, technical) was distilled under reduced pressure. Uroporphyrin I and 'Waldenström porphyrin' were the samples described by Dresel & Tooth (1954). We are grateful to Professor C. Rimington for supplies of PBG hydrochloride, derived either from human acute-porphyria urines or from rabbits with experimental porphyria (cf. Goldberg & Rimington, 1955) and purified by the method of Cookson & Rimington (1954). We thank Dr J. J. Scott for a generous gift of ALA hydrochloride. Serum albumin was 'Dried human albumin for transfusion' supplied by the Lister Institute of Preventive Medicine, London, S.W. 1. Purified human siderophilin was obtained from the Atomic Energy Establishment, Harwell, Berks. Glass-distilled water was used throughout.

Methods

Blood of normal adult domestic fowls was collected as previously described (Dresel & Falk, 1954), and was used for whole-blood experiments after addition of 1 mg. of heparin/25 ml. For 'washed cell' experiments, the cells were centrifuged and washed twice with 0·9% (w/v) NaCl, and the leucocyte layer was removed, unless otherwise stated. The erythrocytes were then resuspended in sufficient 0·9% (w/v) NaCl to restore the original blood volume; thus in the following experiments, '25 ml. of washed cell preparation' means the washed erythrocytes from 25 ml. of blood made to 25 ml. with 0·9% (w/v) NaCl. Heparin (1·5 mg.), penicillin (0·5 mg.) and streptomycin (0·5 mg.) were added to such preparations for incubation; haemolysis during incubation was rarely great and there was no evidence of bacterial contamination. Incubation was carried out in conical 100 ml. flasks, plugged with cotton wool and shaken 90–100 times/min. at 38°. Determinations of radioactivity were made as described by Dresel & Falk (1954). Unless otherwise stated, the S.D. of counting was less than ±5%. Spectrophotometric measurements were made with a Unicam SP. 500 spectrophotometer. Melting points were determined on a Gallenkamp electrically heated micro melting-point apparatus, and were uncorrected. Concentrations of HCl are expressed as percentage (w/v).

Initial extraction procedures. After incubation, the erythrocytes were spun down, the supernatant was removed and the cells were haemolysed completely with distilled water at about 2°. The haemolysed cells and the supernatant were recombined, unless it was desired to determine porphyrins in the cells and the medium separately. In this case the centrifuged cells were washed twice with 0·9% NaCl before haemolysis and the combined supernatant and washings were analysed separately from the haemolysed cells.

Such preparations were then treated with about 10 vol. of ethyl acetate–acetic acid mixture (3:1, by vol.), and set aside overnight. The precipitated protein was filtered off on a sintered-glass funnel and thoroughly washed with the same solvent mixture. The filtrate was used for the following analyses.

* Part 1: Dresel & Falk (1954).

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The extraction of copro- and proto-porphyrins followed essentially the procedure of Schwartz & Wikoff (1952). The filtrate was washed twice with saturated aqueous sodium acetate, the washings were back-extracted with ethyl acetate and the combined ethyl acetate layers were washed once with 3% sodium acetate. The combined aqueous extracts were reserved for the determination of uroporphyrin (see below). The copro- and proto-porphyrins were then extracted completely from the ethyl acetate with 15% HCl. On many occasions the ethyl acetate layer was then tested for the presence of 'porphyrinogens'; after shaking with a solution of I_2 (Schwartz, Zieve & Watson, 1951) no additional porphyrin was found. The ethyl acetate layer was set aside for isolation of the haemin which had remained in solution.

Fractionation and determination of copro- and proto-porphyrins. The porphyrins were transferred quantitatively from the 15% HCl extract to ether, the ether was washed twice with saturated, and once with 3% aqueous sodium acetate and the combined aqueous layers were extracted once with fresh ether. A second ether extraction showed no porphyrin fluorescence. The ether layers were combined and washed once with a little water; after sodium acetate washings as described, no porphyrin was lost in this water washing. The ether solution was extracted exhaustively, first with 0·36% HCl and then with 10% HCl. The porphyrins extracted by 0·36% HCl and by 10% HCl are referred to as coproporphyrin and protoporphyrin respectively, and were determined spectrophotometrically as such, though in each case some other minor components were also present (see below). When relatively large amounts of both porphyrins were present, the extraction with 0·36% HCl was continued until the Soret peak in the extracts had moved to 405–406 mμ., the position of maximum protoporphyrin absorption in this concentration of HCl. The whole of the porphyrin extracted by 0·36% HCl was then transferred to fresh ether and refractionated with 0·36% HCl and 10% HCl.

