Incorporation of haemoglobin haem into the rat hepatic haemoproteins tryptophan pyrrolase and cytochrome *P*-450

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1. After its administration to intact rats, haemoglobin haem was incorporated into hepatic tryptophan pyrrolase as shown by the marked increase in functional constitution of this enzyme. 2. Incorporation of haemoglobin haem into cytochrome *P*-450 was demonstrated in intact rats and in the isolated rat liver perfused with haemoglobin-free medium. In both systems, haemoglobin haem restored cytochrome *P*-450 content and its dependent mixed-function-oxidase activity after substrate-induced destruction of the cytochrome *P*-450 haem moiety. 3. Further confirmation that haemoglobin haem could be incorporated prosthetically into cytochrome *P*-450 was achieved by administration of [³H]haemoglobin to rats and subsequent isolation and characterization of radiolabelled substrate-alkylated products of cytochrome *P*-450 haem. 4. Our findings indicate that, although hepatic uptake of parenteral haemoglobin is slower than that of haem, it appears to serve as an effective haem donor to the intrahepatic 'free' haem pool. Thus parenteral haemoglobin may warrant consideration as a therapeutic alternative to haem in the acute hepatic porphyrias.

INTRODUCTION

Haem (iron-protoporphyrin IX) is the prosthetic moiety of a wide variety of haemoproteins that include haemoglobin, myoglobin, cytochromes *P*-450 and *b*₅, tryptophan pyrrolase, catalase and some of the mitochondrial cytochromes. In the liver, haem required for hepatic haemoprotein formation is believed to be derived largely from a 'free' haem pool (Tschudy & Bonkowsky, 1972; Schmid, 1973; De Matteis, 1973; Granick *et al.*, 1975; Bissell & Hammaker, 1976). This pool consists both of haem newly synthesized in the mitochondria and of haem mobilized in the course of normal hepatic haemoprotein turnover. The possibility that haemoglobin haem dissolved in plasma and/or contained in senescent erythrocytes also may contribute to this hepatocyte pool has not been documented. The size of this pool is finely controlled, in part by haem itself, which regulates its own synthesis and degradation (Tschudy & Bonkowsky, 1972; Schmid, 1973; De Matteis, 1973; Granick *et al.*, 1975; Bissell & Hammaker, 1976). Thus haem in excess of cellular requirements represses δ -aminolaevulinic acid synthetase (ALAS), the rate-limiting enzyme in haem synthesis (Granick, 1966; Marver *et al.*, 1968; Sassa & Granick, 1970), and induces microsomal haem oxygenase, the rate-limiting enzyme in the degradation of haem to bilirubin (Tenhunen *et al.*, 1970).

Circulating endogenous haem or exogenously administered haem is efficiently taken up by hepatocytes and gains access to the 'free' haem pool, where it is

functionally indistinguishable from intracellular haem. Thus administered haem results in repression of ALAS, induction of haem oxygenase, formation of bilirubin and incorporation of haem into hepatic haemoproteins (Tenhunen *et al.*, 1970; Granick *et al.*, 1975; Bissell & Hammaker, 1976, 1977; Correia *et al.*, 1979). Collectively, these properties have provided the rationale for parenteral administration of exogenous haem to patients with acute and often life-threatening attacks of hepatic porphyrias and other situations associated with hepatic haem depletion (Bonkowsky *et al.*, 1971; McColl *et al.*, 1981).

Use of haem in the treatment of acute hepatic porphyrias is occasionally compromised by toxicity; adverse effects of haem administered in the form of haematin include vascular reactions, which range from local irritation to generalized allergic vasculitis, coagulopathy, haemorrhage and thrombosis (Anderson *et al.*, 1942; Dhar *et al.*, 1975; Lamon *et al.*, 1980; McColl *et al.*, 1981; Morris *et al.*, 1981; Glueck *et al.*, 1983). Acute renal failure has been observed after larger doses of haem (Dhar *et al.*, 1978) and is attributed to intravascular polymerization and/or the presence of haem aggregates in the preparation. On the other hand, intravenous infusion of stroma-free haemoglobin solutions has been employed successfully for the resuscitation of patients with severe hypovolaemia, without the adverse effects observed with parenteral haem (Peskin *et al.*, 1969; Elia *et al.*, 1974; Palani *et al.*, 1975; De Venuto *et al.*, 1979; Stone *et al.*, 1979). Thus, provided that haemoglobin is

Abbreviations used: AIA, allylisopropylacetamide; ALA(S), aminolaevulinic acid (synthetase); PCNMA, *p*-chloro-*N*-methylaniline.

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taken up efficiently by the hepatocyte (Garby & O'Hara, 1960; Keene & Jandl, 1965), and is dissociated readily into its constitutive moieties, it should serve as a haem donor to the 'free' haem pool, and hence provide a potential alternative to parenteral haematin for the treatment of porphyric crises. We have investigated this possibility both in intact rats and in isolated perfused rat liver by examining whether haemoglobin can donate its prosthetic haem to the 'free' haem pool, and in turn be incorporated into functional hepatic haemoproteins such as tryptophan pyrrolase and cytochrome *P*-450.

