Regulation of hepatic haem metabolism

Disparate mechanisms of induction of haem oxygenase by drugs and metals

Beth C. LINCOLN,[†] John F. HEALEY[†] and Herbert L. BONKOVSKY^{*†‡}

Departments of ^{*}Biochemistry and [†]Medicine and the [‡]Clinical Research Center, Emory University School of Medicine, Atlanta, GA 30322, U.S.A.

We studied drug- and metal-mediated increases in activity of haem oxygenase, the rate-controlling enzyme for haem breakdown, in chick-embryo hepatocytes *in ovo* and in primary culture. Phenobarbitone and phenobarbitone-like drugs (glutethimide, mephentyoin), which are known to increase concentrations of an isoform of cytochrome *P*-450 in chick-embryo hepatocytes, were found to increase activities of haem oxygenase as well. In contrast, 20-methylcholanthrene, which increases the concentration of a different isoform of cytochrome *P*-450, had no effect on activity of haem oxygenase. Inhibitors of haem synthesis, 4,6-dioxoheptanoic acid or desferrioxamine, prevented drug-mediated induction of both cytochrome *P*-450 and haem oxygenase in embryo hepatocytes *in ovo* or in culture. Addition of haem restored induction of both enzymes. These results are interpreted to indicate that phenobarbitone and its congeners induce haem oxygenase by increasing hepatic haem formation. In contrast, increases in haem oxygenase activity by metals such as cobalt, cadmium and iron were not dependent on increased haem synthesis and were not inhibited by 4,6-dioxoheptanoic acid. We conclude that (1) induction of hepatic haem oxygenase activity by phenobarbitone-type drugs is due to increased haem formation, and (2) induction of haem oxygenase by drugs and metals occurs by different mechanisms.

INTRODUCTION

It is now widely accepted that a major factor in the regulation of hepatic haem metabolism is the size of a small 'regulatory' haem pool (Granick *et al.*, 1975; Bonkovsky, 1982). Evidence for the existence of such a regulatory haem pool, although still indirect, is compelling. A deficiency of haem in this pool is thought to lead to a de-repression of the first and rate-controlling enzyme of haem synthesis, namely 5-aminolaevulinate (ALA) synthase (EC 2.3.1.37). In contrast, an excess of haem provided by the parenteral administration of haem to animals leads to a decrease in ALA synthase and an increase in activity and induction of haem oxygenase (EC 1.14.88.3) (Bonkovsky, 1982; Kikuchi & Yoshida, 1983). In addition, the degree of haem saturation of the enzyme tryptophan dioxygenase (EC 1.13.11.11), a cytoplasmic soluble enzyme that binds haem with a relatively low avidity, has been proposed to reflect the relative size of the hepatocyte regulatory haem pool (Badawy & Evans, 1975; Badawy & Morgan, 1980; Morgan & Badawy, 1980; Sardana & Drummond, 1985), although more recent studies in rats have suggested that tryptophan dioxygenase in intact hepatocytes is always completely saturated with respect to haem (Salter & Pogson, 1986).

The family of haemoproteins collectively called 'cytochrome *P*-450' is the major user of haem synthesized by hepatocytes (for reviews, see Sato & Omura, 1978; Bonkovsky, 1982). Several isoforms of cytochrome *P*-450 are known to be inducible by a variety of drugs and chemicals, including phenobarbitone and 20-methylcholanthrene (Sato & Omura, 1978). In several studies, an inverse relationship has been observed between

concentrations of hepatic cytochrome *P*-450 and haem oxygenase, particularly when haem oxygenase was increased in activity by administration of a variety of metals (Maines & Kappas, 1974, 1977; Sardana *et al.*, 1980; Kikuchi & Yoshida, 1983; Yoshida *et al.*, 1984). It has been pointed out that there is not an obligatory inverse relationship between concentrations of cytochrome *P*-450 and activities of haem oxygenase (Paine & Legg, 1978; Drummond *et al.*, 1982; Kikuchi & Yoshida, 1983). Administration to rats of chemicals capable of increasing the concentration of cytochrome *P*-450, such as phenobarbitone or 20-methylcholanthrene, was found not to affect activity of hepatic haem oxygenase (Schacter & Mason, 1974), although a modest increase in both enzymes was described in rats treated with toxic doses of benzene (Abraham *et al.*, 1986).

