Conversion of 5-aminolaevulinate into haem by homogenates of human liver

Comparison with rat and chick-embryo liver homogenates

Herbert L. BONKOVSKY,*† John F. HEALEY,* Peter R. SINCLAIR*‡ and
Jacqueline F. SINCLAIR*

*Hepatology Laboratory of VA Medical Center, White River Junction, VT 05001, U.S.A., and Departments
of †Medicine and ‡Biochemistry, Dartmouth Medical School, Hanover, NH 03756, U.S.A.

(Received 25 October 1984/14 December 1984; accepted 25 January 1985)

To assess whether the synthesis of haem can be studied in small amounts of human liver, we measured kinetics of the conversion of 5-aminolaevulinate into haem and haem precursors in homogenates of human livers. We used methods previously developed in our laboratory for studies of rat and chick-embryo livers [Healey, Bonkowsky, Sinclair & Sinclair (1981) *Biochem. J.* **198**, 595–604]. The maximal rate at which homogenates of human livers converted 5-aminolaevulinate into protoporphyrin was only 26% of that for rat, and 58% of that for chick embryo. In the absence of added Fe^{2+} , homogenates of fresh human liver resembled those of chick embryos in that protoporphyrin and haem accumulated in similar amounts, whereas fresh rat liver homogenate accumulated about twice as much haem as protoporphyrin. However, when Fe^{2+} (0.25 mM) was added to human liver homogenates, mainly haem accumulated, indicating that the supply of reduced iron limited the activity of haem synthase, the final enzyme in the haem-biosynthesis pathway. Addition of the potent iron chelator desferrioxamine after 30 min of incubation with 5-amino[^{14}C]laevulinate stopped further haem synthesis without affecting synthesis of protoporphyrin. Thus the prelabelled haem was stable after addition of desferrioxamine. Since the conversion of 5-amino[^{14}C]laevulinate into haem and protoporphyrin was carried out at pH 7.4, whereas the pH optimum for rat or bovine hepatic 5-aminolaevulinate dehydratase is about 6.3, we determined kinetic parameters of the human hepatic dehydratase at both pH values. The V_{max} was the same at both pH values, whereas the K_m was slightly higher at the lower pH. Our results indicate that the synthesis of porphyrins and haem from 5-aminolaevulinate can be studied with the small amounts of human liver obtainable by percutaneous needle biopsy. We discuss the implications of our results in relation to use of rat or chick-embryo livers as experimental models for the biochemical features of human acute porphyria.

The liver is an active site of haem biosynthesis. In rats, as much as two-thirds of the haem made by the liver is utilized for formation of cytochrome *P*-450 species (Bonkowsky, 1982), the family of mixed-function oxidases that play an essential role in the metabolism of many endogenous and exogenous compounds, including steroids, drugs and carcinogens (Sato & Omura, 1978). Normally, the process of hepatic haem biosynthesis is tightly regulated. However, a number of genetic and

acquired defects interfere with hepatic haem biosynthesis. Such defects may give rise to the hepatic porphyrias (Bonkowsky, 1982). In some instances, defects in haem synthesis may limit the amount of haem available for cytochrome *P*-450 synthesis, and thus exert far-reaching metabolic consequences.

Most studies of altered hepatic haem synthesis have focused on the activities of individual enzymes of the pathway. However, since some compounds (e.g. ethanol or heavy metals) may affect more than one enzyme (Bonkowsky *et al.*, 1979b, 1983; Bonkowsky, 1982; Doss, 1978;

Abbreviations used: ALA, 5-aminolaevulinate; PBG, porphobilinogen.

Ibrahim *et al.*, 1979, 1981; Maines, 1979; McColl *et al.*, 1980), the overall effect of these compounds on the synthesis of haem has often remained uncertain. Furthermore, since there are up to 100-fold differences among the kinetic constants ($V_{\max}$, K_m) of enzymes of hepatic haem biosynthesis (for review, see Bonkovsky, 1982), the physiological relevance of altered activities of individual enzymes is difficult to assess. Although studies *in vivo* theoretically can circumvent some of the above difficulties, in practice such studies in experimental animals are quite difficult, and in humans often impossible.

