Conversion of 5-aminolaevulinate into haem by liver homogenates

Comparison of rat and chick embryo

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1. We have studied the kinetics of the conversion of 5-aminolaevulinate into haem and haem precursors in homogenates of livers of rats and chick embryos. Homogenates of fresh liver from both species efficiently convert 5-aminolaevulinate into haem. After frozen storage for 1 year, homogenates of rat, but not chick, liver have decreased rates of formation of haem with accumulation of more protoporphyrin. The rate of haem formation after storage is restored by addition of Fe^{2+} and menadione. 2. At all initial concentrations of 5-aminolaevulinate tested ($2\mu\text{M}$ – 1mM), homogenates of rat liver accumulate less protoporphyrin than haem. In contrast, homogenates of chick embryo liver accumulate more protoporphyrin than haem at concentrations of 5-aminolaevulinate greater than $10\mu\text{M}$. Conversion of protoporphyrin into haem by homogenates of fresh or frozen chick embryo liver is not increased by addition of Fe^{2+} . 3. Homogenates of liver from both species accumulate porphobilinogen; the kinetic parameters for this process reflect those of 5-aminolaevulinate dehydratase. 4. The results show that the rate-limiting enzyme for the hepatic conversion of 5-aminolaevulinate into protoporphyrin is porphobilinogen deaminase. In addition, chick liver, compared with rat liver, has only about one-fifth the activity of ferrochelatase, the final enzyme of the haem biosynthetic pathway, which inserts Fe^{2+} into protoporphyrin to form haem. 5. Comparison of these results with previous studies indicates that the homogenate system described here provides physiologically and clinically relevant information for study of hepatic haem synthesis and its control.

Previous studies of the conversion of 5-aminolaevulinate into haem were carried out with haemolysates of avian erythrocytes (Shemin *et al.*, 1950; Falk *et al.*, 1953; Dresel & Falk, 1954). More recently Sinclair *et al.* (1979) reported that homogenates of rat liver efficiently convert 5-aminolaevulinate into haem. We decided to characterize this system more completely since it appeared useful for studying effects on hepatic haem synthesis of compounds thought to inhibit different steps of the pathway. Many studies of inhibition of hepatic haem synthesis have been carried out on individual enzymes of the pathway. The physiological relevance of such studies would be clarified if effects of possible inhibitors on the activity of the entire pathway could be determined simply and reproducibly.

In the present paper we investigated the kinetics of the conversion of 5-aminolaevulinate into haem and haem precursors by homogenates of livers of adult

rats and embryonic chickens. We compared livers from these species because previous studies suggested they were inherently different in their capabilities for haem synthesis (De Matteis, 1973; Marks, 1978). It has been suggested that these differences account for the greater sensitivity of the chick liver system for induction of 5-aminolaevulinate synthase by drugs (De Matteis, 1973; Marks, 1978). We found that homogenates of chick liver, like rat, efficiently convert 5-aminolaevulinate into haem. With both homogenates, as the initial concentration of 5-aminolaevulinate increased, the conversion of porphobilinogen into uroporphyrinogen becomes rate limiting for the formation of protoporphyrin from 5-aminolaevulinate. At high concentrations of 5-aminolaevulinate, the rate of haem formation by homogenates of chick, but not rat, liver is limited by the activity of ferrochelatase. Addition of Fe^{2+} did not inhibit formation of protoporphyrin or haem when iron-catalysed lipid

peroxidation was prevented. The present study characterizes a system that will be useful for examining the effects of various chemicals, including iron, lead and halogenated aromatic compounds, on haem synthesis from 5-aminolaevulinate. Part of this work has appeared in abstract form (Healey *et al.*, 1981).

Materials and methods

Animals

Male Sprague-Dawley rats (180–210g) were housed as previously described (Bonkowsky *et al.*, 1979a) and starved 24 h before killing by decapitation. The livers of three rats were quickly removed, pooled and homogenized (20%, w/v) in ice-cold 0.25 M-sucrose/0.02 M-Tris/HCl, pH 7.40. Livers were homogenized for 15–20 s with a Polytron homogenizer (Brinkmann) at low speed. Homogenates of chick embryo liver were made from 18–19-day-old White Leghorn embryos in the same manner, except that livers from 10 to 20 embryos were used for each pool.

Incubation procedures

Incubations were carried out in the dark at 37°C in open 15 mm × 150 mm test tubes with continuous shaking (120 oscillations/min). Unless otherwise stated, all incubations contained a final homogenate concentration of 7.7% (w/v) in a volume of 0.65 ml. Sucrose/Tris buffer was added as needed to produce this final dilution. Incubations were started by placing the tubes in a shaking water bath and ended by placing the tubes in an ice/water bath.