In previous studies, we and others demonstrated that chick-embryo hepatocytes, *in ovo* or in primary cell culture, provide a useful experimental system for study of the regulation of hepatic haem metabolism. In particular, chick-embryo hepatocyte cultures retain normal inducibility of ALA synthase (Granick *et al.*, 1975), at least two isoforms of cytochrome *P*-450 (Sinclair *et al.*, 1986) and haem oxygenase (Maines & Sinclair, 1977; Sardana *et al.*, 1982).

In the studies reported here, we used chick-embryo hepatocytes *in ovo* and in cell culture to elucidate further the mechanisms underlying the regulation of hepatic haem metabolism, particularly the induction of haem oxygenase. Our results show that phenobarbitone-like drugs, exemplified by glutethimide and mephentyoin, are inducers of haem oxygenase, as well as of the same isoform of cytochrome *P*-450 (Sinclair *et al.*, 1986). Results with inhibitors of haem synthesis show that the

induction of haem oxygenase and cytochrome *P*-450 by mephenytoin is dependent on an increase in haem formation. Induction of haem oxygenase by heavy metals also occurs in chick-embryo hepatocytes *in ovo* or in culture, but this induction, unlike that produced by drugs, is not mediated by haem. A portion of this work has appeared in abstract form (Lincoln *et al.*, 1986) and was presented at the annual meeting of the American Society of Biological Chemists in June 1986.

METHODS

Materials

Fertilized White Leghorn chicken eggs were from Haley Farms, Canton, GA, U.S.A. Haemin, albumin, NADPH, insulin, tri-iodothyronine, cycloheximide, glutethimide, 20-methylcholanthrene, DNAase, dexamethasone and 4,6-dioxoheptanoic acid were from Sigma, St. Louis, MO, U.S.A. Williams E medium, trypsin and antibiotics were from Gibco, Grand Island, NY, U.S.A. Porphyrins were from Porphyrin Products, Logan, UT, U.S.A. Desferrioxamine was from Ciba-Geigy, obtained through the pharmacy at Emory University Hospital. Mephenytoin was a gift from Sandoz, East Hanover, NJ, U.S.A. [^{14}C]Leucine was from New England Nuclear, Boston, MA, U.S.A. Other chemicals were reagent grade from Sigma, or Fisher, Phillipsburg, NJ, U.S.A.

Embryo treatments and sample preparation

Glutethimide and mephenytoin were injected into the egg sac in 0.2 ml of dimethyl sulphoxide. Livers from 17–18-day chick embryos were homogenized (20%, w/v) in cold 0.25 M-sucrose/20 mM-Tris/HCl, pH 7.4, in a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 10 min at 10000 *g* at 4 °C. The resulting supernatant was centrifuged at 105000 *g* for 1 h at 4 °C; the final microsomal pellet was washed and resuspended in 0.1 M-potassium phosphate/20% (v/v) glycerol/1 mM- Na_4EDTA , pH 7.4.

Cell culture

Cell cultures from 16–18-day chick-embryo livers were prepared by digestion with trypsin and DNAase, and maintained in a serum-free medium in the presence of insulin (0.17 μM , for first 24 h only), tri-iodothyronine (1.5 μM) and dexamethasone (0.76 μM) (Sinclair *et al.*, 1979). Any additions were made in dimethyl sulphoxide (1 μl /ml of medium). Controls received dimethyl sulphoxide only. Cells were rinsed, scraped into buffer with a rubber spatula and then homogenized in 0.1 M-potassium phosphate/20% glycerol/1 mM- Na_4EDTA , pH 7.4.

Assays

Cytochrome *P*-450 was measured in microsomal fractions from embryo livers, or in 8700 *g* supernatant of Lubrol-solubilized cell homogenate (Sinclair *et al.*, 1979), by the method of Omura & Sato (1964).