MATERIALS AND METHODS

Materials

Allylisopropylacetamide (AIA) and [^{14}C]AIA (specific radioactivity 7.4 mCi/mmol) were generously donated by Dr. W. Scott and Dr. J. Burns, Hoffman-La Roche, Nutley, NJ, U.S.A. δ -Amino[2,3- ^3H]laevulinic acid hydrochloride ([^3H]ALA; specific radioactivity 30 Ci/mmol) was obtained from Research Products International Corp., Mt. Prospect, IL, U.S.A. Phenylhydrazine hydrochloride was purchased from Eastman-Kodak, Rochester, NY, U.S.A. The sources of all other chemicals used in this study have been described previously (Correia *et al.*, 1979; Farrell & Correia, 1980; Farrell *et al.*, 1981). Other chemicals and solvents were of reagent-grade quality.

Animals

Male Sprague-Dawley rats (225–275 g) were obtained from Simonsen Laboratories, Gilroy, CA, U.S.A., and provided with water and laboratory rat chow (Ralston Purina Co., St. Louis, MO, U.S.A.) *ad libitum*, unless stated otherwise.

Preparation and physicochemical characterization of haemoglobin and haem solutions

Preparation of rat haemoglobin. Whole rat blood was collected from the abdominal aorta in a heparinized syringe. Erythrocytes were sedimented, washed and then lysed in a cell-disruption chamber (Parr Instruments, Moline, IL, U.S.A.; 6.9 MPa (1000 lbf/in 2) of N_2 for 30 min). Haemoglobin was separated from erythrocyte stroma by centrifugation at 27000 *g* for 20 min, filtered through a 0.45 μm -pore-size Millipore filter (Millipore Filter Corp., Bedford, MA, U.S.A.), quantified (see below), and diluted with 0.1 M-phosphate buffer, pH 7.4, to the desired concentration. In all cases, haemoglobin was administered intravenously in a 1 ml volume.

Preparation of haemin. Crystalline equine haemin or radiolabelled rat haemin, prepared as described below, was dissolved in 0.1 M-NaOH and then neutralized by dilution with 0.1 M-phosphate buffer, pH 7.4, to the desired concentration. Solutions were prepared fresh for all experiments, and were protected from light and stored on ice until used.

Preparation of ^3H -labelled haemoglobin and haemin. Crystalline [^3H]haemoglobin was prepared by incubating rat reticulocytes with [^3H]ALA (4 mCi) as described by Goldstein *et al.* (1966). Crystals were collected by centrifugation, washed five times with distilled water and stored at 4 °C until used. Immediately before

[^3H]haemoglobin administration, the crystals were suspended in 0.1 M-phosphate buffer, pH 7.4, containing EDTA (1 mM) and dissolved by titrating to pH 9 with 0.1 M-NaOH. Undissolved haemoglobin was removed by centrifugation at 27000 *g* for 10 min. Any methaemoglobin that might have been formed during synthesis and storage was converted into oxyhaemoglobin by gel filtration through a sodium dithionite reducing column (Dixon & McIntosh, 1967). After such treatment, 2–8% methaemoglobin remained in the haemoglobin solution, whereas freshly prepared native haemoglobin contained less than 1%.

[^3H]Haemin was extracted and crystallized from [^3H]haemoglobin by the method of Labbe & Nishida (1957). The crystals were dried overnight under vacuum, and their purity was verified by t.l.c., equine haemin being used as the standard. With a mobile phase of hexane/propan-1-ol/acetic acid (20:10:3, by vol.), [^3H]haemin migrated in a single band with the same R_F value (0.33) as the haemin standard.

Quantification of haemin and haemoglobin. Haemin and haemoglobin concentrations were assayed by the pyridine haemochromogen method (Falk, 1964; Waterman, 1978). The concentration of haemin was obtained directly, whereas haemoglobin concentration required dividing the value obtained for the pyridine haemochromogen by 4 (i.e., 4 mol of haem per mol of haemoglobin). Specific radioactivity of haemin or haemoglobin was determined, from 50–100 μl portions of known haem concentration, by liquid-scintillation spectrometry.

Physicochemical characterization of haemoglobin. The physicochemical authenticity of the radiolabelled haemoglobin preparation was verified by comparing the spectral binding characteristics of various ligands to those of an authentic haemoglobin standard. The spectra of the oxy, deoxy and carbonmonoxy forms of radioisotopically labelled haemoglobin, obtained by exposing haemoglobins to O_2 , sodium dithionite or carbon monoxide respectively were found to be identical with those of native haemoglobin treated in a comparable manner (results not shown).

Oxygen binding was evaluated by analysing the oxygen dissociation curves for the labelled and native haemoglobins, obtained by using an Aminco Hem-O-Scan oxygen dissociation analyser as described by the manufacturers (American Instrument Co., catalogue no. J-4-9675A; instruction 1054-B). The P_{50} values (mean $\pm$ s.d.) were found to be 2.17 ± 0.15 kPa (16.3 ± 1.1) and 2.63 ± 0.16 kPa (19.7 ± 1.2 mmHg), and the Hill coefficients (*h*) 1.76 ± 0.03 and 2.02 ± 0.06 for the labelled and native haemoglobin solutions respectively. Because of the close similarity of these values to those of native haemoglobin, the synthetic haemoglobin was assumed to be of adequate purity.