We have developed and characterized simple procedures for studying the conversion of 5-amino[4- 14 C]laevulinate ([14 C]ALA) into haem and haem precursors by homogenates of rat and chick-embryo livers (Healey *et al.*, 1981). Using these procedures, we demonstrated a decreased rate of haem synthesis in liver homogenates of rats treated with large doses of iron-dextran, owing to decreased activity of ALA dehydratase, and we characterized the non-linear relationship between decreases in activity of this enzyme and in the overall rate of hepatic haem synthesis (Bonkovsky *et al.*, 1983).

Although the rat and chick embryo are widely used for studies of experimental hepatic porphyria (Bonkovsky, 1982; Tschudy & Bonkovsky, 1972), it is not entirely clear how the process of hepatic haem synthesis in such animals resembles or differs from that in humans. To help to resolve this uncertainty, we now report results for the conversion of [14 C]ALA into haem and haem precursors in homogenates of normal human livers. We found that the maximal rate of conversion of ALA into haem by homogenates of normal human liver is much less than that of rat liver, but similar to that of chick-embryo liver.

Methods

Patients studied

Both hepatic ALA dehydratase and conversion of ALA into haem were studied in homogenates of liver biopsies from four men, aged 38, 58, 68 and 70 years. None of the subjects had a personal or family history of porphyria or other disorder of haem synthesis, and all had normal excretions of urinary and faecal porphyrins and normal concentrations of erythrocyte porphyrin. Patient 1 had Crohn's disease and iron-deficiency anaemia (blood haemoglobin concentration, 12 g/dl); patient 2 had carcinoma of the distal oesophagus; and patients 3 and 4 had chronic cholecystitis. Within 1 week of liver biopsy, all patients had normal serum aspartate aminotransferase and

alanine aminotransferase, alkaline phosphatase, bilirubin, protein electrophoresis, prothrombin time, partial thromboplastin time, and white-blood-cell and platelet counts.

To provide an adequate amount of liver for detailed kinetic studies of the conversion of ALA into haem and for comparative studies of fresh and frozen-stored homogenates, wedge biopsies were obtained at the time of laparotomies, done for reasons unrelated to our study. During the week before, and during surgery, patients received no drugs known to induce cytochrome *P*-450 or enzymes of haem synthesis (e.g. barbiturates or hydantoins). Patients gave voluntary informed consent to participate in this study under a protocol approved by the Committee for the Protection of Human Subjects of the Dartmouth-Hitchcock Medical Center and by the Research and Development Committee of the VA Medical Center.

Owing to limitation in the amount of liver available, we were unable to perform kinetic studies of ALA dehydratase with the livers from the above four patients. Therefore more detailed studies of ALA dehydratase were carried out with five livers obtained from young, previously healthy, accident victims who had sustained fatal head injuries. Kidneys from these patients were used as donor organs for transplantation; livers were removed at the same times as the kidneys, placed into ice-cold 0.15M-NaCl and, in a cold-room, were cut into pieces that weighed 3–10 g. The pieces were snap-frozen in liquid N_2 and stored at -80°C for 1–2 years before study. In preliminary experiments, we found that the rates of conversion of [14 C]ALA into protoporphyrin or haem by homogenates of these livers were only 25–35% of those in homogenates of fresh liver. Therefore the frozen liver pieces, which provided abundant amounts of homogenate, were used only for comparative studies of effects of pH on ALA dehydratase.

Handling of human liver specimens

Fresh livers. Biopsies (0.5–2.5 g wet wt.) were placed immediately into ice-cold saline (0.15M-NaCl). A portion was fixed in 10% (v/v) formaldehyde in normal saline and processed for routine histological examination. Sections of 4 μm thickness were stained with haematoxylin and eosin, trichrome stain, and with Prussian Blue stain for iron (Luna, 1975). The livers of patients 1 and 2 were normal histologically; those of patients 3 and 4 showed mild chronic portal fibrosis. The livers of patients 1 and 4 were deficient in storage iron, as shown by absence of stainable iron.