Optical densities were measured at the peak of the Soret band and, with coproporphyrin, at 380 and 430 mμ. for correction by the method of Rimington & Sveinsson (1950). For calculations the following extinction coefficients were used: $E^{1\%}_{1\,cm.}$ (coproporphyrin), 6667 (Rimington, unpublished); $E^{1\%}_{1\,cm.}$ (protoporphyrin), 4900 (Grinstein & Wintrobe, 1948).

Determination of uroporphyrin. This method is a modification of that described by Dresel & Tooth (1954). The pH of the sodium acetate washings (see above) was adjusted to 3·0–3·2, and the washings were extracted repeatedly with ethyl acetate until such extracts gave non-fluorescent aqueous layers on shaking with 2% HCl. The combined ethyl acetate layers were then extracted to completion with 2% HCl. Some brown material, with considerable absorption in the region of 400 mμ., accompanied the porphyrin in such 2% HCl extracts, and a spectrophotometric correction factor for this was established as follows. Readings were taken at 405 mμ. (the Soret peak of uroporphyrin in 2% HCl) and at 397 mμ. The average quotient d_{405}/d_{397} for the brown impurity (I) was 0·88, while for pure uroporphyrin (U) in 2% HCl d_{397}/d_{405} was 0·29. Thus

$$d_{405}\text{ found} = dU_{405} + 0{\cdot}88dI_{397};$$

$$d_{397}\text{ found} = 0{\cdot}29dU_{405} + dI_{397}.$$

Hence $$dU_{405} = (d_{405} - 0{\cdot}88d_{397})/0{\cdot}745.$$

From this corrected density value, uroporphyrin concentrations were calculated by using the extinction coefficient $E^{1\%}_{1\,cm.} = 6517$ (Rimington & Sveinsson, 1950).

*cyclo*Hexanone is a convenient solvent for the extraction of uroporphyrins (cf. Kennedy, 1954) and it has sometimes been used in place of ethyl acetate. The pH of the sodium acetate washings was adjusted to 1·5–1·8 with conc. HCl; passage of the porphyrin into *cyclo*hexanone from this aqueous layer was usually complete after two extractions with solvent. After the addition of an equal volume of ether to the *cyclo*hexanone, the uroporphyrin is readily extracted with 5% HCl. It was found that the same factor as that given above could be applied to correct for contamination with brown pigments. The final calculated value was multiplied by 1·05, since the extinction coefficient of uroporphyrin in 5% HCl is 95% of that in 2% HCl.

With the procedures described above, recoveries of 90–100% were obtained when 'Waldenström porphyrin' or uroporphyrin I, in amounts ranging from 15 to 100 μg., were added to ethyl acetate–acetic acid filtrates prepared as described. If, however, the porphyrin was added to the haemolysate before precipitation of the protein with the ethyl acetate–acetic acid mixture, recoveries were much lower because of considerable adsorption of uroporphyrin on the precipitated protein. Then the average recovery of uroporphyrin I was 44·5±10·7% (14 experiments, addition 14–207 μg.), and of 'Waldenström porphyrin' 43·2±11·1% (24 experiments, addition 8–205 μg.). The recovery was slightly lower at low levels of added uroporphyrin than at high levels: the average recovery in 10 experiments (4 experiments with uroporphyrin I and 6 experiments with Waldenström porphyrin), in which less than 30 μg. of porphyrin was added, was 32·7±9·2%, and the average recovery in all the remainder was 47·7±8·4%. In spite of considerable efforts no better method could be found. The experimental uroporphyrin values were therefore multiplied by 2 and all values recorded in this and the two following papers (Dresel & Falk, 1956; Falk, Dresel, Benson & Knight, 1956) were corrected in this manner. This is not an ideal method, but it was considered adequate for the present purpose, particularly since the uroporphyrin fraction contains a porphyrin of unknown constitution (see p. 78).

Determination of haem synthesis. After complete extraction of the free porphyrins, the ethyl acetate solution containing the porphyrin-free haem was evaporated to dryness *in vacuo*. The dry residue was dissolved quickly in the minimum volume (about 10 ml.) of 0·1N-NaOH, and to this solution about 10 vol. of 10% oxalic acid in methanol was added at once. The haem was then converted into crystalline protoporphyrin dimethyl ester by the method of Grinstein (1947) and the ester was converted into its crystalline copper complex for radioactivity determination (cf. Dresel & Falk, 1954). Occasionally the protoporphyrin ester failed to crystallize; on chromatography with benzene on a column of Al_2O_3 (grade V; Nicholas, 1951), the protoporphyrin ester was eluted before interfering materials and was then always crystallizable. The amount of haem synthesized was calculated from the radioactivity of the protoporphyrin ester copper complex prepared from haem on the basis of the following considerations and experiments.