Determination of hepatic uptake rates of haemoglobin and haem

The extent of incorporation of haem into cytochrome *P*-450 is a function not only of the amount of haem injected but also of the rate of its hepatic uptake. Thus the relative efficacy of parenteral haemoglobin as a substitute for haem would be determined in part by the extent to which its haem moiety is incorporated into the 'free' haem pool, as well as by its rate of hepatic uptake

Fig. 1. Total hepatic uptake and biliary secretion of radioactivity administered as [³H]haemoglobin or native haemoglobin + [³H]haem

For experimental details, see the Materials and methods section and Table 1. Radioactivity is expressed in haem equivalents/liver or /bile output rather than as a percentage of radioactivity injected (Table 1). The values are obtained by dividing the radioactivity in liver or bile by the specific radioactivity of the haem administered either as [³H]haemoglobin or [³H]haem. ● and ○ represent [³H]haemoglobin and native haemoglobin + [³H]haem respectively. Continuous and broken lines respectively represent radioactivity present in the liver and secreted in the bile at the time points indicated.

relative to that of haem in the form of methaemalbumin or methaemoglobin. It is apparent that hepatocyte uptake of methaemoglobin haem and haem prosthetically bound to haemoglobin occurs by different mechanisms (Muller-Eberhard, 1970; Bissell *et al.*, 1972; Bunn, 1972; Kino *et al.*, 1980). Circulating methaemoglobin haem dissociates readily from globin, is bound by the haem-binding protein haemopexin, and is believed to be transported in this form to the liver (Muller-Eberhard, 1970; Bissell *et al.*, 1972; Bunn, 1972). In contrast, haemoglobin is bound by serum haptoglobin and the entire haemoglobin-haptoglobin complex is internalized by the hepatocyte (Bissell *et al.*, 1972; Kino *et al.*, 1980), whereupon haem is liberated by an as-yet-unidentified mechanism to the 'free' haem pool.

In these experiments, the relative uptake of haemoglobin (and/or methaemoglobin) and haem into the liver was determined *in vivo* in phenobarbital-pretreated rats provided with a bile-duct cannula. To determine the relative hepatic uptake of haemoglobin and of contaminating methaemoglobin and their relative contributions to the 'free' haem pool, it was necessary to employ haemoglobin that was quantitatively matched in haem content to labelled haemoglobin, but which contained radioactive haem in amounts equivalent to that of any methaemoglobin contaminating the isotopic preparation.

Table 1. Percentage of radioactivity in liver and bile after injection of [³H]haemoglobin or native haemoglobin + [³H]haem

In a typical experiment, 1 ml of [³H]haemoglobin (470 nmol in haem equivalents; 7.4% methaemoglobin content) was injected intravenously. Native haemoglobin (0.7% methaemoglobin content) was similarly injected in 470 nmol of haem equivalents containing 42.6 nmol of added [³H]haem of specific radioactivity similar to that of the [³H]haemoglobin haem (45 630 d.p.m./nmol). Values are means ± s.d. from three individual experiments.

Injected preparation	Observation time (min) ...	[³ H]Haemoglobin (percentage of radioactivity injected)				Native haemoglobin + [³ H]haem (percentage of radioactivity injected)			
		15	30	60	120	15	30	60	120
Liver		5.8 ± 0.9	12.6 ± 1.7	16.7 ± 4.9	28.6 ± 10.9	19.0 ± 6.9	21.5 ± 12.4	27.9 ± 9.2	38.8 ± 6.6
Bile		0.2 ± 0.1	0.5 ± 0.3	2.9 ± 1.7	17.2 ± 2.7	0.5 ± 0.2	2.6 ± 1.4	10.1 ± 3.6	24.4 ± 7.5
Total		6.0	13.1	19.6	45.8	19.5	24.1	38.0	63.2
Ratio of bile/liver radioactivity ...		0.03	0.04	0.17	0.60	0.02	0.12	0.36	0.62

Scheme 1. Experimental protocol for structural and functional reconstitution of cytochrome *P*-450 by haemoglobin haem in isolated perfused rat liver

This was achieved by adding radiolabelled haem (isolated from the labelled haemoglobin and therefore of identical specific radioactivity) to native, unlabelled, haemoglobin. Indeed, haem added in this manner was spectrally indistinguishable from methaemoglobin and could be quantified by the standard assay (see above).

Hepatic uptake of [^3H]haem administered as [^3H]haemoglobin or native haemoglobin + [^3H]haem (methaemoglobin control) was monitored on the basis of total radioactivity recovered from liver homogenates and bile over a 2 h period. Since the amount of [^3H]haem administered as haemoglobin was 18 times that of the methaemoglobin control (470 and 26 nmol respectively), the total radioactivity recovered from liver and bile (expressed in [^3H]haem equivalents) was far greater in the animals that received [^3H]haemoglobin than in the [^3H]methaemoglobin-treated control (Fig. 1). However, when hepatic uptake of [^3H]haem was expressed as a percentage of administered radioactivity, it was evident that the total hepatic (liver + bile) uptake of haem as methaemoglobin is more efficient than that of haemoglobin haem (38% as against 19.6% at 1 h respectively; Table 1). Moreover, because haem catabolized to bilirubin originates from the 'free' haem pool, bilirubin excretion into the bile also reflects haem incorporated into the hepatic 'free' haem pool. Thus, as shown by the appearance of radioactivity (predominantly as bilirubin) in bile, methaemoglobin [^3H]haem was incorporated into the 'free' haem pool to a larger extent than was haemoglobin [^3H]haem (Table 1). Hence haemoglobin can contribute its prosthetic haem to the 'haem' pool, but it does so somewhat more slowly than non-prosthetically bound haem preparations.