The remainder of each biopsy was blotted, weighed and homogenized (at low speed for 15–20 s in a Polytron homogenizer) in 9 vol. of 0.25M-

sucrose/0.02M-Tris/HCl, pH 7.4. To prevent lipid peroxidation, immediately after homogenization, butylated hydroxytoluene dissolved in ethanol (10mg/ml) was added to a final concentration of 45 μ M. Portions of this homogenate were frozen (-60°C) for later assays, and other portions were used immediately, after suitable dilution with sucrose/Tris buffer. After dilution, additional butylated hydroxytoluene was added to maintain a concentration of 45 μ M.

Frozen livers. Pieces were thawed and 10% (w/v) homogenates were prepared in the sucrose/Tris (pH 7.4) buffer. Portions of the homogenates were centrifuged (10000g for 10 min and then 100000g for 60 min) to obtain a soluble fraction. Portions of the whole homogenates and of the soluble fractions were frozen (-20°C) until they were assayed 1–2 weeks later.

Handling of rats and rat livers

Mature male Sprague-Dawley rats (200–300g) (Charles River Laboratories, Cambridge, MA, U.S.A.) were housed as previously described (Bonkowsky *et al.*, 1979a) and starved for 24h before being killed by decapitation. Liver homogenates were prepared as described previously (Healey *et al.*, 1981) and portions were frozen (-60°C) until used.

Assay of ALA dehydratase

Fresh livers. ALA dehydratase was assayed by incubation in a metabolic shaker (120 oscillations/min) of 0.5ml liver homogenates at 37°C with various concentrations of ALA in a total volume of 0.65ml. For routine determinations, 5mM-ALA and 10mM-dithiothreitol were used. Addition of dithiothreitol increased activity by 1, 5 and 24% respectively, in the three human homogenates studied (patients 1–3). Incubations were ended by transfer of the tubes to an ice/water bath and addition of 1.3ml of 7.5% (w/v) trichloroacetic acid containing 0.05M-HgCl₂. Tubes were centrifuged for 10min at 1100g, and 0.5ml of the supernatant was added to an equal volume of modified Ehrlich's reagent (Urata & Granick, 1963). After 15min, an absorbance spectrum was run from 500 to 650nm against a reference sample from homogenate to which trichloroacetic acid had been added before incubation. The concentration of PBG was determined by using $\Delta\epsilon_{555-650} = 61\text{mm}^{-1}$ for the coloured adduct (Mauzerall & Granick, 1956).

Frozen livers. Frozen samples were thawed at 37°C , and assayed by the method of Sassa (1982); sucrose/Tris buffer (final pH 7.4) was compared with 0.2M-sodium phosphate (final pH 6.35).

Conversion of [^{14}C]ALA into porphyrins and haem

Incubations were carried out and products isolated and analysed as described previously (Healey *et al.*, 1981), with the following modifications: (i) owing to the limited amount of fresh liver available, more dilute homogenates (0.7–3.1%, w/v) were usually used; and (ii) the ether extract containing haem was washed twice rather than once with 4ml of 2M-HCl. Incubations were performed in the dark at 37°C in open test tubes (15mm $\times$ 150mm) with continuous shaking (120 oscillations/min).

Other assays

Urinary ALA, porphobilinogen (Mauzerall & Granick, 1956) and porphyrins (Schwartz *et al.*, 1960), faecal porphyrins (Schwartz *et al.*, 1960) and erythrocyte porphyrins (Piomelli, 1973) were measured by standard procedures. The porphyrins made during incubations of homogenates with 1mM-ALA (unlabelled) were extracted into 4 vol. of 1M-HClO₄/methanol (1:1, v/v), the mixture was centrifuged (1000g, 10min), and porphyrins were determined in the supernatant by the method of Granick *et al.* (1975). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Chemicals

5-Amino[4- ^{14}C]laevulinic acid hydrochloride (51.1 Ci/mol in 0.1M-HCl) was obtained from New England Nuclear (Boston, MA, U.S.A.). Crystalline ALA was from Sigma (St. Louis, MO, U.S.A.). Solutions of ALA were prepared as described by Healey *et al.* (1981).