For each homogenate studied, incubations were run in duplicate. Values of the duplicates agreed within ± 2% of the mean value.

Each incubation was performed on at least two different occasions with different homogenate pools. Results obtained from the different pools varied by less than 10% of the mean value. For homogenates of three rat livers run separately for 30 min, the coefficient of variation of radioactivity in haem was 7% and in protoporphyrin was 2.6%. Results of studies in which unlabelled products were measured had similar small variances.

Isolation and analysis of products

Unlabelled products. For determination of porphobilinogen, 0.25 ml of incubated homogenates was added to 0.05 ml of 2.5 M-HClO₄. This mixture was vortex-mixed vigorously and centrifuged (1100g for 10 min). Of the clear supernatant 0.2 ml was removed and mixed with an equal volume of modified Ehrlich's reagent (Urata & Granick, 1963).

After 15 min at room temperature, the sample was scanned from 500 to 650 nm with an Aminco DW2 spectrophotometer. A portion of homogenate kept on ice and treated identically was used for the reference blank. Porphobilinogen concentration was determined by using a $\Delta \epsilon(555-650 \text{ nm})$ of 61 000.

For total porphyrin determination, 0.2 ml of sample was mixed vigorously with 1.0 ml of 1 M-HClO₄/methanol (1:1, v/v). The mixture was centrifuged (1100g, 10 min) and porphyrins were determined in the supernatant by the method of Granick *et al.* (1975). A Perkin-Elmer 512 spectrofluorimeter equipped with a Hamamatsu R-928 photomultiplier tube was used for porphyrin determinations. In some experiments, chloranil (Eastman-Kodak, Rochester, NY, U.S.A.) was dissolved in the HClO₄/methanol (0.1 or 0.2 mM) to ensure complete oxidation of porphyrinogens to porphyrins (Schwartz *et al.*, 1976). Chloranil had no effect on the type or quantity of porphyrin detected.

Haem was determined by the pyridine haemochromogen method of Falk (1964).

Labelled products. Haem and protoporphyrin were isolated by a modification of the method of Sinclair *et al.* (1979). Before extraction, to each 0.65 ml sample were added 0.1 ml of human erythrocyte lysate (65 nmol of haem) and 0.05 ml of carrier protoporphyrin IX (8 nmol). All extractions were carried out under subdued lighting conditions. Location of protoporphyrin was followed by fluorescence using a Wood's lamp.

Haem and porphyrins were extracted with 3.5 ml of freshly prepared acetone/conc. HCl (20:1, v/v), which was added while vortex-mixing vigorously. After centrifugation (1100g, 10 min) the supernatant was decanted into a 25 mm × 200 mm test tube. To the supernatant, 9.0 ml of diethyl ether (anaesthesia grade) and 4.0 ml of 2 M-HCl was added. After vigorous mixing, the diethyl ether layer containing the haemin was removed to a scintillation vial. The aqueous layer, containing the porphyrins, was saved for protoporphyrin isolation. The diethyl ether was washed again with 4.0 ml of 2 M-HCl to remove any remaining porphyrin, and the diethyl ether was evaporated to dryness at 40°C under a stream of air. Radioactivity of haem was determined after dissolving the haem in 10 ml of scintillation fluid. Recovery of haem (from haemolysate + homogenate) by this procedure was $74 \pm 1\%$ (mean ± S.D., $n = 4$). Unless otherwise indicated, radioactivity in haem was not corrected for recovery.

Labelled protoporphyrin was isolated as follows. To the aqueous layer containing porphyrins, 9.0 ml of diethyl ether and 8.0 ml of 1.5 M-sodium acetate were added. Solid NaHCO₃ was added in small quantities with intermittent mixing, until all the protoporphyrin moved into the diethyl ether layer

(pH 4.5–5.0). The diethyl ether layer was removed, and an additional 3.0 ml portion of diethyl ether and more NaHCO_3 were added to the residual aqueous fraction to ensure complete extraction of protoporphyrin. The diethyl ether layers were combined in a scintillation vial, washed twice with 7.0 ml of 0.1 M- NaHCO_3 , and once with 10.0 ml of 0.12 M-HCl to remove coprotoporphyrin. The diethyl ether was evaporated to dryness and the radioactivity was determined after the residue was dissolved in 10 ml of scintillation fluid. Recovery of protoporphyrin was $68\% \pm 7\%$ (mean $\pm$ S.D., $n = 3$). As with haem, radioactivity in protoporphyrin was not corrected for recovery, unless otherwise indicated.

Determinations of radioactivity were carried out in a Searle Mark III scintillation counter with external standardization. Counting efficiencies for haem ranged from 70 to 80% and, for protoporphyrin, from 87 to 93%.