Incorporation of haemoglobin haem into hepatic tryptophan pyrrolase

Freshly prepared haemoglobin (1 ml, 350–400 nmol) was administered as an intravenous bolus to rats, and control animals received an equivalent volume of 0.1 M-phosphate buffer. Hepatic holo- and total tryptophan pyrrolase activity was determined as described previously (Badawy & Evans, 1975; Bissell & Hammaker, 1977; Correia & Burk, 1978), and percentage haem saturation of the enzyme was calculated as follows:

$$\text{Percentage saturation} = \frac{\text{Holoenzyme activity}}{\text{Total enzyme activity}} \times 100$$

Incorporation of haemoglobin haem into hepatic cytochrome *P*-450

Three criteria were used to determine whether haemoglobin haem was incorporated into cytochrome *P*-450: (1) a spectrally measured increase in hepatic content of cytochrome *P*-450, after its destruction by the 'suicide' inactivator AIA and its reconstitution by haemoglobin haem; (2) restoration of mixed-function-oxidase activity of this reconstituted cytochrome; and (3) incorporation of haemoglobin haem into AIA porphyrin adducts, which are formed exclusively from the prosthetic haem moiety of cytochrome *P*-450 during its oxidative metabolism of AIA and as such serve as reporters of this event.

The first two criteria were examined in isolated rat liver perfused in a recirculating system with a haemoglobin-free O_2 carrier, thereby circumventing haemoglobin loss by the kidneys and maximizing hepatic uptake of haemoglobin. The third criterion was examined both in isolated perfused rat livers and in intact, nephrectomized, rats. In these studies, except where otherwise indicated, phenobarbital-pretreated rats were employed because AIA has been shown to selectively destroy the phenobarbital-inducible cytochrome *P*-450 isoenzymes (Farrell & Correia, 1980, and references therein).

Isolated perfused liver

Livers of male Sprague-Dawley rats (235–275 g), pretreated with phenobarbital [80 mg/kg, i.p. (intraperitoneally) daily, for 5 days] were perfused as detailed previously (Farrell *et al.*, 1981; Gollan *et al.*, 1981) with a haemoglobin-free medium consisting of perfluorotributylamine emulsion as oxygen carrier in a Krebs-Ringer bicarbonate solution (Oxypherol; Alpha Therapeutic Corp., Los Angeles, CA, U.S.A.). The experimental design for these studies (Scheme 1) was as follows. Rats were paired on the basis of body weight, and two perfusions, a haemoglobin treatment and a 0.89% NaCl control, were performed on the same day. After a 15 min equilibration period, haemoglobin (570–580 nmol) or 0.89% NaCl (control) was added to the perfusion medium by slow infusion over 15 min. Next, AIA was added to the perfusate as a bolus [90 μmol in Oxypherol (5 ml) containing 8.3 μmol of bovine serum albumin (fraction V)]. This concentration of AIA was previously determined to be optimal for

cytochrome *P*-450 destruction in the isolated perfused rat liver (Farrell *et al.*, 1981). Control experiments for the reconstitution of cytochrome *P*-450 were performed in an identical manner, except that either haemoglobin or AIA was omitted. All experiments were terminated 2.5 h from the start of perfusion (i.e. 2 h after the addition of AIA).

Spectral and functional reconstitution of cytochrome *P*-450

At the end of each perfusion, the liver was flushed through the portal-vein cannula with 60 ml of ice-cold 0.15 M-KCl. Microsomal cytochrome *P*-450 content was determined by the CO+dithionite-CO difference spectrum by using an absorption coefficient of $100 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Estabrook *et al.*, 1972). The protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. The functional restoration of cytochrome *P*-450-dependent mixed-function-oxidase activity was determined by assaying microsomal *N*-demethylation of two prototype substrates, ethylmorphine and PCNMA, as detailed previously (Farrell & Correia, 1980).

Formation of radiolabelled AIA-porphyrin adducts

Preliminary experiments were conducted in isolated rat livers as described above, except that [^3H]haemoglobin was used. Although radiolabelled AIA-porphyrin adducts were isolated from the perfused liver (results not shown), it was theoretically possible that [^3H]haemoglobin might be oxidized to methaemoglobin under the relatively high oxygen partial pressure of the fluorocarbon-containing perfusate and that the formation of radiolabelled adducts might merely indicate the incorporation of the readily dissociable haem from methaemoglobin generated in the perfusate.