Tris was from Sigma and butylated hydroxytoluene was from Aldrich (Milwaukee, WI, U.S.A.). Porphyrins were from Porphyrin Products (Logan, UT, U.S.A.). Other chemicals were of analytic grade, supplied by Sigma or Fisher Chemical Co. (Phillipsburg, NJ, U.S.A.). Solutions of FeSO₄ were prepared in 1mM-HCl just before use, and 0.05ml was added to 0.65ml of homogenate mixtures.

Repetition of experiments

With one exception experiments were carried out two or more times with closely similar results. Owing to the limited amounts of human liver available, the experiment in which desferrioxamine was added to human liver homogenate was done only once.

Statistics

Differences between mean values were tested for significance by use of Student's two-tailed *t* test (Dixon & Massey, 1969).

Results and discussion

ALA dehydratase

We routinely carried out assays for ALA dehydratase and conversion of [^{14}C]ALA into haem at pH 7.4. However, the pH optima for the dehydratase from rat or bovine liver or from human erythrocytes are about 1 pH unit lower than 7.4 (Sassa, 1982). Therefore, we determined the effect of pH on kinetic parameters of the dehydratase from human liver. In order to have sufficient material for these studies, we used homogenates of livers from kidney donors and carried out kinetic studies of ALA dehydratase, both at pH 7.4, the pH used for studies of the conversion of ALA into haem, and at pH 6.35 (Sassa, 1982). Results are summarized in Table 1. There was no significant difference between K_m or $V_{\max}$ values determined with whole homogenates or soluble fractions. The K_m values were similar to those reported for ALA dehydratase from other mammalian sources (Sassa, 1982). These results show that activity of the dehydratase from human liver is nearly the same at pH 7.4 as at pH 6.35.

Activity of ALA dehydratase was readily measured in small amounts (5 mg wet wt.) of homogenates of human livers. Formation of the product PBG increased linearly as the concentration of homogenate (0–20 mg wet wt. of liver/reaction) and the time of incubation (0–60 min) were increased. In the four homogenates of fresh liver from patients 1–4 respectively, the dehydratase activities were 1.5, 2.25, 2.3 and 2.4 μmol of PBG/h per g wet wt. of liver. These values are within the range of those reported previously for human liver (summarized by Bonkovsky, 1982) and are similar to those reported by Kodama *et al.* (1983), if one assumes that there is approx. 150 mg of protein in the soluble fraction from 1 g wet wt. of liver. The activities of ALA dehydratase are also similar to values that we obtained previously from homogenates of normal rat liver (Healey *et al.*, 1981; Bonkovsky *et al.*, 1983). In contrast, the $V_{\max}$ of

ALA dehydratase in homogenates of chick-embryo liver is about 50% less (Healey *et al.*, 1981). Activity of the dehydratase decreased by 20–35% when 2 or 4% (w/v) homogenates of the human livers were stored frozen (-60°C) for 22–53 days.