Special chemicals

5-Amino[4- ^{14}C]laevulinic acid hydrochloride (51.5 Ci/mol in 0.1 M-HCl) was obtained from New England Nuclear (Boston, MA, U.S.A.). Crystalline 5-aminolaevulinic acid hydrochloride was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Unlabelled solutions of 5-aminolaevulinic acid were prepared in 0.01 M-HCl and precise concentrations were determined spectrophotometrically by the method of Mauzerall & Granick (1956). All solutions of 5-aminolaevulinic acid were stored at 4°C. Immediately before addition to homogenate, 1 vol. of radioactive 5-aminolaevulinic acid was diluted with 19 vol. of 0.15 M-NaCl and unlabelled 5-aminolaevulinic acid was added to achieve the final desired specific radioactivity. 5-Aminolaevulinic acid was always added to tubes for incubations in a volume of 50 μl .

Protoporphyrin IX (crystalline) was obtained from Porphyrin Products (Logan, UT, U.S.A.). Carrier protoporphyrin was prepared by dissolving a few crystals in 1.0 ml of 0.1 M-NaOH and diluting to 15 ml with 0.1 M- NaHCO_3 .

Unconjugated bilirubin was obtained from Pfanstiehl Chemicals (Waukegan, IL, U.S.A.). A carrier solution was freshly prepared (1 mg/ml in 0.1 M- Na_2CO_3) and kept on ice in the dark. A volume of 25 μl was added to samples. Spectra of unconjugated bilirubin in ethanol/sucrose in Tris buffer (120:13, v/v) had an absorbance maximum at 455 nm ($\epsilon = 60000$).

Menadione (Sigma Chemical Company, St. Louis, MO, U.S.A.) was prepared fresh in sucrose/Tris buffer, pH 7.4, and added to homogenates in a volume of 25 μl .

FeSO_4 (ACS grade; Fisher Scientific Company, Medford, MA, U.S.A.) was dissolved in 1 mM-HCl and added to homogenates in a volume of 25 μl .

Results

Kinetics of protoporphyrin and haem synthesis from labelled 5-amino[4- ^{14}C]laevulinate by liver homogenates

The time course of the incorporation of 5-aminolaevulinate into protoporphyrin and haem by homogenates is shown in Fig. 1. The rate of haem synthesis by homogenates of rat liver (Fig. 1a) was linear for 30 min, after which the rate declined. As the rate of accumulation of label in haem decreased, the rate of accumulation of label in protoporphyrin increased in a reciprocal manner. The total amount of protoporphyrin synthesized, as estimated by the sum of the haem and protoporphyrin radioactivity [$\Sigma(\text{haem} + \text{protoporphyrin})$], was linear to 90 min.

Since a decrease in the rate of accumulation of radioactivity in the isolated haem fraction at the later times may have resulted from haem breakdown, the stability of labelled haem was established. This was done in separate experiments in which the concentration of homogenate was 15% (w/v) rather than 7.7% (w/v). The concentration was doubled to enhance incorporation of 5-aminolaevulinate into haem and to increase the likelihood that breakdown of haem would be detected. It was found that when haem was pre-labelled by prior incubation of homogenates (30 min) with 3 μM -5-aminolaevulinate, 90% of the label was incorporated into haem. No radioactivity was lost from haem during a further 60 min of incubation. Thus, for at least 90 min of incubation, labelled haem is stable. We therefore conclude that the decreased rate of accumulation of radioactivity in haem at the later times (Fig. 1a) was not a result of haem breakdown, but a result of decreasing conversion of protoporphyrin into haem. This conclusion is supported by experiments in which Fe^{2+} was added (see below).

We also determined whether the isolated haem fraction was contaminated with bile pigment. Unconjugated bilirubin, if formed, would be extracted with haem into diethyl ether. Biliverdin or conjugated bilirubin, the other products of physiological breakdown of haem, are not extracted with haem into diethyl ether but remain in the aqueous phase. In some experiments, unconjugated bilirubin (40 nmol) was added to homogenates of rat liver that had been incubated for 30 min with 5-amino[^{14}C]laevulinate. These samples were extracted twice with 3.0 ml of ethanol before treatment with acetone/HCl. Extraction with ethanol removed more than 98% of the carrier bilirubin but did not affect the radioactivity in the final haem fraction. This result indicates that the haem fraction does not contain labelled bile pigment.