Radioactivity of glycine in the system. After incubation for 20 hr. with 0·0556M added glycine, the radioactivity of the recovered glycine was 94 and 96% of that added initially to whole blood and washed cells respectively; the small

dilution was probably due to endogenous glycine (cf. Dresel & Falk, 1954). For the present calculations it was assumed that the radioactivity of the glycine throughout the incubation was the same as that found at the end.

Radioactivity of newly synthesized protoporphyrin. If eight α-C atoms of glycine are incorporated into each molecule of protoporphyrin synthesized (Muir & Neuberger, 1950; Wittenberg & Shemin, 1950), the activity of the newly synthesized protoporphyrin as the dimethyl ester (mol.wt. 590) should be $(8 \times 75)/590 \times$ activity of α-^{14}C-glycine used (mol.wt. 75), both materials being counted at infinite thickness (I.T.). It was found experimentally that the radioactivity of protoporphyrin dimethyl ester copper complex (at I.T.) is not the theoretical 90%, but 85% of the activity of the protoporphyrin ester from which it is made, probably owing to self-absorption. Thus the radioactivity of the copper complex derived from newly synthesized protoporphyrin should correspond to $85 \times 1{\cdot}02 = 86{\cdot}5\%$ of that of the glycine.

This was confirmed experimentally as follows. After incubation of 500 ml. of whole blood with radioactive glycine (conditions similar to those in Fig. 1), the free protoporphyrin was extracted as described above and converted into its crystalline dimethyl ester. This was recrystallized to constant m.p. (229°); paper chromatography (Nicholas & Rimington, 1949) revealed only a dicarboxylic porphyrin; the material corresponded spectrophotometrically to pure protoporphyrin (cf. Falk *et al.* 1956). After dilution, by weighing, with pure crystalline protoporphyrin ester the material was converted into the crystalline copper complex. The radioactivity of this, corrected for the dilution, was 86·3% of that of the glycine isolated at the end of the incubation. This supports the assumption that, under the conditions of our experiments, practically all the newly synthesized haem is derived from the glycine.

Calculation of the quantity of haem synthesized. The amount of haem synthesized is equivalent to $(X/Y) \times P$ μg. of protoporphyrin, where X is the radioactivity of the isolated haem, determined as the protoporphyrin ester copper complex, Y is the calculated radioactivity of newly synthesized haem (as the protoporphyrin ester copper complex), and P is the total haem content of the system, which was determined as pyridine haemochromogen essentially by the method of Rimington (1942). Figures are expressed as μg. of protoporphyrin, using the extinction coefficient $\epsilon_{mM} = 34{\cdot}7$ (Dr K. G. Paul, private communication). During these haemochromogen determinations considerable and rapid fading of the absorption was partially overcome by increasing the concentration both of pyridine and of NaOH. The possible error, however, was of the order of ±10%.

RESULTS

Haem and free porphyrin formation from glycine

Incubation with whole blood. The rates of synthesis of haem and of free porphyrin were linear for at least 24 hr. (Fig. 1). The free porphyrin recovered was protoporphyrin (for detailed identification see Falk *et al.* 1956) and no significant amounts of coproporphyrin or uroporphyrin were detectable. Fig. 2 shows that 0·056M glycine is near the concentration above which free porphyrin synthesis in the whole-blood preparation, incubated for 16·5 hr., is at its maximum and is independent of glycine concentration. With glycine as the only added substrate for porphyrin synthesis, the maximum rate of haem synthesis (Fig. 1) is therefore less than one-third of that of the total porphyrin synthesized, the remaining two-thirds accumulating in the form of free protoporphyrin. It would be of interest to study the relative amounts of haem and free protoporphyrin formed from low concentrations of added glycine, but such a study would require careful determinations of the dilutions of the radioactivity of added glycine by endogenous glycine, and was not performed. However, from the results

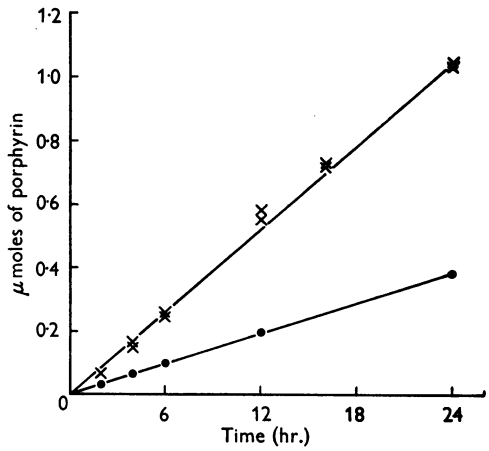

Fig. 1. Rate of synthesis of haem and free porphyrins from glycine in whole blood. Blood (25 ml.) was incubated in a final volume of 30 ml. with 0·056M glycine (10·7 μc). ●, Newly synthesized haem; ×, free protoporphyrin.