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

Effect of homogenate concentration. Previously we showed that, at 7.7% (w/v) final concentration of rat or chick-embryo liver homogenate, incorporation of [^{14}C]ALA into protoporphyrin was linear with time for at least 90 min (Healey *et al.*, 1981). In that study, we did not test lower concentrations of homogenates; our routine assay contained 50 mg wet wt. of liver in a total volume of 0.65 ml. However, owing to the limited amounts obtainable from a portion of a needle biopsy (10–30 mg wet wt.), it would be preferable if smaller amounts of human liver could be used. Therefore we compared the effect of lower homogenate concentrations on conversion of [^{14}C]ALA into protoporphyrin and haem by rat and human livers. Fig. 1 shows the effect of liver homogenate concentration on the conversion of ALA into haem and protoporphyrin by human and rat liver. The rates of conversion were slower at low than at high (20–50 mg/0.65 ml) concentrations. Among possible explanations for these findings are: (i) that, at low homogenate concentrations, PBG deaminase, the rate-limiting enzyme for conversion of ALA into protoporphyrin and haem (Healey *et al.*, 1981), was not rapidly saturated with its substrate PBG, and therefore its rate was sub-maximal; (ii) that activity of human hepatic haem synthase (ferrochelatase) increased as homogenate concentrations were increased (Bonkovsky *et al.*, 1975); or (iii) that, at low homogenate concentrations, there was more oxidative or other damage to these (or other) enzymes of haem synthesis. In any case, for human liver, the minimal concentration of homogenate

Table 1. Kinetic parameters of ALA dehydratase in homogenates and soluble fractions of normal human livers. Frozen samples were thawed at 37°C , and assays performed by the method of Sassa (1982), comparing 0.25 M-sucrose/10 mM-Tris buffer (final pH 7.4) with 0.1 M-sodium phosphate (final pH 6.35). Values are means $\pm$ S.D. for n experiments. Soluble fractions are supernatants after centrifugation at 100000g for 60 min.

		Buffer and pH			
		Sucrose/Tris, pH 7.4		Sodium phosphate, pH 6.35	
	n	K_m (mM-ALA)	$V_{\max}$ (nmol of PBG/h per mg of protein)	K_m (mM-ALA)	$V_{\max}$ (nmol of PBG/h per mg of protein)
Homogenates	5	0.20 ± 0.05	5.60 ± 1.30	0.36 ± 0.06	6.60 ± 1.90
Soluble fractions	4	0.26 ± 0.12	12.30 ± 3.40	0.28 ± 0.09	16.90 ± 3.40

Fig. 1. Effect of homogenate concentration on the conversion of $[^{14}\text{C}]\text{ALA}$ into protoporphyrin and haem by homogenates of rat and human livers

Liver homogenates from a rat (a) or from patient 4 (b) were prepared as described in the Methods section. Appropriate dilutions of each were incubated with $[^{14}\text{C}]\text{ALA}$ ($0.2\mu\text{Ci}$, $39\mu\text{M}$) for 30 min, after which products were separated and counted for radioactivity as described previously (Healey *et al.*, 1981). Means of duplicate determinations which varied by less than $\pm 5\%$ are plotted. Symbols: radioactivity accumulating in haem ($\circ$), in protoporphyrin ($\triangle$), and sum of that in haem + protoporphyrin [$\Sigma(\text{haem} + \text{protoporphyrin})$] ($\square$).

needed for accurate measurement of the rate of conversion of $[^{14}\text{C}]\text{ALA}$ into haem and protoporphyrin is about 20 mg of liver/0.65 ml (Fig. 1b), and at this concentration the conversion of ALA into

Fig. 2. Effect of ALA concentration on conversion of $[^{14}\text{C}]\text{ALA}$ into protoporphyrin and haem by homogenates of human liver

Liver homogenates from patient 2 (a) and patient 1 (b) were incubated for 30 min with various concentrations of $[^{14}\text{C}]\text{ALA}$. For the first three points, undiluted $[^{14}\text{C}]\text{ALA}$ was used. For the fourth point, unlabelled ALA was added to $0.2\mu\text{Ci}$ of $[^{14}\text{C}]\text{ALA}$ and results were corrected for dilution of radioactivity. In (a) 20 mg of liver was used per tube; in (b), owing to a limitation in available liver, 10 mg was used per tube. Means of duplicate determinations, which varied less than $\pm 5\%$, are plotted. Symbols are as in Fig. 1.

$\Sigma(\text{haem} + \text{protoporphyrin})$ proceeds linearly for at least 1 h.