In contrast with the above findings with homogenates of rat liver, the rate of haem synthesis by

Fig. 1. Time course of formation of radioactive protoporphyrin and haem from 5-amino[4- ^{14}C]laevulinate by homogenates of (a) adult rat liver and (b) embryonic-chick liver

Homogenates were prepared as described in the Materials and methods section. Incubation mixtures contained 0.26 μCi of 5-amino[4- ^{14}C]laevulinate diluted with unlabelled 5-aminolaevulinate to a final concentration of 24 μM . Protoporphyrin (Δ) and haem ($\circ$) were extracted after incubation and the radioactivity in each fraction was determined as described in the Materials and methods section. The sum of the radioactivity in the two fractions $\Sigma(\text{haem} + \text{protoporphyrin})$ ($\square$) represents the total amount of protoporphyrin synthesized at each time point. Each point represents the mean of two separate incubations of the same homogenate. The duplicate values fall within the area of the symbols.

homogenates of chick embryo liver was linear for 90 min (Fig. 1b). The rate of haem synthesis, however, was lower, at 30 min being only 34% that

Fig. 2. Effects of increasing concentrations of 5-aminolaevulinate on the accumulation of protoporphyrin and haem by homogenates of (a) adult rat liver and (b) embryonic-chick liver

Homogenate of rat liver was incubated for 15 min and homogenate of chick embryo liver for 30 min, under conditions as in Fig. 1. Symbols are as in Fig. 1. For the first three points, undiluted 5-amino[4- ^{14}C]laevulinate was used. For the other points, unlabelled 5-aminolaevulinate was added to 0.26 μCi of 5-amino[^{14}C]laevulinate and results were corrected for dilution of the radioactivity.

of homogenates of rat liver. Production of $\Sigma(\text{haem} + \text{protoporphyrin})$ was also linear for 90 min and, in contrast with Fig. 1(a), the rate of protoporphyrin formation exceeded the rate of conversion of protoporphyrin into haem. In homogenate of chick embryo liver, protoporphyrin was being made faster than it could be converted into haem.

Similar results were obtained when homogenates were incubated with increasing initial concentrations of 5-aminolaevulinate (Fig. 2). In this

experiment, incubation times were chosen so that comparable amounts of $\Sigma(\text{haem} + \text{protoporphyrin})$ would be produced in both chick and rat systems. In homogenates of both rat and chick liver, the rates of haem synthesis were saturable with respect to 5-aminolaevulinate. Relative to haem, little protoporphyrin accumulated in the rat homogenate (Fig. 2a). This was also true for homogenate of chick liver incubated with low concentrations of 5-aminolaevulinate ($<2\mu\text{M}$), but at concentrations in excess of $10\mu\text{M}$, the rate of synthesis of protoporphyrin exceeded the rate of conversion of protoporphyrin into haem. These data suggest that the activity of either protoporphyrinogen oxidase or ferrochelatase in chick is less than that in rat. Since activity of hepatic protoporphyrinogen oxidase in chick embryos is similar to that in rats (N. Jacobs & J. Jacobs, personal communication), we conclude that accumulation of protoporphyrin in homogenate of chick liver is due to limiting activity of ferrochelatase. Furthermore, since the rates of $\Sigma(\text{haem} + \text{protoporphyrin})$ and haem synthesis (Fig. 2) are saturable functions of the initial concentration of 5-aminolaevulinate, giving typical hyperbolic plots, one can determine the maximum rate of formation of $\Sigma(\text{haem} + \text{protoporphyrin})$ and haem from 5-aminolaevulinate by linear transformation of these data ($v/[S]$ versus v). One can also determine the initial concentration of 5-aminolaevulinate that produces half-maximal rates of synthesis of these products. Values of these kinetic parameters appear in Table 1.

The effect of storage (at -60°C) of rat liver homogenate on the time course of conversion of 5-aminolaevulinate into haem can be evaluated by comparing Fig. 1(a) with Fig. 3. Even after a year of storage, the initial rate (0–30 min) of protoporphyrin and haem synthesis remained the same as for fresh samples. After 30 min the rate of haem synthesis decreased but was restored by addition of FeSO_4 (see below). In contrast with this effect of storage on homogenates of rat liver, similar storage had no effect on rates of protoporphyrin and haem accumulation by homogenates of chick embryo livers (results not shown).

Effects of added FeSO_4 on haem synthesis

Figs. 1 and 3 showed that the rate of formation of haem from protoporphyrin by homogenates of rat liver decreased during incubation. Since Fe^{2+} is required for haem formation from protoporphyrin, we tested whether the conversion of protoporphyrin into haem could be restored by the addition of FeSO_4 . The results are shown in Fig. 4. In these experiments, menadione (0.5 mM) was also added to prevent iron-catalysed lipid peroxidation (Levin *et al.*, 1973). Comparison with the appropriate controls (Figs. 1a and 3) reveals that accumulation of

Fig. 3. Effect of long-term storage of rat liver homogenate on the incorporation of 5-amino ^{14}C laevulinate into protoporphyrin and haem

Frozen (-60°C , 1 year) samples of the same homogenate as used in Fig. 1(a) were used. Homogenate was stored at a concentration of 20% (w/v) and diluted to 10% (w/v) just before use. Symbols and procedure are as in Fig. 1.