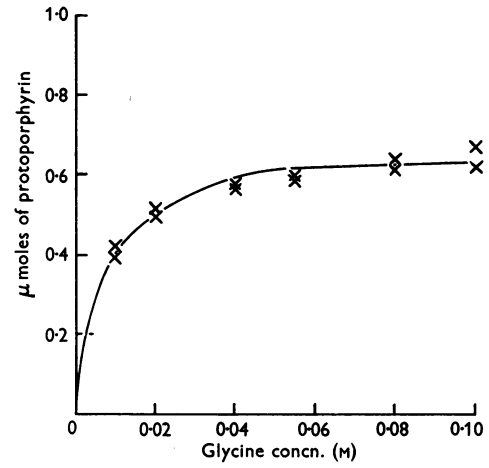

Fig. 2. Effect of glycine concentration on synthesis of free protoporphyrin in whole blood. Blood (25 ml.) was incubated in a final volume of 30 ml. for 16·5 hr.

obtained in the presence of lead (see p. 77), it might be inferred that, when the capacity for total porphyrin synthesis is limited, haem is formed in preference to free protoporphyrin.

Incubation with washed cells. It would appear from Fig. 3 that, although the initial rates of formation of free protoporphyrin may be similar in washed cells and in whole blood, the rate of synthesis falls off very much more rapidly with the former than with the latter. The results from which the curves are drawn were obtained with two different batches of blood, but the respective capacities for free protoporphyrin formation were not significantly different (cf. Fig. 3, 16 hr.). Some slight degree of haemolysis was noted in the washed-cell preparation, but the haemoglobin recovered from the saline medium after incubation was less than 1 % of the total. Haem synthesis was not determined, but the relationship of haem to free porphyrin synthesis in washed cells can be deduced from the results shown in Table 2. After incubation for 4 hr. the ratio of haem to free protoporphyrin was about 1:2·5, which is similar to that found with whole blood (cf. Fig. 1).

Distribution of free protoporphyrin between cells and medium. When whole blood was incubated with glycine, about two-thirds of the free protoporphyrin formed escaped into the serum, whereas with washed cells the free protoporphyrin was retained almost entirely inside the cells (Fig. 4). Serum proteins are known to have an affinity for protoporphyrin (Holden, 1937). From Fig. 5 it will be seen that a distribution of the free porphyrin between the cells and medium similar to that found in the whole-blood preparation could, in fact, be obtained in the washed-cell preparation by the addition of purified human serum albumin. Further, the addition of serum albumin during incubation also resulted in a significant increase in the total amount of free protoporphyrin formed. It would appear, therefore, that one of the factors limiting the formation of free porphyrin in washed cells is its excessive accumulation inside the cells. At the highest albumin concentration tested, the

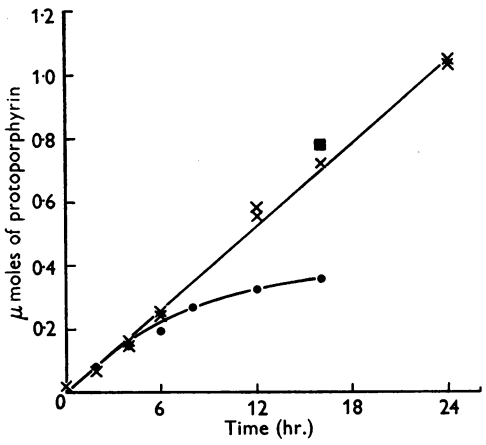

Fig. 3. Comparison of free protoporphyrin formation in whole blood and washed cells. Incubation as described in Fig. 1. ×, 25 ml. of whole blood, expt. no. 98; ■, 25 ml. of whole blood, expt. no. 104; ●, 25 ml. of washed-cell preparation, expt. no. 104.

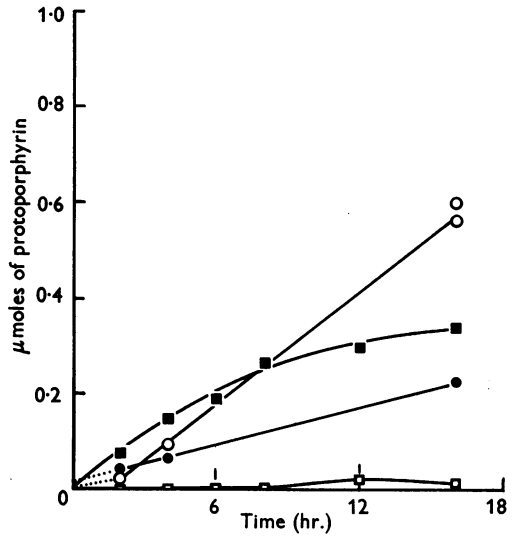

Fig. 4. Distribution of free protoporphyrin between cells and medium. Preparations (25 ml.) incubated in a final volume of 30 ml. with 0·056 M glycine. Free protoporphyrin in whole blood: ●, inside cells; ○, outside cells. Free protoporphyrin in washed-cell preparation: ■, inside cells; □, outside cells.