Haem oxygenase was measured by a modification of the method of Tenhunen *et al.* (1969). The rate of bilirubin formation at 37 °C was assessed by the increase in absorbance at 470 nm versus 540 nm. In addition to the sample, the 1.0 ml reaction mixture contained 0.085 M-potassium phosphate, pH 7.4, 5 mM-desferrioxamine, 42 μM -haemin and 25 μM -bovine serum albumin added as a haem-albumin solution, biliverdin reductase (0.05 ml

Fig. 1. Time course of effect of glutethimide on haem oxygenase (□) and cytochrome *P*-450 (○) in (a) intact chick-embryo liver and (b) cultured chick-embryo hepatocytes

(a) Embryos were injected with glutethimide (6 mg/egg). Each point represents the mean $\pm$ S.E.M. ($n = 3-5$). (b) Cultured hepatocytes were exposed to 50 μM -glutethimide. Each point represents the average of two plates, whose individual values differed by less than 10% from the mean.

of a 105000 *g* supernatant of a 20% homogenate of perfused rat liver), and 1 mM-NADPH (all final concns.). The absorption coefficient for bilirubin obtained with standards was 66 $\text{mm}^{-1} \cdot \text{cm}^{-1}$. The reaction mixture with samples from cell cultures contained 0.1–0.2 mg of homogenate protein, and those from embryo livers contained 0.05–0.4 mg of microsomal protein.

Porphyrins were determined by the method of Grandchamp *et al.* (1980). Homogenates of embryo livers were diluted with 4 vol. of a mixture of 70% (w/v) HClO_4 /water/methanol (1:10:10, by vol.), with further dilutions as necessary. Cell homogenates in medium were diluted with an equal volume of HClO_4 /methanol (1:9, v/v). The porphyrin patterns in some samples were verified by h.p.l.c. (Bonkovsky *et al.*, 1986).

The rate of protein synthesis in cultured hepatocytes

Fig. 2. Dose-response of haem oxygenase (a, c) and cytochrome *P*-450 (b, d) to glutethimide, mephentoin, phenobarbitone and 20-methylcholanthrene in intact chick-embryo liver (a, b) and in cultured chick-embryo hepatocytes (c, d)

(a), (b), Representative experiment of a 24 h exposure of cultured chick hepatocytes to drugs. Each point represents a single plate. (c), (d), 24 h exposure of chick embryos to drugs. Each point represents the average of several experiments: for glutethimide (glut) $n = 3-4$; phenobarbitone (PB) $n = 2$; mephentoin (meph) $n = 4$; 20-methylcholanthrene (20-MC) $n = 4$.

was estimated by the incorporation of [U - ^{14}C]leucine ($344 \mu\text{Ci}/\mu\text{mol}$; $0.05 \mu\text{Ci}/3.5 \text{ cm}$ plate) into protein over 2 h. After the 2 h exposure, the cells were rinsed with 0.15 M-NaCl, and the protein was precipitated with 5% (w/v) trichloroacetic acid. The protein precipitate was rinsed with 0.15 M-NaCl and scraped into 0.1 M-NaOH/0.1% (w/v) deoxycholate; portions were used for measurement of radioactivity and protein concentration.

Ouchterlony analysis

Rabbit antiserum was produced against a form of cytochrome *P*-450 purified from glutethimide-induced chick-embryo livers (Sinclair *et al.*, 1986). The Ouchterlony double diffusion followed the method of Thomas *et al.* (1976).

Other methods

Proteins were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Experiments were repeated two to four times; unless otherwise noted, representative data from a single experiment are presented in the Figures.

RESULTS

Glutethimide increased both cytochrome *P*-450 and haem oxygenase in embryo liver *in ovo* and in cultured chick-embryo hepatocytes (Fig. 1). In embryo liver *in ovo* (Fig. 1a), concentrations of cytochrome *P*-450 rose within 4 h after the drug was administered, and continued to rise throughout the 48 h induction period, reaching values greater than 10-fold above controls. In contrast, the activity of haem oxygenase remained at control values for 10 h and then increased rapidly to maximal values about 5-fold above control. In cultured hepatocytes exposed to $50 \mu\text{M}$ -glutethimide (Fig. 1b), concentrations of cytochrome *P*-450 rose throughout the 24 h induction period, despite an increase in haem oxygenase activity. The activity of haem oxygenase was significantly increased ($P < 0.05$) by 12 h. Measurements of concentrations of cytochrome *P*-450 and haem oxygenase activity in embryo liver *in ovo* were with microsomal fractions, and those in cultured hepatocytes were with homogenate, which accounts for the apparent difference in absolute values.