Table 1. Kinetic parameters of protoporphyrin and haem formation from 5-amino[4- ^{14}C]laevulinate (ALA) by liver homogenates

Values of the parameters were derived from Eadie–Hofstee plots of the data from Fig. 2.

Source of homogenate		Initial concentration of ALA producing half-maximal rates of formation (μM)	Maximal rate of formation (nmol of ALA equiv./g of liver per h)*
Rat	$\Sigma(\text{haem} + \text{protoporphyrin})$	5.8	151
	Haem formation	5.1	129
Chick	$\Sigma(\text{haem} + \text{protoporphyrin})$	6.9	69
	Haem formation	1.9	26

* 1 nmol of porphyrin or haem is equivalent to 8 nmol of ALA.

Fig. 4. Effect of ferrous sulphate and menadione on protoporphyrin and haem synthesis from 5-amino[4-¹⁴C]-laevulinate

The time course of incorporation of 5-amino[4-¹⁴C]-laevulinate into protoporphyrin (Δ) and haem ($\circ$) was repeated as in Fig. 1(a) and Fig. 3, but after addition of FeSO_4 ($50\ \mu\text{M}$) and menadione ($0.5\ \text{mM}$). The data in part (a) were obtained with homogenate of fresh rat liver and those in part (b) with homogenate of the same liver stored for 1 year at -60°C . The homogenate used was from the same livers as those of Figs. 1(a) and 3. Symbols are as in Fig. 1.

radioactivity in haem is increased in the presence of FeSO_4 and menadione at the expense of radioactivity that previously accumulated as protoporphyrin. Addition of menadione without FeSO_4

Fig. 5. Time course of the accumulation of porphobilinogen and total porphyrin from 5-aminolaevulinate by homogenate of (a) rat liver and (b) embryonic-chick liver

Homogenates (20%, w/v) were frozen at -60°C several weeks before use. After incubation of homogenates with 5-aminolaevulinate ($1\ \text{mM}$), porphobilinogen ($\bullet$) and total porphyrin ($\blacktriangle$) determinations were carried out as described in the Materials and methods section. For ease of comparison, results are expressed as equivalents of 5-aminolaevulinate/g wet wt. of liver. One nmol of porphobilinogen is equivalent to 2 nmol of 5-aminolaevulinate, and 1 nmol of porphyrin is equivalent to 8 nmol of 5-aminolaevulinate. A single determination of porphobilinogen and total porphyrin was made from each incubated sample. Points show the means of values from two incubations of the same homogenate.

decreased radioactivity in haem by 15–20%; however, radioactivity in Σ (haem + protoporphyrin) was decreased by only 4–8%. The decrease in haem formation produced by menadione alone may have been due to diversion of reducing equivalents from reduction of endogenous Fe^{3+} .

Addition of FeSO_4 (50 or 250 μM) and menadione to chick homogenates had negligible effects on the accumulation of radioactivity in haem or protoporphyrin.

Formation of porphobilinogen and total porphyrins from 5-aminolaevulinate

When incubations were carried out with saturating quantities of 5-aminolaevulinate, up to 90% was not converted into haem or protoporphyrin (see Fig. 2). Therefore, studies were conducted to see if other haem precursors accumulated. In these experiments, an initial concentration of 1 mM-5-aminolaevulinate was used because of the high K_m and $V_{\max}$ of liver 5-aminolaevulinate dehydratase (Shemin, 1972; De Matteis, 1975; Tait, 1978). Fig. 5 shows the results of such an experiment. Homogenates of both rat and chick livers accumulated 20–40 times as much porphobilinogen as porphyrins. Furthermore, the conversion of porphobilinogen into protoporphyrin occurred with little accumulation of intermediate tetrapyrroles, since protoporphyrin represented 80–100% of the porphyrin that accumulated.

The accumulation of porphobilinogen and porphyrins with increasing amounts of 5-aminolaevulinate is shown in Table 2. At all concentrations of 5-aminolaevulinate, porphobilinogen and protoporphyrin accumulated. Total porphyrin accumulation reached a maximum value at low concentrations of

5-aminolaevulinate (15 μM , rat; 30 μM , chick), whereas the rate of porphobilinogen accumulation increased markedly as the initial concentration of 5-aminolaevulinate increased from 10 to 300 μM . As before (Fig. 5), 80–100% of the porphyrin was protoporphyrin.