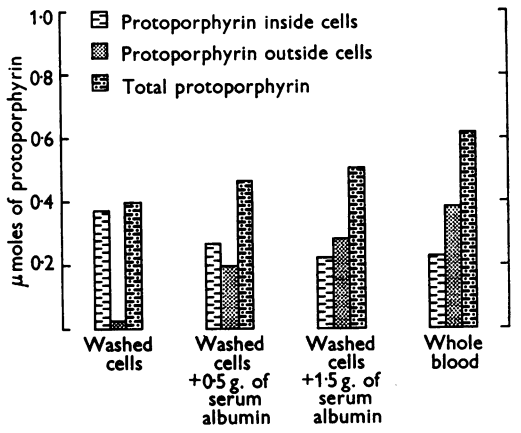

Fig. 5. Effect of serum albumin on distribution of free protoporphyrin between cells and medium. Preparations incubated for 15 hr. as described in Fig. 4.

amount of protoporphyrin recovered from inside the cells was of the same low order as when the plasma was present. Even so the total protoporphyrin in the whole-blood preparation was greater than in the albumin experiment. It seems thus that other factors may be present in the plasma, which also have some stimulating effect on porphyrin production.

Effect of anaerobiosis and 2:4-dinitrophenol (DNP). The effect of anaerobiosis on the formation of free porphyrins from glycine in whole blood is shown in Table 1. No increase in free protoporphyrin occurred; some coproporphyrin was formed, but the yield was only 6% of the protoporphyrin formed aerobically, a result not unlike that found in the haemolysed preparation with PBG as substrate (Falk *et al.* 1953). No significant amount of uroporphyrin was observed in the present experiments, in which precursors converted into porphyrin by iodine were not sought. DNP (10^{-3} M), tested under conditions similar to those described in Table 1, caused a 96% inhibition of free protoporphyrin synthesis. No coproporphyrin was formed (cf. similar inhibitions in washed cells and haemolysate preparation, Dresel & Falk, 1954).

Effect of iron. $FeSO_4$ was tested at concentrations of 2×10^{-4} and 2×10^{-5} M. 2×10^{-3} M-$FeSO_4$ caused complete agglutination of the cells. The lower concentrations of Fe^{2+} caused some increase in haem (Table 2), and this was accompanied by an approximately equivalent fall in the free protoporphyrin. The rather poor agreement in some cases can probably be attributed to experimental inaccuracies resulting from the very small differences studied. The effect of asparagine was tested, since, according to Yoshioka, Oguchi, Kuse & Seno (1950), this compound forms with ferrous ions a complex which is very effective in alleviating iron-deficiency symptoms *in vivo*. In our system, addition of asparagine did not appear to affect the efficiency of utilization of Fe^{2+} for haem synthesis.

The effect of siderophilin on the utilization of Fe^{2+} was studied in the second experiment illustrated in Table 2. The concentration of siderophilin chosen corresponds approximately to that quoted for human blood (Drabkin, 1951). Furthermore, it may be calculated from the figures given by Drabkin that 2×10^{-5} M-$FeSO_4$ should have been just sufficient to saturate the added siderophilin. This was further suggested by the fact that the intensity of the pink colour developed on adding the siderophilin was approximately the same with both Fe^{2+} concentrations. From the figures in Table 2 it will be seen that the stimulation, if significant, of haem synthesis by 2×10^{-5} M-$FeSO_4$ was unchanged by the addition of siderophilin.

With 2×10^{-4} M-$FeSO_4$, siderophilin may have reduced the stimulation due to iron, but the effect cannot be considered significant, particularly when it is remembered that there should have been a large excess of free Fe^{2+} present. The haem and

Table 1. *Effect of anaerobiosis on formation of free porphyrins*

Whole blood (25 ml.) was incubated for 16 hr. in a final volume of 30 ml. In the last experiment nitrogen was passed through the blood for about 30 min., when practically no oxyhaemoglobin (HbO_2) could be seen spectroscopically. The flask was then closed with a mercury seal; at the end of incubation no HbO_2 could be detected.