Effect of increasing concentrations of ALA on conversion of [^{14}C]ALA into protoporphyrin and haem

As shown in Fig. 2, the conversion of [^{14}C]ALA into labelled protoporphyrin and $\Sigma(\text{haem} + \text{protoporphyrin})$ was maximal at an initial concentration of $12\mu\text{M}$ -ALA. These curves are similar to those previously obtained with homogenates of normal rat and chick-embryo liver (Healey *et al.*, 1981).

From studies with fresh homogenates of human livers of the type shown in Fig. 2, using graphical methods (Segal, 1975), we determined the V_{max} for conversion of ALA into total protoporphyrin [i.e. $\Sigma(\text{haem} + \text{protoporphyrin})$] and the concentration of ALA producing a half-maximal rate of formation of products. The results are summarized in Table 2, which also lists the values for these kinetic parameters previously found (by the same methods) for homogenates of rat and chick-embryo

liver homogenates. Table 2 shows that, although the ALA concentration required for a half-maximal rate of conversion of ALA into protoporphyrin by human liver was slightly lower than for rat or chick liver, the maximal rate at which ALA could be converted into protoporphyrin by human liver was only 26% and 58% of that by rat and chick liver, respectively. When high concentrations (1–5 mM) of unlabelled ALA were added to human liver homogenates, a marked accumulation of PBG was found (linearly increasing with amount of liver used and duration of incubation), but relatively little accumulation of porphyrins. This is similar to results obtained previously with rat or chick-embryo liver homogenates (Healey *et al.*, 1981), and is due to the 100-fold greater V_{max} for ALA dehydratase (3000–5000 nmol of ALA equivalent/h per g of liver) compared with that of PBG deaminase (17–40 nmol of ALA equivalents/h per g of liver) (see results above, and Bonkovsky, 1982). These findings further indicate that the activity of PBG deaminase is severely limited in

Table 2. Kinetic parameters of formation of $\Sigma(\text{haem} + \text{protoporphyrin})$ from 5-amino[4- ^{14}C]laevulinate by liver homogenates
For details see the text.

Source of homogenate	Initial concn. of ALA producing half-maximal rates of formation (μM)	Maximal rate of formation (nmol of ALA equivalents/h per g of liver)*	Reference
Human	$3.2 \pm 1.7^\dagger$	$40 \pm 3^\dagger$	The present paper
Rat	5.8	151	Healey <i>et al.</i> (1981)
Chick embryo	6.9	69	Healey <i>et al.</i> (1981)

* 1 nmol of haem or protoporphyrin = 8 nmol of ALA equivalents.

† Mean $\pm$ 1 S.D., $n = 4$.

Table 3. Effect of FeSO_4 on conversion of [^{14}C]ALA into protoporphyrin and haem by fresh and stored homogenates of human liver

Liver homogenates (4%, w/v) were prepared and incubated for 60 min with [^{14}C]ALA (0.2 μCi ; 39 μM), as described in the Methods section, in the absence or presence of FeSO_4 (0.25 mM). Products were separated and counted for radioactivity as described previously (Healey *et al.*, 1981). Values are average of duplicate determinations that differed by less than 7%. For ease of comparison, data are expressed as percentages of total radioactivity recovered in protoporphyrin + haem. The absolute number of d.p.m. recovered ranged from 6000 to 12000.

Homogenate ...	Radioactivity recovered (% of total)			
	Fresh liver		Stored liver	
	Protoporphyrin	Haem	Protoporphyrin	Haem
Patient 1	56	44	68	32
+ FeSO_4	19	81	18	83
Patient 2	50	50	64	36
+ FeSO_4	9	91	13	87
Patient 3	38	62	55	45
+ FeSO_4	8	92	14	86
Patient 4	—	—	72	28
+ FeSO_4	—	—	21	79

Fig. 3. Effect of desferrioxamine on the incorporation and accumulation of radioactivity in protoporphyrin and haem by homogenates of rat and human livers incubated with $[^{14}\text{C}]\text{ALA}$