Since the rate of conversion of porphobilinogen into porphyrins or haem was much lower than the rate of formation of porphobilinogen, the apparent K_m and $V_{\max}$ of 5-aminolaevulinate dehydratase could be determined. The apparent K_m of 5-aminolaevulinate dehydratase was similar (100–140 μM) for homogenates of both rat and chick livers. However, the apparent $V_{\max}$ values were different (3700 nmol of 5-aminolaevulinate equiv./g wet wt. of liver per h for homogenate of rat liver and 1800 nmol for homogenate of chick liver). All measurements of 5-aminolaevulinate dehydratase activity were performed under the same conditions as experiments involving haem formation.

Discussion

In the present experiments, by using a combination of radiochemical and chemical assays, we have shown that hepatic haem synthesis from 5-aminolaevulinate can be studied simply and relatively inexpensively *in vitro*. To aid discussion the haem biosynthetic pathway is outlined in Scheme 1.

Studies with homogenate of rat liver

Our results indicate that homogenates of rat liver incorporate exogenously added 5-aminolaevulinate into haem with little accumulation of protoporphyrin, in agreement with the previous findings by

Table 2. Accumulation of porphobilinogen (PBG), protoporphyrin (Proto) and haem from 5-aminolaevulinate (ALA) in liver homogenates

Final volume of incubation was 0.65 ml. Values for haem and protoporphyrin were corrected for recoveries.

Source of homogenate	Original ALA added (nmol)	Accumulation (nmol-equiv. of ALA/30 min)				Proportion of added ALA in products (%)
		PBG (a)	Proto (b)	Haem (c)	Total (a + b + c)	
Rat	0.0	0.0	0.0	0.0	0.0	—
	11.4	7.4	0.8	3.1	11.3	99
	22.8	15.9	0.8	3.1	19.8	87
	45.6	30.5	0.8	3.1	34.4	76
	183.0	64.6	0.8	3.1	68.5	37
	365.0	73.8	0.8	3.1	77.7	21
	730.0	81.4	0.8	3.1	85.3	12
Chick	0.0	0.0	0.0	0.0	0.0	—
	11.4	6.2	0.92	1.15	8.3	73
	22.8	11.7	0.92	1.15	13.8	60
	45.6	18.0	0.92	1.15	20.1	44
	183.0	34.0	0.92	1.15	36.1	20
	365.0	39.0	0.92	1.15	41.1	11
	730.0	41.0	0.92	1.15	43.1	6

Scheme 1. *Haem biosynthetic pathway*

Numbers represent the following enzymes: (1), 5-aminolaevulinate synthase; (2), 5-aminolaevulinate dehydratase; (3), porphobilinogen deaminase; (4), uroporphyrinogen III co-synthase; (5), uroporphyrinogen decarboxylase; (6), coproporphyrinogen oxidase; (7), protoporphyrinogen oxidase; (8), ferrochelatase.

Sinclair *et al.* (1979). Similarly, small amounts of 5-aminolaevulinate injected into the intact animal are rapidly and efficiently converted into haem by intact rat liver (De Matteis & Gibbs, 1977; Bonkowsky *et al.*, 1980). Cultured rat hepatocytes also convert 5-aminolaevulinate into haem (Grandchamp *et al.*, 1980) without appreciable accumulation of porphyrinogen or porphyrin intermediates (D. M. Bissel, personal communication). It is clear, however, that when high concentrations ($>15\mu\text{M}$) of 5-aminolaevulinate are presented to homogenates of rat liver, their capacity to convert 5-aminolaevulinate into porphyrins or haem may be exceeded (Fig. 2a, Table 2). Increasing amounts of porphobilinogen, but little porphyrin, accumulate in homogenates incubated under these conditions (Table 2, Fig. 5).

The formation of unporphyrinogen III from porphobilinogen requires two enzymes: porphobilinogen deaminase (Scheme 1, enzyme 3) and uroporphyrinogen III co-synthase (enzyme 4). In the absence of uroporphyrinogen III co-synthase, uroporphyrinogen I is formed. This isomer cannot be converted into haem (Battersby *et al.*, 1980). We detected little or no accumulation of uroporphyrinogen or uroporphyrin, indicating that the activity of uroporphyrinogen III co-synthase was greater than the activity of porphobilinogen deaminase. This conclusion is in agreement with results previously obtained by others who studied activities of the individual enzymes (Meyer & Schmid, 1978). The accumulation of porphobilinogen, but not other intermediates, in the haem biosynthetic pathway (Fig. 5a and Table 2) indicates that porphobilinogen deaminase is the rate-limiting enzyme in rat hepatic haem synthesis under conditions of excess 5-aminolaevulinate.