Other inducers of cytochrome *P*-450 also increased haem oxygenase in cultured hepatocytes (Fig. 2a).

Table 1. Effects of chemicals on porphyrin accumulation by chick-embryo hepatocytes

Embryo or cultured hepatocytes were treated as shown for 24 h. Values represent means $\pm$ S.D.; *significantly different from control ($P < 0.05$).

Hepato-cytes	Treatment	Uroporphyrin (pmol/mg of protein)	Coproporphyrin (pmol/mg of protein)	Protoporphyrin (pmol/mg of protein)	Total porphyrin (pmol/mg of protein)
<i>In ovo</i>	Control ($n = 22$)	0.4 ± 0.4	0.4 ± 0.5	0.9 ± 0.4	2.0 ± 1.0
	Glutethimide (6 mg/egg) ($n = 16$)	3 ± 6	3 ± 10	238 ± 280	$247 \pm 283^*$
	Phenobarbitone (8 mg/egg) ($n = 16$)	0.4 ± 1	0.7 ± 0.6	1.0 ± 0.1	2 ± 1
	Mephénytoin (6 mg/egg) ($n = 4$)	0.6 ± 0.03	0.06 ± 0.11	2.0 ± 1.6	2.9 ± 1.2
In culture	Control ($n = 19$)	5 ± 10	18 ± 16	12 ± 9	36 ± 26
	Glutethimide ($50 \mu\text{M}$) ($n = 14$)	$527 \pm 341^*$	$67 \pm 28^*$	10 ± 13	$606 \pm 354^*$
	Mephénytoin ($200 \mu\text{M}$) ($n = 3$)	$95 \pm 26^*$	32 ± 11	19 ± 5	$145 \pm 26^*$

Table 2. Effect of actinomycin D and cycloheximide on concentration of cytochrome *P*-450, haem oxygenase activity and porphyrin accumulation produced by glutethimide and methenytol in cultured chick-embryo hepatocytes

Cultured chick-embryo hepatocytes were treated as shown for 24 h. Each value represents the average of two measurements, which varied by $< 15\%$ from each other for cytochrome *P*-450 and haem oxygenase.

Treatment	Addition	Cytochrome <i>P</i> -450 (pmol/mg of protein)	Haem oxygenase (pmol of bilirubin/min per mg of protein)	Total porphyrin (pmol/mg of protein)	Protein synthesis (d.p.m./min per mg of protein)
Control	None	24	56	29	21
	Actinomycin D ($0.78 \mu\text{M}$)	18	63	25	7
	Cycloheximide ($0.2 \mu\text{M}$)	20	47	22	8
Glutethimide ($50 \mu\text{M}$)	None	114	100	712	22
	Actinomycin D ($0.78 \mu\text{M}$)	11	46	20	4
	Cycloheximide ($0.2 \mu\text{M}$)	58^*	54^*	23	10
Mephénytoin ($200 \mu\text{M}$)	None	181	142	308	20
	Actinomycin D ($0.78 \mu\text{M}$)	11	43	19	6
	Cycloheximide ($0.2 \mu\text{M}$)	62^*	50	24	9

* $1 \mu\text{M}$ -Cycloheximide lowered values to that of the uninduced control.

Mephénytoin and glutethimide produced similar increases of haem oxygenase activity; phenobarbitone increased haem oxygenase activity only at relatively high concentrations. In contrast, 20-methylcholanthrene had no effect on haem oxygenase activity. All four drugs increased cytochrome *P*-450 in cultured hepatocytes (Fig. 2b), although the potency of phenobarbitone was less than that of the other chemicals.

In contrast with the findings in cultured cells, in embryo liver *in ovo* (Fig. 2c) phenobarbitone did not detectably increase the activity of haem oxygenase. All of these compounds increased cytochrome *P*-450 in embryo liver *in ovo* (Fig. 2d); as in culture, phenobarbitone was a relatively weak inducer of cytochrome *P*-450.