Homogenates of rat and human liver from patient 4 were prepared and incubated with $[^{14}\text{C}]\text{ALA}$ (rat, $0.2\mu\text{Ci}$, $39\mu\text{M}$; human $0.2\mu\text{Ci}$, $5.8\mu\text{M}$) as described in the Methods section. (A lower concentration of ALA was used with human liver, so that radioactivity of products would be higher.) After 30 min (arrow), desferrioxamine was added (0.5mM final concn.) and the incubation continued for an additional 30 or 60 min. Products were separated and counted for radioactivity as described by Healey *et al.* (1981). (a), Rat liver; (b), human liver (both

human liver homogenates and is the rate-limiting step between ALA and protoporphyrin or haem (De Matteis, 1975; Elder, 1976; Brodie *et al.*, 1977; Doss, 1978; Bonkowsky, 1982).

Effect of added Fe^{2+} or dithiothreitol on conversion of $[^{14}\text{C}]\text{ALA}$ into protoporphyrin and haem

Previously, we reported that addition of Fe^{2+} did not affect the conversion of $[^{14}\text{C}]\text{ALA}$ into protoporphyrin or haem by homogenates of chick-embryo liver or in homogenates of fresh rat liver. However, in homogenates of rat liver that had been stored frozen, addition of Fe^{2+} increased $[^{14}\text{C}]\text{haem}$ formation without affecting the rate of formation of $\Sigma([^{14}\text{C}]\text{haem} + \text{protoporphyrin})$ (Healey *et al.*, 1981). This result indicated that Fe^{2+} became limiting for haem synthase after frozen storage of rat liver homogenates. In the present study, we found that added Fe^{2+} increased labelled haem formation in both fresh and stored human liver homogenates. Without addition of extra Fe^{2+} , the fresh human liver homogenates accumulated relatively more radioactivity in protoporphyrin than did the homogenates of rat liver that we studied previously (Healey *et al.*, 1981). This was true particularly for human liver from patient 1, the subject with iron deficiency (Table 3). The conversion of labelled protoporphyrin into haem was increased by incubation with Fe^{2+} (Table 3), indicating that the limiting factor in protoporphyrin conversion was the concentration of Fe^{2+} , rather than the amount of active haem synthase. In this regard, homogenates of human liver resemble those of rat liver more closely than those of chick liver, since, in the latter, addition of Fe^{2+} did not increase conversion of protoporphyrin into haem because the synthase itself is limiting (Healey *et al.*, 1981).

The apparent limitation of the rate at which protoporphyrin can be converted into haem by iron-deficient human liver raises the question of whether hepatic haem or haemoprotein concentrations remain normal in iron deficiency. We are unaware of studies specifically addressing this question in humans; however, a few studies have been carried out in iron-deficient rats and mice (for reviews, see Becking, 1976; Jacobs, 1977). Results

20mg wet wt. per tube). Means of duplicate determinations, which varied by less than $\pm 5\%$, are plotted. In separate experiments, it was found that desferrioxamine did not affect extraction of either haem or protoporphyrin. The same results as shown here were also obtained when the final concentration of desferrioxamine was 0.1, 0.25 or 2.5mM. Symbols are as in Fig. 1; open symbols, incubations without desferrioxamine; solid symbols, with desferrioxamine.

indicate that contents of hepatic haemoproteins remain normal or nearly normal after 6–7 weeks' feeding of an iron-deficient diet, but some decrease after 14 weeks' feeding of the iron-deficient diet in rats (Bailey-Wood *et al.*, 1975). Marked species differences have been noted (Becking, 1976); thus the relevance of these findings to human disease is uncertain. In cultured chick-embryo hepatocytes, an iron-deficient state produced by addition of desferrioxamine decreases haem synthesis, haem concentrations and drug-mediated induction of cytochrome(s) *P*-450 (Sinclair *et al.*, 1984; P. R. Sinclair, H. L. Bonkovsky & J. F. Sinclair, unpublished work).