Similarly, under conditions where there is high induction of 5-aminolaevulinate synthase, porphobilinogen accumulates in the liver (Bonkowsky *et al.*, 1981) and is excreted in the urine (Tschudy & Bonkowsky, 1972). This occurs in a variety of experimentally-produced porphyrias in animals and in the acute hepatic porphyrias in man (Tschudy & Bonkowsky, 1972; Meyer & Schmid, 1978; Tschudy & Lamon, 1980), where activity of hepatic 5-aminolaevulinate synthase may be increased more than 10-fold. Under these conditions, activity of porphobilinogen deaminase may become rate-limiting for haem synthesis and constitute a 'physiological bottleneck' that results in overexcretion of 5-aminolaevulinate and porphobilinogen as these accumulated haem precursors leak out of hepatocytes (De Matteis, 1975; Elder, 1976; Brodie *et al.*, 1977; Doss, 1978).

The maximal rate of formation of $\Sigma(\text{haem} + \text{protoporphyrin})$ from 5-aminolaevulinate by homogenate of rat liver (Table 1) is similar to the value for activity of porphobilinogen deaminase previously found in mouse liver (180 nmol of 5-aminolaevulinate equiv./g wet wt. of liver per h; Miyagi *et al.*, 1971). Strand *et al.* (1970) reported a lower value for activity of the enzyme in homogenates of rat liver (42 nmol/g wet wt. of liver per h). However, they appear to have underestimated the activity because they used the rate of porphyrin accumulation as the assay. Here we have shown that the conversion of porphyrin into haem may be considerable (Table 2). In contrast, Miyagi *et al.* (1971) included EDTA in their assays, which, by chelating iron, inhibited the conversion of protoporphyrin into haem. We conclude that the maximal rate of formation of $\Sigma(\text{haem} + \text{protoporphyrin})$ from 5-aminolaevulinate under our conditions reflects the activity of porpho-

bilinogen deaminase. Based on our data, the activity of this enzyme in rat liver is very similar to that of mouse liver.

From the data of Fig. 5 and Table 2, one can also determine the $V_{\max}$ and apparent K_m for 5-aminolaevulinate dehydratase (Scheme 1, enzyme 2). The values derived from our data for rat liver ($V_{\max} = 3700 \text{ nmol}$ of 5-aminolaevulinate equiv./g wet wt. of liver per h; $K_m = 136 \mu\text{M}$) are in close agreement with values reported previously (Shemin, 1972; Tait, 1978).

The rate of haem synthesis from protoporphyrin by homogenate of rat liver declined after 30 min of incubation (Fig. 1a), a decline that was more obvious in homogenates of frozen liver (Fig. 3). When incubations were supplemented with Fe^{2+} and menadione, linearity of the rate of haem synthesis was restored (compare Fig. 1a with 4a and Fig. 3 with 4b). The increase in haem was accompanied by a corresponding decrease in protoporphyrin. These results indicate that the availability of Fe^{2+} became limiting for haem synthesis at later times when extra Fe^{2+} was not added.

Others have reported that $70\text{--}300 \mu\text{M-Fe}^{2+}$ inhibits activity of uroporphyrinogen III co-synthase (Kushner *et al.*, 1972) and uroporphyrinogen decarboxylase (Kushner *et al.*, 1975) in extracts of pig liver. More recently, Blekkenhorst *et al.* (1979) reported that $10\text{--}2000 \mu\text{M-Fe}^{2+}$ stimulated, rather than inhibited, uroporphyrinogen decarboxylase in extracts of rat liver. This controversy has not been resolved, and the physiological relevance of the findings is unclear. In our studies, $50 \mu\text{M-}$ or $250 \mu\text{M-FeSO}_4$ did not affect $\Sigma(\text{haem} + \text{protoporphyrin})$ synthesized from 5-aminolaevulinate so that neither inhibition nor activation of these enzymes by Fe^{2+} seems important in determining the rate of conversion of 5-aminolaevulinate into haem.

Effects of iron on hepatic haem metabolism are clinically relevant since activity of 5-aminolaevulinate synthase in human liver is correlated directly with liver iron concentrations (Bonkowsky & Pomeroy, 1977; Bonkowsky *et al.*, 1979b), and since haemosiderosis plays a role in the pathogenesis of porphyria cutanea tarda (Meyer & Schmid, 1978; Tschudy & Lamon, 1980), and perhaps certain other hepatic porphyrias as well (Stein *et al.*, 1972). Iron-loading of rats increases the rate of hepatic haem turnover (Ibrahim *et al.*, 1979; Bonkowsky *et al.*, 1980), and we have preliminary evidence that it also decreases activity of 5-aminolaevulinate dehydratase (J. F. Healey, H. L. Bonkowsky, P. R. Sinclair & J. F. Sinclair, unpublished work).