To exclude this possibility, these experiments were also performed in phenobarbital-pretreated intact rats. To minimize the renal loss of haemoglobin and thereby maximize hepatic uptake, the kidneys were surgically ligated and the animals allowed to recover for 1 h before administration of AIA or vehicle. The experimental protocol was as follows. Nephrectomized rats were divided into two groups of four rats. Two rats from each group were injected subcutaneously with AIA (200 mg/kg); the other two received saline and served as the non-AIA-treated controls. After 30 min all four animals of one group received intravenous [^3H]haemoglobin (471 μM in haem equivalents), while the other group received an equivalent amount (475 μM , total, in haem equivalents) of native haemoglobin with [^3H]haemin added to control for the [^3H]methaemoglobin contamination in the labelled-haemoglobin preparation. At 2 h after haemoglobin administration the animals were killed by decapitation and the livers removed and weighed before and after perfusion with ice-cold KCl (0.15 M). Portions (2–5 g) of liver were suspended (1:10, w/v) in 0.1 M-phosphate buffer, pH 7.4, and used for preparation of microsomes (microsomal fractions) and CO-binding particles (Bissell & Hammaker, 1977; Correia *et al.*, 1979). Cytochromes *P*-450 and *b*₅, total haem, total radioactivity and haem-specific radioactivity were assayed in homogenates, microsomes and/or CO-binding particles as described (Correia *et al.*, 1979). The rest of the AIA-treated livers were used for the isolation of AIA-porphyrin adducts.

Isolation and purification of AIA-porphyrin adducts

AIA-porphyrin adducts in the form of zinc complexes were isolated from livers of rats given AIA and further purified by sequential h.p.l.c. as described previously (Correia *et al.*, 1981). The porphyrin adducts were considered pure when a constant specific radioactivity was attained. The identity of the zinc complex was established by its characteristic absorption spectrum, and confirmed by its subsequent conversion into the zinc-free derivative (Ortiz de Montellano *et al.*, 1979; Correia *et al.*, 1981). The average recovery of AIA-porphyrin adducts, calculated by the amount isolated after t.l.c., was determined as follows. Purified radiolabelled AIA-porphyrin adducts (zinc complex) were added to liver homogenates obtained from AIA-treated rats and the adducts extracted and isolated as described above. On the basis of total radioactivity recovered, the amount of AIA-porphyrin adducts isolated after t.l.c. was 35% (mean value from five experiments).

Isolation of labelled adducts from SKF 525-A-treated rats

Nephrectomized rats (not pretreated with phenobarbital) were administered SKF 525-A (60 mg/kg, i.p.) 1 h before subcutaneous injection of AIA (200 mg/kg). At 30 min after AIA administration, radiolabelled haemoglobin (610 nmol in haem equivalents; specific radioactivity 44275 d.p.m./nmol of haem) was injected intravenously. In control experiments, animals were injected with saline instead of haemoglobin, AIA or SKF 525-A. Animals were killed 2 h after administration of labelled haemoglobin or saline, and the AIA-porphyrin adducts were quantified in their livers as described above.

Statistical analysis

Differences between the values obtained in studies of cytochrome *P*-450 reconstitution in isolated perfused livers (conducted in pairs) were analysed for statistical significance employing the Student's paired *t* test. Values from all other experiments were analysed by using the unpaired *t* test.

RESULTS AND DISCUSSION

Incorporation of haemoglobin haem into hepatic tryptophan pyrrolase

Hepatic cytosolic tryptophan pyrrolase is believed to be in dynamic equilibrium with the 'free' haem pool, such that minor fluctuations in the size of this pool are reflected by enzymic activity of the haemoprotein. This relationship exists because normally the haemoprotein is present partly as the functional holoenzyme and partly as the haem-free apoenzyme (Feigelson & Greengard, 1961; Badawy & Evans, 1975; Bissell & Hammaker, 1977; Badawy, 1978). The apoenzyme can be functionally constituted *in vivo* by various experimental manoeuvres that expand the 'free' haem pool (Bissell & Hammaker, 1977). Thus increased tryptophan pyrrolase activity resulting from haem saturation of the apoenzyme in haemoglobin-treated rats would constitute direct evidence that haemoglobin haem was indeed incorporated into the 'free' haem pool and then into this haemoprotein.

At 30 min after intravenous administration of haemoglobin to rats, haem saturation of tryptophan pyrrolase

Fig. 2. Reconstitution of hepatic tryptophan pyrrolase after administration of haemoglobin to rats

Freshly prepared haemoglobin (350–400 nmol) was administered intravenously to rats in a 1 ml volume (for details see the Materials and methods section). Control animals received an equivalent volume of 0.1 M-phosphate buffer. Values are means $\pm$ S.D. from three individual experiments. ○ and ● represent haemoglobin-treated and control animals respectively.

was significantly ($P < 0.05$) increased above that of the control animals (Fig. 2). This functional haem saturation of the enzyme continued to increase progressively to a maximal level of 80–90% over the ensuing 3 h. These findings clearly indicate that haemoglobin haem is incorporated into apo-(tryptophan pyrrolase) and results in a functional haemoprotein. Moreover, the observed increase in haem saturation of hepatic tryptophan pyrrolase cannot be ascribed to methaemoglobin formed on storage of the haemoglobin (Stowell & Morland,

1983; Badawy *et al.*, 1983); the injected haemoglobin was freshly prepared and contained less than 1% methaemoglobin, and the livers were assayed for tryptophan pyrrolase activity promptly after killing the animals and perfusion *ex situ*. The rate of haemoglobin haem incorporation into tryptophan pyrrolase is comparable with that of haemin administered i.p. (Badawy & Evans, 1975), but is somewhat slower than that reported for methaemalbumin administered intravenously (Yamamoto *et al.*, 1981). These findings demonstrate that intravenously administered haemoglobin haem is readily available to the 'free' haem pool, although in comparison with haem, its hepatic uptake and subsequent dissociation within the hepatocyte may occur somewhat more slowly.