Stability of labelled haem

Previously we showed that labelled haem, formed by rat liver homogenates under our usual conditions of incubation, was not degraded (Bonkovsky *et al.*, 1983), as indicated by the finding that haem prelabelled with a trace amount of [^{14}C]ALA remained stable for at least 1 h of incubation. Because of the lower rate of haem formation from ALA by human than by rat liver homogenate (see above), we used a different approach, as shown in Fig. 3, to determine whether the labelled haem formed by human liver homogenate was also stable. In this experiment, we allowed formation of [^{14}C]protoporphyrin and [^{14}C]haem to proceed for 30 min, at which time we added desferrioxamine to the incubations. This addition stopped further incorporation of radioactivity into haem; instead, radioactivity accumulated as protoporphyrin. The rate of incorporation of radioactivity into haem by the human liver homogenate in the absence of desferrioxamine decreased, probably because of insufficient endogenous supply of Fe^{2+} . The critical point, however, is that the labelled haem formed before addition of desferrioxamine remained the same in both rat (Fig. 3a) and human (Fig. 3b) liver homogenates. Thus our previous conclusion for rat liver homogenates (Bonkovsky *et al.*, 1983) is confirmed, and we conclude that the haem formed by human liver homogenates is also not degraded. These results provide more evidence that our simple system of homogenate incubation is accurate for studying conversion of ALA into haem, without effects of haem breakdown.

Experimental models for human hepatic haem synthesis

Comparison of ALA conversion into protoporphyrin and haem by homogenates of rat, chick-embryo and human livers reveals both similarities and differences. In liver homogenates from all species, activity of PBG deaminase is rate-limiting between ALA and protoporphyrin. However, the

maximal rates of conversion of ALA into haem by homogenates of human and chick livers are about the same (33 and 26 nmol of ALA equivalents/h per g wet wt of liver respectively) because, under our experimental conditions, the activity of human hepatic PBG deaminase is approximately the same as that of chick-embryo liver haem synthase (Healey *et al.*, 1981) (when both are expressed on the basis of ALA equivalents utilized to form products). In contrast, the rate of conversion of ALA into haem by homogenates of rat liver is 5-fold higher (Healey *et al.*, 1981). Thus, as an experimental model for haem metabolism, chick-embryo liver resembles human liver more closely than does rat liver. However, the chick-embryo model is not ideal, because the rate-limiting step of haem synthesis from ALA in chick-embryo liver is haem synthase rather than PBG deaminase. Furthermore, since persons with genetic deficiencies of PBG deaminase activity (i.e. carriers of the genetic defect for acute intermittent porphyria) have an even greater limitation in haem synthesis, neither rat nor chick embryo is a sufficiently sensitive model for human acute intermittent porphyria. To make the chick-embryo system more sensitive, we and others have inhibited haem synthase when testing drugs considered for administration to porphyric patients (Anderson, 1978; Bonkovsky *et al.*, 1980; Anderson *et al.*, 1982; Shedlofsky & Bonkovsky, 1984). The ideal animal model would have a V_{max} of hepatic PBG deaminase (and of other enzymes of the haem-biosynthetic pathway) as similar to that of human liver as possible. The methods described here, in addition to providing a relatively simple means for studying the entire pathway between ALA and haem in human liver, can be used to seek a new animal model that resembles the human situation more closely.

We thank Professor U. A. Meyer and Dr. P. Meier for providing frozen livers from human kidney donors. We thank Professor E. L. Smith for helpful comments concerning the manuscript, and Mrs. D. Burke for typing it. A portion of the work was carried out during a sabbatical leave provided to H. L. B. by the U.S. Veterans Administration. Travel funds for the sabbatical were provided by the Roche Foundation for scientific exchange with Switzerland. This work was supported by medical research funds from the Veterans Administration and by grants from the U.S. National Institutes of Health (CA 25012 and HLAM 28177).

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