On the basis of immunochemical cross-reactions of embryo liver microsomal proteins, glutethimide and phenobarbitone induce the same form of cytochrome *P*-450. 20-Methylcholanthrene is known to induce a different form of cytochrome *P*-450 (Sato & Omura, 1978; Sinclair *et al.*, 1986).

The effects of glutethimide and mephénytoin on porphyrin concentrations are shown in Table 1. Glutethimide was the most efficacious inducer of porphyrins both in embryo liver *in ovo* and in cultured hepatocytes. Particularly after treatment with glutethimide there was wide variation in porphyrin accumulations among embryos and cultures, which did not correlate with either cytochrome *P*-450 concentrations or haem oxygenase activities. The most striking finding was the difference in patterns of porphyrin accumulation between embryo liver and cultured hepatocytes treated with glutethimide: uroporphyrin was the predominant porphyrin that accumulated in cultured hepatocytes, whereas protoporphyrin was the predominant porphyrin that accumulated in embryo liver *in ovo*. Similar differences in porphyrin accumulation were previously described (Feroli *et al.*, 1984). Thus the cultured hepatocytes differ from hepatocytes *in ovo* in response to these drugs with respect to both porphyrin accumulation and haem oxygenase induction.

Table 3. Effects of chemicals and heavy metals on concentrations of cytochrome *P*-450 and activity of haem oxygenase in cultures of chick-embryo hepatocytes

Cultured chick hepatocytes were treated as shown for 24 h. Values are means $\pm$ S.D. ($n = 4$); *significantly different from control ($P < 0.05$).

Treatment	Cytochrome <i>P</i> -450 (pmol/mg of protein)	Activity of haem oxygenase (pmol of bilirubin/min per mg of protein)
Control	24 $\pm$ 5	34 $\pm$ 2
20-Methylcholanthrene (4 μ M)	43 $\pm$ 5*	45 $\pm$ 15
Glutethimide (50 μ M)	117 $\pm$ 7*	83 $\pm$ 8*
Mephénytoin (200 μ M)	134 $\pm$ 12*	66 $\pm$ 10*
CoCl ₂ (100 μ M)	14 $\pm$ 7	260 $\pm$ 26*
CdCl ₂ (2 μ M)	24 $\pm$ 3	291 $\pm$ 13*

Fig. 3. Effect of cadmium on mephénytoin-mediated increases in (a) cytochrome *P*-450 and (b) haem oxygenase in cultured hepatocytes

Cultured chick hepatocytes were treated for 24 h. Final concns: mephénytoin (meph), 200 μ M; cadmium, 2 μ M. Results are averages of two measurements which differed from each other by $< 10\%$.

In cultured hepatocytes, cycloheximide and actinomycin blocked the increases in cytochrome *P*-450, haem oxygenase and porphyrins by glutethimide and mephénytoin (Table 2). Although protein synthesis was markedly decreased by cycloheximide and actinomycin, cytochrome *P*-450, haem oxygenase and porphyrin concentrations did not fall below control values.

A comparison of the effects of several chemicals on cytochrome *P*-450 and haem oxygenase in cultured hepatocytes is shown in Table 3. 20-Methylcholanthrene increased only cytochrome *P*-450; cobalt and cadmium increased only haem oxygenase; glutethimide and mephénytoin increased both cytochrome *P*-450 and haem oxygenase. These results suggest that cytochrome *P*-450 and haem oxygenase are independently regulated. To emphasize further the independence of cytochrome *P*-450 and haem oxygenase regulation, cultured cells were exposed to both mephénytoin and cadmium (Fig. 3). Mephénytoin-mediated induction of cytochrome *P*-450 in the presence of cadmium was similar to that of mephénytoin alone, despite a greater than 10-fold increase over control in haem oxygenase activity. Induction of haem oxygenase in the presence of both cadmium and mephénytoin appeared to be additive.