Incorporation of haemoglobin haem into hepatic cytochrome P-450

Addition of AIA to the perfusate of isolated livers from phenobarbital-pretreated rats resulted in a mean decrease of 35% in cytochrome P-450 content and a 60% decrease in its ethylmorphine *N*-demethylase activity (Table 2), in agreement with our previous findings (Farrell & Correia, 1980; Farrell *et al.*, 1981). PCNMA *N*-demethylase activity was not significantly altered, consistent with the observation that AIA-mediated inactivation is somewhat selective for particular phenobarbital-inducible cytochrome P-450 species (Farrell & Correia, 1980; Ortiz de Montellano & Correia, 1983, and references cited therein). Inclusion of haemoglobin in the perfusate of AIA-treated livers produced a modest but significant increase in hepatic cytochrome P-450 content and a significant restoration of ethylmorphine *N*-demethylase activity (Table 2). These parameters were not increased significantly when haemoglobin was added to the perfusate of livers that had not been pretreated with AIA. Such increases might have been even greater had benzphetamine *N*-demethylation also been monitored at the time these studies were conducted. Recent findings indicate that exogenous haem restores benzphetamine *N*-demethylase activity to a much greater extent than that of ethylmorphine *N*-demethylase (Bonkowsky *et al.*, 1984; Bornheim *et al.*, 1985).

Table 2. Effects of haemoglobin (Hb) on hepatic microsomal cytochrome P-450 content and *N*-demethylase activity in isolated rat livers treated with AIA

Freshly prepared rat haemoglobin (560–580 nmol) was infused into the perfusate for 15 min, followed by the addition of AIA (90 μ mol). Livers were perfused for 2 h after the addition of AIA (see the Materials and methods section). Values are means $\pm$ S.D.; *n*, number of independent paired observations; NS, not significant.

Preparation	Cytochrome P-450 (nmol/mg of protein)	Increase (%)	<i>N</i> -Demethylase activity (nmol of formaldehyde formed/15 min per mg of protein)			
			Ethylmorphine	Increase (%)	PCNMA	Increase (%)
AIA-treated +Hb	1.34 $\pm$ 0.24	20.7 ($P < 0.005$)	194 $\pm$ 48	40.1 ($P < 0.05$)	89 $\pm$ 14	27.1 (NS)
–Hb	1.11 $\pm$ 0.13 (<i>n</i> = 11)		138 $\pm$ 42 <i>n</i> = 5		70 $\pm$ 25 (<i>n</i> = 5)	
Untreated +Hb	1.97 $\pm$ 0.15	13.9 (NS)	387 $\pm$ 23	9.3 (NS)	89 $\pm$ 16	1.1 (NS)
–Hb	1.73 $\pm$ 0.16 (<i>n</i> = 5)		354 $\pm$ 70 (<i>n</i> = 3)		88 $\pm$ 11 (<i>n</i> = 3)	

Table 3. Hepatic cytochromes *P*-450 and *b*₅, haem content and radioactivity after administration of [³H]haemoglobin ([³H]Hb) or native haemoglobin + [³H]haem (Hb + [³H]haem) to AIA-treated rats

Rats were killed 2 h after administration of either [³H]Hb (471 nmol of haem equivalents/ml; specific radioactivity 45630 d.p.m./nmol) or native haemoglobin (475 nmol of haem equivalents/ml containing 33.4 nmol of [³H]haem; specific radioactivity 49810 d.p.m./nmol), which was added to compensate for the [³H]methaemoglobin content of the [³H]Hb preparation (see the Materials and methods section for the rationale). Liver preparations were assayed as described in the Materials and methods section. 'Haem-specific radioactivity' represents that radioactivity in the cellular preparation intrinsically associated with the haem moiety as determined after isolation and purification of haem. Values presented are from a typical experiment and represent the mean result for two animals.

Liver fraction	Cytochrome <i>P</i> -450 (nmol/g of liver)		Cytochrome <i>b</i> ₅ (nmol/g of liver)		Haem (nmol/ g of liver)		Haem-specific radioactivity (d.p.m./g of liver)	
	Control	AIA	Control	AIA	Control	AIA	Control	AIA
Homogenate								
[³ H]Hb	50.4	15.7	—	—	151.0	91.0	361 000	171 300
Hb + [³ H]haem	39.4	13.0	—	—	143.8	85.3	42 700	18 300
Microsomes								
[³ H]Hb	26.2	6.3	8.5	8.1	67.6	28.1	127 700	43 400
Hb + [³ H]haem	27.2	6.5	8.2	7.0	54.8	26.1	16 200	10 600
Co-particles								
[³ H]Hb	23.3	5.8	1.6	1.1	45.6	15.1	105 400	26 400
Hb + [³ H]haem	19.4	6.2	2.0	1.6	41.2	16.2	17 100	7 000

Table 4. Conversion of hepatic cytochrome *P*-450 haem to AIA-porphyrin adducts in rats treated with labelled haemoglobin ([³H]Hb) or native haemoglobin + labelled haem (Hb + [³H]haem)

Rats 1, 2 and 3 were administered [³H]Hb (471 nmol of haem equivalents/ml), whereas rats 4 and 5 received native Hb (475 nmol of haem equivalents/ml containing 33.4 nmol of [³H]haem, corresponding to the [³H]methaemoglobin content of the [³H]Hb preparation). Animals were killed 2 h later and their livers assayed as described in the Materials and methods section.