Gas phase	Addition	Porphyrins found (μmoles) Proto-porphyrin	Copro-porphyrin
Air	—	0·024	0·00
Air	Glycine (0·056 M)	0·727	<0·011
N_2	Glycine (0·056 M)	0·022	0·044

Table 2. *Effect of ferrous sulphate on the synthesis of haem and free porphyrin in washed cells*

Washed cells were incubated for 4 hr. with 0·056 M [α-^{14}C]glycine, containing 10·7 μc. Heparin, penicillin and streptomycin were not added. In Expts. 1 and 2 there were used 25 and 19 ml. of washed cells in final volumes of 30 and 23 ml. respectively. Asparagine was added at a concentration of 2×10^{-3} M, and siderophilin at a concentration of 1 mg./ml.

	Additions	Newly synthesized porphyrin (μmoles) as Haem	Protoporphyrin	Δ haem (μmoles)	Δ protoporphyrin (μmoles)
1	None	0·054	0·150	—	—
	2×10^{-5} M-$FeSO_4$	0·066	0·141	+0·012	−0·009
	2×10^{-4} M-$FeSO_4$	0·080	0·118	+0·026	−0·032
	Asparagine	0·052	0·146	−0·002	−0·004
	Asparagine plus 2×10^{-5} M-$FeSO_4$	0·061	0·135	+0·007	−0·015
	Asparagine plus 2×10^{-4} M-$FeSO_4$	0·087	0·131	+0·033	−0·019
2	None	0·063	0·116	—	—
	2×10^{-5} M-$FeSO_4$	0·070	0·112	+0·007	−0·004
	2×10^{-4} M-$FeSO_4$	0·096	0·108	+0·033	−0·008
	Siderophilin	0·066	0·123	+0·003	+0·007
	Siderophilin plus 2×10^{-5} M-$FeSO_4$	0·071	0·120	+0·008	+0·004
	Siderophilin plus 2×10^{-4} M-$FeSO_4$	0·084	0·119	+0·018	+0·003

free-protoporphyrin figures in this experiment refer only to what was recovered from the cells after incubation. This separate analysis was done in order to avoid the inclusion of any haem formed as an artifact outside the cells, should siderophilin, like serum albumin, have promoted the diffusion of free protoporphyrin out of the cells. Since the free protoporphyrin recovered from the cells was, if anything, slightly higher in the flasks incubated with siderophilin, this seems unlikely. The amounts of free porphyrin found outside the cells were insignificant and were not determined quantitatively. A determination of the radioactivity of the haem present in the medium was not considered worthwhile, because of the experimental difficulties involved.

Inhibition by lead. The parallel formation of large amounts of haem and free protoporphyrin in bird red cells offered an opportunity for a study of the effect of lead on the incorporation of iron into haem. It was found that the synthesis of free protoporphyrin is inhibited at lower concentrations of lead than is the synthesis of haem (Fig. 6). Thus, with 10^{-5} M lead acetate, where protoporphyrin synthesis was inhibited by 50%, haem synthesis was reduced by only about 9%, and with 3×10^{-5} M lead acetate, when free protoporphyrin accumulation was almost completely suppressed, haem formation was inhibited by only about 64%. It seems clear, therefore, that in this system lead inhibits some step in the synthesis of a precursor of haem, rather than the incorporation of iron into the porphyrin nucleus. It may also be concluded that haem is formed in preference to free protoporphyrin when the synthesis of porphyrin is limited.

Formation of haem and free porphyrin from ALA and PBG. As was found with haemolysate preparations (cf. Dresel & Falk, 1956), ALA is readily converted by whole-blood and washed-cell preparations into protoporphyrin and traces of uroporphyrin and coproporphyrins (Table 3). The yields of these two porphyrins would probably have been greater if higher concentrations of ALA had been tested. PBG, on the other hand, gave rise only to traces of protoporphyrin in these preparations, the major porphyrin formed then being uroporphyrin. It seems probable that this porphyrin is formed non-enzymically outside the cells (see Discussion). The behaviour of PBG here differs markedly from its behaviour in haemolysate preparations, where proto-, copro- and uroporphyrins are freely formed from PBG, and the yields are identical with those obtained from equivalent amounts of ALA (Dresel & Falk, 1956). It seems, then, that PBG does not penetrate the chicken red cell to any significant extent under these conditions.

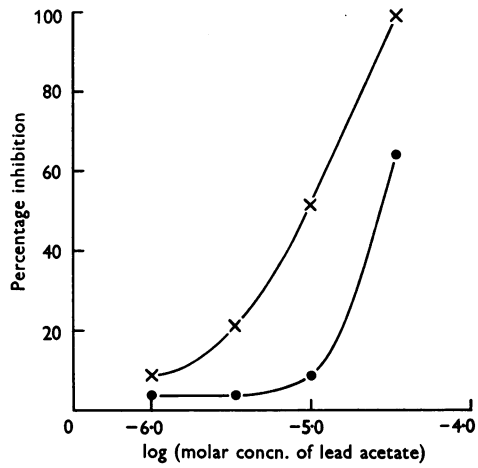

Fig. 6. Effect of lead acetate on synthesis of haem and protoporphyrin from glycine. Incubation for 16 hr. as described in Fig. 1. ●, Inhibition of haem synthesis; ×, free protoporphyrin synthesis.