The relationship between the induction of cytochrome *P*-450 and haem oxygenase activity in cultured hepatocytes was further probed by the addition of inhibitors of haem synthesis. 4,6-Dioxoheptanoic acid is an inhibitor of ALA dehydratase (Tschudy *et al.*, 1981), and desferrioxamine blocks conversion of protoporphyrin into haem (Shedlofsky *et al.*, 1987). These inhibitors had little effect on porphyrin accumulation or concentrations of cytochrome *P*-450 in control cells (Figs. 4a and 4b). However, in cells treated with mephénytoin, the inhibitors of haem synthesis prevented induction of both cytochrome *P*-450 and haem oxygenase by mephénytoin (Figs. 4b and 4c); as expected, 4,6-dioxoheptanoic acid blocked porphyrin accumulation, whereas desferrioxamine resulted in protoporphyrin accumulation (Fig. 4a).

Exposure of control cells to 10 μ M-haem increased concentrations of cytochrome *P*-450 ($P < 0.01$), suggesting the presence of some apo-cytochrome *P*-450. Haem is a potent inducer of haem oxygenase, as shown with both control and mephénytoin-treated cells (Fig. 4c). The addition of haem plus 4,6-dioxoheptanoic acid or desferrioxamine fully restored the mephénytoin-mediated increase in cytochrome *P*-450, implying that mephénytoin alone increased both haem synthesis and apoprotein synthesis. 4,6-Dioxoheptanoic acid and desferrioxamine had no effect on the haem-mediated induction of haem oxygenase, in either control or mephénytoin-treated cells.

Fig. 4. Effect of 4,6-dioxoheptanoic acid, desferrioxamine, haem and mephenytoin on (a) porphyrins, (b) cytochrome *P*-450 concentrations and (c) haem oxygenase activity in cultured hepatocytes

Cultured chick-embryo hepatocytes were exposed to various treatments as shown. Final concentrations were: 2 mM-4,6-dioxoheptanoic acid (4,6-DHA); 10 μ M-haemin (haem); 10 μ M-desferrioxamine (des); 200 μ M-mephenytoin (meph). Each bar represents the average of two measurements, which varied by < 10% of the mean.

In contrast with the major effects on drug-mediated increases in haem oxygenase, 4,6-dioxoheptanoic acid had little effect on metal induction of haem oxygenase (Fig. 5a).

DISCUSSION

A major finding of this paper is that the activity of haem oxygenase and the concentration of cytochrome *P*-450 are increased in chick-embryo hepatocytes by phenobarbitone, mephenytoin and glutethimide. These chemicals increase the same form of cytochrome *P*-450 in chick-embryo hepatocytes (Sinclair *et al.*, 1986). Synthesis of new RNA and protein is required in order for these drug effects to occur, as shown by the inhibitions produced by actinomycin D and cycloheximide (Table 2). Induction of the apoprotein moiety of cytochrome *P*-450 is believed secondarily to produce a partial depletion of the regulatory haem pool, and in turn to cause a depression of ALA synthase (Granick *et al.*, 1975; Bonkovsky, 1982). However, the possibility that induction of ALA synthase occurs by a mechanism indepen-

dent of changes in a regulatory haem pool has not been excluded. The experiments with inhibitors of haem synthesis and haem (Fig. 4) indicate that phenobarbitone-like chemicals increase synthesis of both the haem and the apoprotein moieties of cytochrome *P*-450, thereby increasing concentrations of the holo-cytochrome. The experiments further suggest that induction of haem oxygenase activity by phenobarbitone-like chemicals is a secondary effect, dependent on increased haem synthesis (Lincoln & Bonkovsky, 1987). 4,6-Dioxoheptanoic acid and desferrioxamine, both potent inhibitors of haem synthesis with different sites of action, completely prevented increases in haem oxygenase in the presence of phenobarbitone-like drugs, effects that were reversed by the addition of haem (Fig. 4). That haem is involved in these drug effects is further supported by examination of the time courses in Fig. 1, in which an increase in cytochrome *P*-450 concentrations preceded the increase in haem oxygenase activity.