Treatment	Loss of haem* (nmol/g of liver)	Loss of haem-specific radioactivity† (d.p.m./g of liver)	Loss of [³ H]haem‡ (nmol/g of liver)	AIA adducts recovered§ (nmol/g of liver)	Radioactivity of adducts (d.p.m./g of liver)	[³ H]AIA adducts formed (nmol/g of liver)	Recovery of AIA adducts (% of lost [³ H]haem)
[³ H]Hb							
Rat 1	67.0	169 700	3.72	36.4	57 670	1.28	34.4
Rat 2	62.6	166 800	5.43	24.9	33 050	1.04	19.1
Rat 3	53.4	130 500	4.25	27.4	45 750	1.28	30.1
Hb + [³ H]haem							
Rat 4	61.4	24 880	0.50	32.1	14 350	0.35	70.0
Rat 5	53.9	24 050	0.48	46.2	12 910	0.47	97.9

* 'Loss of haem' represents the total amount of haem that was lost from the rat liver due to AIA treatment. It reflects the difference between the haem content of liver homogenates of rats infused with [³H]Hb or Hb + [³H]haem, but *not* pretreated with AIA (control), and that of corresponding rats treated with AIA.

† Loss of haem-specific radioactivity similarly represents the difference in the radioactivity of haem isolated from the liver homogenates of untreated rats and AIA-treated rats after haemoglobin infusion.

‡ Determined by dividing the haem-specific radioactivity by the specific radioactivity of [³H]haemoglobin haem (rat 1 = 45 630 d.p.m./nmol and rats 2 and 3 = 30 700 d.p.m./nmol) or [³H]haem isolated from [³H]haemoglobin (49 810 d.p.m./nmol).

§ Determined spectrophotometrically by using a millimolar absorption coefficient of 125 mm·cm⁻¹ at 432 nm; values presented were corrected for 35% recovery of AIA adducts after t.l.c., on the basis of control experiments using known amounts of purified zinc-complexed [³H]adducts.

|| Determined by dividing radioactivity of adducts by the specific radioactivity of [³H]haemoglobin haem or [³H]haem isolated from [³H]haemoglobin, as in footnote ‡.

Moreover, that haemoglobin was not protecting cytochrome *P*-450 from AIA-mediated inactivation, either by limiting its hepatic uptake or impairing its metabolism and elimination, was suggested by preliminary studies in which [¹⁴C]AIA disappearance from the

perfusate was monitored (results not shown). However, in keeping with the restoration of mixed-function-oxidase activity, [¹⁴C]AIA disappearance actually increased, although the values obtained from haemoglobin-perfused versus control livers were not statistically different. This

Table 5. Relative formation of radiolabelled AIA-porphyrin adducts in rats from haem prosthetically bound to haemoglobin (Hb) and that present as contaminating methaemoglobin (MetHb)

The values listed below were calculated directly from data given in Tables 1, 3, and 4 and preliminary experiments.

	Amount of [³ H]haem injected (nmol/g of liver)	Hepatic uptake of [³ H]haem† (nmol/g of liver)	[³ H]AIA adducts formed (nmol/ g of liver)	[³ H]AIA adducts (% of hepatic [³ H]haem uptake‡)	Contribution of radioactive haem to [³ H]AIA adducts (%)
[³ H]Hb	42.0	19.2	1.20§	6.3	100
Hb + [³ H]haem	2.4	1.53	0.41§	26.8	34.2¶
MetHb	2.9*	1.83	0.49	—	40.8**

* [³H]Hb amount injected multiplied by the percentage of methaemoglobin initially present in the labelled preparation plus that determined to be formed intravascularly under simulated conditions after 2 h; total = 7.54%.

† Amount of [³H]haem injected multiplied by the mean total (liver + bile) percentage of [³H]haem uptake, i.e. 45.8% for [³H]Hb or 63.2% for Hb + [³H]haem (mean values for three expts; detailed in Table 1).

‡ Percentage of total [³H]adducts formed, divided by the total hepatic uptake of [³H]haem.

§ Mean values from Table 4.

|| Value calculated from hepatic uptake of [³H]haem (1.83 nmol/g of liver) and its percentage conversion (26.8) to radiolabelled AIA adducts.

¶ Contribution by MetHb haem initially present.

** Contribution by MetHb haem possibly present 2 h after administration, i.e. initial plus that potentially formed intravascularly.

is in contrast with our previous observation that hepatic [¹⁴C]AIA disappearance was significantly enhanced by the addition of haem to the perfusate (Farrell *et al.*, 1979); this apparent discrepancy may be explained by the somewhat slower release of haemoglobin haem into the hepatic 'free' haem pool.