Table 3. *Conversion of ALA and PBG into free porphyrins*

15 ml. preparation made up to 20 ml. with saline and incubated for 16 hr. All figures are expressed as 'μmoles porphyrin equivalents' (cf. Dresel & Falk, 1956).

Expt. no.	Preparation	Addition	Porphyrins formed: Proto-	Copro-	Uro-	Constituents of 'uroporphyrin fraction'*
140	Whole blood	ALA, 3·40	0·219	0·002	0·005	—
	Washed-cell preparation	ALA, 3·40	0·249	0·002	0·007	—
210	Whole blood	—	0·018	0·000	0·000	—
	Whole blood	PBG, 10·25	0·034	0·001	1·35	I⩾III
136	Washed-cell preparation	ALA, 0·083	0·059	0·001	0·011	III = *pseudo*Uroporphyrin
	Washed-cell preparation	ALA, 2·49	0·144	0·001	0·015	III = *pseudo*Uroporphyrin
	Washed-cell preparation	PBG, 1·87	0·007	0·000	0·145	I⩾III > *pseudo*Uroporphyrin
	Washed-cell preparation	PBG, 9·33	0·012	0·000	0·855	I⩾III > *pseudo*Uroporphyrin
138	Washed-cell preparation	PBG, 2·08	Trace	0·000	0·169	I⩾III > *pseudo*Uroporphyrin
	Second washings of cells	PBG, 2·08	0·000	0·000	0·143	III > I
	Second washings of cells, boiled	PBG, 2·08	0·000	0·000	0·248	III > I

* This analysis was carried out by the 'dioxan paper chromatography' method described by Falk *et al.* (1956). >, Greater than; ⩾, much greater than; ⫸, very much greater than.

Nature of the porphyrins formed. The detailed results of our studies of the nature of the porphyrins formed in these experiments are given in a later paper of this series (Falk *et al.* 1956). In brief, the results show that in the 'protoporphyrin' fractions described above, most of the material is, in fact, protoporphyrin.

The 'uroporphyrin' fraction from ALA with whole-cell preparations (Table 3), like that from ALA or PBG with the haemolysate (cf. Dresel & Falk, 1956), consists of uroporphyrin III and *pseudo*uroporphyrin (cf. Falk *et al.* 1956) in about equal amounts. The possibility that material referred to as uroporphyrin III may contain uroporphyrin IV cannot at present be excluded (cf. Falk *et al.* 1956).

It is well known that PBG can be condensed to uroporphyrins by purely chemical means. In aqueous acid or alkali, mixtures of the uroporphyrin isomers are readily obtained (cf. Cookson & Rimington, 1954), but no *pseudo*uroporphyrin is formed. Similarly, on incubation of PBG with the second washings of the cells (corresponding approximately to the incubation medium), or with these washings boiled, or with the intact washed cells suspended in saline, mixtures of uroporphyrin isomers are formed (cf. Table 3). But only where PBG was incubated with the intact cells was there a trace of *pseudo*uroporphyrin formed. In this case there was also a trace of protoporphyrin, and this and the trace of *pseudo*uroporphyrin were probably due to enzymic conversion of a small amount of PBG which penetrated the cells. Both porphyrins are formed freely from PBG when the cell-wall barrier is removed by haemolysis.

DISCUSSION

Determination of haem synthesis. The use of the isotope-tracer technique for the study of haem synthesis necessitates the complete separation of protoporphyrin from haemin. Contamination of the haemin with as little as 0·1 % of protoporphyrin could make considerable difference to its radioactivity, since most of the protoporphyrin is newly synthesized and highly labelled, while most of the haemin is derived from preformed haemoglobin. In our experiments the newly synthesized, labelled haem is only about 0·1 % of the total haem present. About 10 % of the protoporphyrin present in whole chicken blood after incubation with glycine may be recovered from crystalline haemin prepared according to Fischer (1941). Thorough washing of the crystals failed to remove the porphyrin; removal was effected only by extraction with HCl of a solution of the crystals in ethyl acetate. Protoporphyrin ester prepared from blood by the method of Grinstein (1947) may also include some of the free protoporphyrin originally present; only 80–90 % of this was recovered in the acetone supernatant after precipitation of the proteins from incubated blood. A complete separation of free porphyrin from haemin is effected during the determination of free porphyrins as described under Methods. The porphyrin-free haemin which remains in the ethyl acetate solution after this extraction was used in our studies.