Our current results also clearly show that there is a second mechanism for the induction of haem oxygenase that is not dependent on increased regulatory haem. This

Fig. 5. Effect of heavy metals (Co, Cd, Fe) and 4,6-dioxoheptanoic acid on (a) cytochrome *P*-450 and (b) haem oxygenase in cultured hepatocytes

Cultured chick-embryo hepatocytes were exposed to various chemicals as shown, for 24 h. Final concentrations of chemicals were: 2 mM-4,6-dioxoheptanoic acid (4,6-DHA), 100 μ M- CoCl_2 , 2 μ M- CdCl_2 , and ferric nitrilotriacetate (Fe; 100 μ M- Fe^{3+} + 200 μ M-nitrilotriacetic acid). Each bar represents the average of two measurements which varied by < 10% from the mean.

conclusion is supported by the experiments with metals that produce marked increases in activity of haem oxygenase, with no concomitant increases, and indeed moderate decreases, in concentrations of cytochrome *P*-450 (Fig. 5b). Furthermore, the metal induction of haem oxygenase occurred even when haem synthesis was blocked by the addition of 4,6-dioxoheptanoic acid or desferrioxamine (Fig. 5).

The observation that iron increased activity of haem oxygenase, even when haem formation was blocked by the presence of 4,6-dioxoheptanoic acid (Fig. 5a), provides evidence that iron itself, as well as its protoporphyrin chelate haem, is a proximate mediator of the induction of hepatic haem oxygenase. The greater induction of haem oxygenase activity by ferric nitrilotriacetate than by other iron salts (Sardana *et al.* 1982) is probably due to greater cellular iron uptake of the iron-nitrilotriacetate complex (Shedlofsky *et al.* 1983). Thus iron, acting as a heavy metal, analogous to cobalt or cadmium, increases the activity of haem oxygenase by one mechanism, whereas haem increases this activity by another mechanism. The present results in chick-embryo hepatocytes support the concept of at least two disparate mechanisms for induction of haem oxygenase, previously suggested from studies in rats (Kikuchi & Yoshida, 1983).

Glutethimide and mephenytoin were found to increase porphyrin accumulation both in hepatocytes *in ovo* and in culture (Table 1). The increase in porphyrins *in ovo* was primarily observed for glutethimide-treated eggs and was principally protoporphyrin (Table 1). In cultures, all the drugs tested produced an increase in porphyrin accumulation, and the majority of this porphyrin was uro- rather than proto-porphyrin. These findings are similar to those previously described (Ferioli *et al.*, 1984). As discussed elsewhere (Bonkovsky *et al.*, 1987), the predominance of uroporphyrin accumulation in the cultures is probably related to an increased propensity for uroporphyrinogen to be oxidized to the corresponding porphyrin, which is not a substrate for uroporphyrinogen decarboxylase, the enzyme that converts uroporphyrinogen via stepwise decarboxylation into coproporphyrinogen. Despite porphyrin accumulation, treatment of hepatocytes with glutethimide and similar chemicals actually produced an excess of haem beyond that required for cytochrome *P*-450 induction, as shown by an apparent increase in haem saturation of tryptophan dioxygenase (B. C. Lincoln, A. Alvarado, N. Setty & H. L. Bonkovsky, unpublished work), the ability to sustain cytochrome *P*-450 induction in the presence of elevated haem oxygenase activity (Fig. 3), and increased haem degradation (Lincoln & Bonkovsky, 1987). The fact that haem excess occurred is evidence against the view that an increase in ALA synthesis, and thus haem synthesis, produced by glutethimide and similar drugs is related solely to increased haem demand by cytochrome *P*-450.

In conclusion, glutethimide and mephenytoin induce cytochrome *P*-450, and secondarily haem oxygenase activity owing to increased haem formation. In contrast, metal induction of haem oxygenase activity occurs independent of increased haem formation.

This work was supported by grants from N.I.H. (DK38825 and RR00039). We thank Ms. Pat Gulliford for assistance with typing the manuscript.