Studies with homogenate of chick embryo liver

In homogenates of chick, unlike rat, haem synthesis from 5-aminolaevulinate proceeds linearly

for at least 90 min (Fig. 1b), and in addition, the maximal rate of total protoporphyrin production is three times that of haem production (Table 1). With saturating concentrations of 5-aminolaevulinate ($> 10 \mu\text{M}$), substantial accumulation of protoporphyrin occurs (Fig. 2b). This accumulation was not prevented by addition of Fe^{2+} and menadione. At low concentrations of 5-aminolaevulinate (less than $4 \mu\text{M}$), however, little protoporphyrin accumulates as was seen with homogenate of rat liver. Thus, in homogenate of chick liver at low 5-aminolaevulinate concentrations, protoporphyrin is utilized almost as quickly as it is made. As concentrations of 5-aminolaevulinate are increased beyond $10 \mu\text{M}$, the rate of protoporphyrin synthesis continues to increase, whereas the rate of haem synthesis from protoporphyrin does not (Fig. 2b).

At very high concentrations of 5-aminolaevulinate (approx. 1 mM), the accumulation of porphobilinogen and porphyrin is similar in homogenates of both rat and chick liver (Fig. 5 and Table 2). Large amounts of porphobilinogen accumulate, indicating that the activity of porphobilinogen deaminase is limiting. For the reasons already discussed, the maximal rate of formation of protoporphyrin from 5-aminolaevulinate by chick liver, as by rat liver, reflects the activity of porphobilinogen deaminase (Table 1). As in homogenate of rat liver, 80–100% of the porphyrin that accumulates is protoporphyrin. We conclude that, for homogenates of chick embryo liver at high concentrations of 5-aminolaevulinate, porphobilinogen deaminase limits the rate of protoporphyrin synthesis, but ferrochelatase limits the overall rate of haem synthesis. In rat liver under similar conditions, the rates of both protoporphyrin and haem synthesis are limited by activity of porphobilinogen deaminase.

Compatible with these results is the finding that direct assays of the activity of ferrochelatase of chick liver give values only 15–25% of those of rat liver (De Matteis, 1973; Cole *et al.*, 1979). In our system, where protoporphyrin is generated from 5-aminolaevulinate, the maximal activity of ferrochelatase of chick embryo liver homogenate is 20% of that in homogenate of rat (see Table 1). This estimate may be somewhat high since the rat enzyme may not be saturated with respect to protoporphyrin (Honeybourne *et al.*, 1979; Table 2).

The difference between the activities of rat and chick ferrochelatase may help account for the differences observed in the sensitivity of induction of 5-aminolaevulinate synthase in these two species (De Matteis, 1973; Marks, 1978; Cole *et al.*, 1979). It is thought that, *in vivo*, the activity of hepatic 5-aminolaevulinate synthase, hence the rate of hepatic haem synthesis, is primarily controlled by a hypothetical 'regulatory haem pool' (Scheme 1). Depletion of this haem pool (e.g. by administration

of certain drugs) leads to de-repression of the synthesis of 5-aminolaevulinate synthase. Chick liver, because of less ferrochelatase activity, possesses less ability than rat liver to rapidly replenish its 'regulatory haem pool', and is more sensitive to drugs that induce 5-aminolaevulinate synthase. Our results are consistent with this hypothesis.

The results also help to explain why patients with acute hepatic porphyrias, who have genetically determined defects in various enzymes of the haem biosynthetic pathway, are uniquely susceptible to drugs that deplete the 'regulatory haem pool'. Like the chick, they have an inherent limitation in their ability to replenish this pool. Of the acute hepatic porphyrias, acute intermittent porphyria is the most virulent. Probably this is because the genetic defect in acute intermittent porphyria is in porphobilinogen deaminase (Meyer & Schmid, 1978; Tschudy & Lamon, 1980), which, as shown above, is the rate-limiting enzyme between 5-aminolaevulinate and haem.

In conclusion, we have characterized a liver homogenate system that converts 5-aminolaevulinate into haem at a constant rate that is easy to determine. This system can now be used for the study of agents considered to inhibit steps of haem synthesis. In contrast with studies of individual enzymes, the system we have developed avoids some of the problems associated with the use of intact animals and facilitates study of the entire pathway from 5-aminolaevulinate to haem. We believe this system will help define whether the interference in haem synthesis produced by various chemical agents is of physiological relevance.

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