Synthesis of haem and porphyrins. The capacity of normal adult chicken blood for the synthesis of protoporphyrin and haem *in vitro* is remarkable. If 70 ml. is taken as the approximate total blood volume of the fowl (A. Gajdos, private communication), 30 days as the average life span of the chicken erythrocyte (Hevesy, 1948), and a haemoglobin concentration as 10 g./100 ml. of blood (E. I. B. Dresel, unpublished results), it can be calculated that the fowl synthesizes every day about 8 mg. of protoporphyrin in the form of haemoglobin. Under our best conditions, 70 ml. of whole chicken blood synthesized, from glycine, 2·2 mg. of protoporphyrin and haem in 24 hr. This indicates considerable retention by the peripheral blood cells of their capacity for synthesis, most of which appears normally to occur in the marrow and the spleen (Jordan, 1942). The synthesis in the peripheral blood may be thought to be due solely to those immature cells which are present, but this would not be consistent with the constant rate of synthesis observed over a period of 24 hr. (Fig. 1), because there is considerable maturation of immature chicken cells under similar incubation conditions (Wright & Alstyne, 1931).

This intact-cell preparation, in which both haem and free protoporphyrin are formed freely, offered a suitable system for studies of factors influencing haem formation. The addition of ferrous iron causes some increase of haem with a corresponding decrease of protoporphyrin. We found no effect with either siderophilin, the normal iron-transporting protein of the serum, or asparagine, which according to Yoshioka *et al.* (1950) stimulates the utilization of iron for erythropoiesis on parenteral injection. The similarity of the uptake by duck erythrocytes of ferrous and of ferric iron has been discussed by Sharpe, Krishnan & Klein (1952); in our experiments only ferrous iron was added. The question whether the increase in haem which we find on the addition of ferrous sulphate is enzymic or not is discussed in detail in the next paper (Dresel & Falk, 1956).

The finding that serum proteins promote the diffusion of free protoporphyrin from the erythrocyte might suggest that some free protoporphyrin should be present in normal plasma. The normal concentrations of free erythrocyte protoporphyrin are relatively low, both in the mammal and in the

bird (Schwartz & Wikoff, 1952), perhaps because of diffusion of the porphyrin into the plasma, and its rapid excretion by the liver (Falk, 1954).

A comparison of the respective activities for haem and protoporphyrin synthesis in whole blood and the haemolysate preparation (cf. Dresel & Falk, 1954) indicates that, after 2 hr. with optimum glycine concentration, when maximum rates of formation in both preparations are still found, in the haemolysate haem formation is 75%, and free protoporphyrin formation is about 20%, of that observed in whole blood. In the haemolysate yeast juice may cause stimulation of haem synthesis up to sixfold, (Dresel & Falk, 1954), so that the retention of activity after haemolysing is remarkable. The synthetic capacity of the haemolysate preparation, however, falls off very rapidly, whereas that of whole blood is stable for at least 24 hr.

SUMMARY

1. Methods are described for the quantitative determination of the haem, protoporphyrin, coproporphyrin and uroporphyrin formed in chicken-blood preparations.

2. In whole blood, the rate of synthesis of porphyrins from glycine was constant for at least 24 hr. at 38°. Only one-third of the total porphyrin synthesized was present as haem, the remainder being recovered as protoporphyrin.

3. In washed cells, haem and protoporphyrin were again formed from added glycine, but the activity of the tissue fell off more rapidly.

4. The distribution of the free protoporphyrin between the cells and medium was found to depend on the presence or absence of protein in the external medium. In washed cells almost all the protoporphyrin was retained inside the cells, whereas in whole blood, or in a washed-cell preparation with added serum albumin, about two-thirds of the protoporphyrin was found in the external medium.

5. Anaerobic incubation resulted in the formation of only small amounts of coproporphyrin and of no protoporphyrin from glycine. 2:4-Dinitrophenol (10^{-3}M) completely inhibited protoporphyrin synthesis from glycine in whole blood.

6. Added ferrous sulphate caused some increase in haem, which was accompanied by an approximately equivalent fall in free protoporphyrin. Neither asparagine nor siderophilin had any significant effect on the utilization of iron for haem synthesis.

7. Lead was found to inhibit porphyrin formation from glycine, but did not apparently interfere specifically with the incorporation of iron into porphyrin.

8. δ-Aminolaevulic acid was utilized by whole blood and washed-cell preparations for porphyrin synthesis. Porphobilinogen, on the other hand, does not appear to penetrate the red cell to any significant extent.

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