REFERENCES

- Abraham, N. G., Lutton, J. D., Freedman, M. L. & Levere, R. D. (1986) *Am. J. Med. Sci.* **292**, 81–86
- Badawy, A. A.-B. & Evans, M. (1975) *Biochem. J.* **150**, 511–520
- Badawy, A. A.-B. & Morgan, C. J. (1980) *Biochem. J.* **186**, 763–772
- Bonkovsky, H. L. (1982) in *A Textbook of Liver Disease* (Zakim, D. & Boyer, T. D., eds.), pp. 351–393, Saunders, Philadelphia
- Bonkovsky, H. L., Wood, S. G., Howell, S. K., Sinclair, P. R., Lincoln, B. C., Healey, J. F. & Sinclair, J. F. (1986) *Anal. Biochem.* **155**, 54–64
- Bonkovsky, H. L., Sinclair, P. R., Bement, W. J., Lambrecht, R. W. & Sinclair, J. F. (1987) *Ann. N.Y. Acad. Sci.*, in the press
- Drummond, G. S., Rosenberg, D. W. & Kappas, A. (1982) *Biochem. J.* **202**, 59–66
- Ferioli, A., Harver, C. & de Matteis, F. (1984) *Biochem. J.* **224**, 769–777
- Grandchamp, B., Deybach, J. C., Grelier, M., deVerneuil, H. & Nordmann, Y. (1980) *Biochim. Biophys. Acta* **629**, 577–586
- Granick, S., Sinclair, P., Sassa, S. & Griening, G. (1975) *J. Biol. Chem.* **250**, 9215–9225

- Kikuchi, G. & Yoshida, T. (1983) *Mol. Cell. Biochem.* **53/54**, 163–183
- Lincoln, B. C. & Bonkovsky, H. L. (1987) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **46**, 2264
- Lincoln, B., Healey, J., Sinclair, P., Frezza, J., Sinclair, J. & Bonkovsky, H. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **45**, 1763
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Maines, M. D. & Kappas, A. (1974) *J. Biol. Chem.* **250**, 4171–4177
- Maines, M. D. & Kappas, A. (1977) *Science* **198**, 1215–1221
- Maines, M. D. & Sinclair, P. (1977) *J. Biol. Chem.* **252**, 219–223
- Morgan, C. J. & Badawy, A. A.-B. (1980) *Biochem. J.* **186**, 993–996
- Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2370–2385
- Paine, A. J. & Legg, R. F. (1978) *Biochem. Biophys. Res. Commun.* **81**, 672–679
- Salter, M. & Pogson, C. I. (1986) *Biochem. J.* **240**, 259–263
- Sardana, M. K. & Drummond, G. S. (1985) *Biochem. Pharmacol.* **35**, 473–478
- Sardana, M. K., Sassa, S. & Kappas, A. (1980) *J. Biol. Chem.* **255**, 11320–11323
- Sardana, M. K., Sassa, S. & Kappas, A. (1982) *J. Biol. Chem.* **257**, 4806–4811
- Sato, R. & Omura, T. (1978) *Cytochrome P-450*, pp. 14–16, Academic Press, New York
- Schacter, B. A. & Mason, J. I. (1974) *Arch. Biochem. Biophys.* **160**, 274–278
- Shedlofsky, S. I., Sinclair, P. R., Sinclair, J. F. & Bonkovsky, H. L. (1983) *Biochem. Pharmacol.* **33**, 1487–1491
- Shedlofsky, S. I., Bonkovsky, H. L., Swim, A. T., Sinclair, P. R., Healey, J. F. & Robinson, J. M. (1987) *Biochem. J.* **248**, 229–236
- Sinclair, J. F., Sinclair, P. R. & Bonkovsky, H. L. (1979) *Biochem. Biophys. Res. Commun.* **86**, 710–717
- Sinclair, P., Frezza, J., Sinclair, J., Bement, J., Healey, J. & Bonkovsky, H. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **45**, 1663
- Tenhunen, R., Marver, H. S. & Schmid, R. (1969) *J. Biol. Chem.* **244**, 6388–6394
- Thomas, P. E., Lu, A. Y. H., Ryan, D., West, S. B. & Kawale, K. J. (1976) *J. Biol. Chem.* **251**, 1385–1391
- Tschudy, D. P., Hess, R. A. & Frykholm, B. C. (1981) *J. Biol. Chem.* **256**, 9915–9923
- Yoshida, T., Arakaki, M., Kumakawa, J. & Kuroiwa, Y. (1984) *J. Pharmacobio-Dyn.* **7**, 112–119

Received 15 June 1987/15 September 1987; accepted 26 October 1987