Confirmation that the prosthetically bound haem of haemoglobin is incorporated into hepatic cytochrome *P*-450 in intact rats was obtained by means of a probe, i.e., the generation of AIA adducts (Correia *et al.*, 1979, 1981). These adducts are formed solely from the prosthetic haem moiety of cytochrome *P*-450 and AIA during cytochrome *P*-450-dependent oxidative metabolism of AIA by a process termed 'suicidal inactivation' (Correia *et al.*, 1981; Ortiz de Montellano & Correia, 1983). With this probe (Tables 3 and 4), it was shown that the administration of radiolabelled haemoglobin to rats results in hepatic formation of radiolabelled AIA-porphyrin adducts. This finding confirms that labelled haem present in the haemoglobin preparation was incorporated prosthetically into cytochrome *P*-450 before generation of the AIA-porphyrin adduct. Because radiolabelled haem may have been both tightly (prosthetically) and loosely (i.e. methaemoglobin) bound to haemoglobin, this observation did not unambiguously identify the specific source of labelled haem in the adduct. However, inclusion of the native haemoglobin + [³H]haem control enabled precise quantification of the incorporation of loosely bound haem into the adducts and verification that prosthetic haemoglobin haem had indeed been incorporated into cytochrome *P*-450 (Table 4). By using this approach, we observed that, over the initial 2 h period, loosely bound haem is incorporated more efficiently (70–98%) than was prosthetic haemoglobin haem (20–35%) into the labelled adducts (Table 4). This difference may be due simply to the relatively slower hepatic uptake of haemoglobin relative to that of haem during the initial period (Scheme 1, Table 1). Also, once taken up by the hepatocytes, the efficiency of haemoglobin haem incorporation into the 'free' haem pool and its utilization (i.e. catabolism to

bilirubin) are low initially but appear to increase gradually over 2 h (Table 1). Thus it is not surprising that, during the initial 2 h period, loosely bound haem would be incorporated into the adducts to a greater extent than would prosthetic haemoglobin haem (Table 4). However, when the relative incorporation of prosthetic and loosely bound haem into radiolabelled adducts is computed on the basis of labelled haem taken up either as 'haemoglobin' or 'methaemoglobin' by the liver, it is evident that loosely bound haem (i.e. initial and intravascularly generated methaemoglobin) accounts for only 41% of radiolabel in the AIA-porphyrin adducts, whereas 59% is derived from prosthetically bound haemoglobin haem. Thus, despite the fact that hepatic uptake of parenteral haemoglobin is slower than that of haem, it serves as an effective haem donor to the intrahepatic 'free' haem pool.

Because proof that haemoglobin haem is incorporated into cytochrome *P*-450 is based on the isolation of radiolabelled adducts, it was important to establish that these adducts were indeed derived from haemoglobin haem incorporated prosthetically into cytochrome *P*-450 and not directly from haemoglobin itself. The latter theoretically is possible because haemoglobin has been shown to catalyse some drug oxidations, with an efficiency often comparable with that of cytochrome *P*-450 (Juchau & Symms, 1972; Mieyal *et al.*, 1976; Blisard & Mieyal, 1979). Thus labelled adducts also could have been generated by direct oxidative alkylation of radiolabelled haemoglobin by AIA. This possibility was examined in animals that had been pretreated with SKF 525-A, an inhibitor of cytochrome *P*-450-dependent oxidations (Anders & Mannering, 1966; Jenner & Netter, 1972), but not of those catalysed by haemoglobin (Juchau & Symms, 1972). In these studies phenobarbital pretreatment was omitted to minimize the cytochrome *P*-450-dependent pathway of AIA-porphyrin adduct generation. The potential formation of any additional hepatic AIA-porphyrin adducts derived from AIA-mediated alkylation of cytochrome *P*-450 isoenzyme(s) was further minimized by SKF 525-A treatment of rats.

Thus radiolabelled adducts isolated from these rats after administration of labelled haemoglobin should reflect predominantly direct AIA-mediated haem alkylation of haemoglobin itself and, to a lesser extent, that of any residually active cytochrome *P*-450 that remained refractory to SKF 525-A inhibition. This combined contribution was determined to be 0.08 nmol/g of liver, or 7% of the total hepatic content of labelled AIA-porphyrin adducts generated by phenobarbital-pretreated rats given radiolabelled haemoglobin (Tables 4 and 5).

Collectively, these observations suggest that haemoglobin haem, both in the tightly bound (ferrous haemoglobin) and loosely bound (methaemoglobin) form is capable of a significant contribution to the hepatic 'free' haem pool. Moreover, parenterally administered haemoglobin is almost as efficient as haem in reconstituting hepatic tryptophan pyrrolase, but it is less so in the reconstitution of hepatic cytochrome *P*-450. This differential effect may be explained by the slower donation of haemoglobin haem to the hepatic 'free' haem pool, and the relatively lower utilization of hepatic haem by tryptophan pyrrolase in comparison with cytochrome *P*-450. Indeed, if the neurological dysfunction observed during acute attacks of hepatic porphyria is due to impaired tryptophan catabolism, as our recent findings intimate (Litman & Correia, 1983, 1985), then such a discrepancy should not compromise the therapeutic potential of haemoglobin as a haem substitute. Thus the administration of stroma-free haemoglobin may serve not only as a 'slow-release' form of haem but, more importantly, as an effective clinical alternative to haem therapy in the acute hepatic porphyrias.

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