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EFFECT OF COBALT PROTOPORPHYRIN ON HEPATIC DRUG-METABOLIZING ENZYMES

SPECIFICITY FOR CYTOCHROME P-450

STEPHEN M. SPAETHE* and DAVID J. JOLLOW†
Department of Pharmacology, Medical University of South Carolina, Charleston, SC 29425, U.S.A.

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Abstract—Cobaltic protoporphyrin IX (cobalt protoporphyrin) is known to cause an extensive and long-lasting depletion of hepatic cytochrome P-450 in rats, and it has been used to evaluate the role of hepatic cytochrome P-450 in xenobiotic metabolism and toxicity. To examine the specificity of cobalt protoporphyrin for hepatic cytochrome P-450, cobalt protoporphyrin was administered to rats and hamsters, and its effects on cytochrome P-450-dependent and non-P-450-dependent phase I and phase II metabolism were determined. Cobalt protoporphyrin pretreatment depleted hepatic cytochrome P-450 in both species and lowered their V_{max} values for the hepatic microsomal metabolism of ethylmorphine, aminopyrine, ethoxyresorufin and ethoxycoumarin, without change in their K_m values. In the rat, cobalt protoporphyrin treatment lowered both the V_{max} and the K_m for microsomal metabolism of aniline. *In vivo* hepatic cytochrome P-450-dependent metabolism, as measured by antipyrine clearance, was decreased in both species. UDP-Glucuronyltransferase, phenolsulfotransferase and glutathione-*S*-transferase were unaffected, as was hepatic glutathione. Modest effects of cobalt protoporphyrin were seen on the hepatic microsomal flavoprotein mixed-function oxidase (hamster only), cytochrome P-450 reductase, cytochrome b_5 (rat only), UDPGA (rat only), and glycogen, and on blood glucose (rat). In *in vivo* studies with hamsters given a low dose of acetaminophen, cobalt protoporphyrin suppressed the apparent rate constants for the cytochrome P-450-dependent pathways of acetaminophen metabolism but had no effect on acetaminophen glucuronidation and sulfation. Polyacrylamide gel electrophoresis analysis indicated that cobalt protoporphyrin markedly reduced the levels of the cytochrome P-450 holoenzyme but did not alter either the content or profile of the cytochrome P-450 apoenzyme. Collectively, the data indicate that cobalt protoporphyrin shows relatively high selectivity for the hepatic cytochrome P-450 system, and support the use of this compound as a tool for resolution of the role of hepatic cytochrome P-450 in xenobiotic metabolism and toxicity.

It has been known for over a decade that a variety of metal ions such as those of cobalt, nickel and tin have the ability to induce heme oxygenase in the liver [1]. In turn, enhancement of heme oxygenase results in an increase in heme degradation, a decrease in liver cytochrome P-450 levels, and a decrease in cytochrome P-450-dependent metabolism of drugs and other xenobiotic substances [1, 2]. The ability of cobalt chloride to suppress hepatic cytochrome P-450 has subsequently found use in the evaluation of the role of cytochrome P-450 in the metabolism and toxicity of a variety of xenobiotics [3–11]. Cobalt chloride, however, has several disadvantages when used as an experimental tool for this purpose. First, the duration of the effect on heme oxygenase is relatively brief (48–72 hr) [2]. Second, cobalt chloride lacks specificity in that, in addition to suppression of cytochrome P-450, it causes an enhancement of hepatic glutathione levels [11–13], an enhancement of acetaminophen glucuronidation [11], and a suppression of acetaminophen sulfation [11].

It was later realized that the incorporation of cobalt ion into an organic chelate greatly increased the duration of the metal ion effect on heme oxygenase and cytochrome P-450 levels. Thus, an equimolar dose of cobaltic protoporphyrin IX (cobalt protoporphyrin) was found to depress cytochrome P-450 levels in the liver profoundly for 3–5 weeks [14], in contrast to the 48–72 hr depression after cobalt chloride [2]. This long duration of effect has, for example, been of particular value in the study of the role of cytochrome P-450 activities in steroid metabolism [15].

Little is known, however, about the specificity of the effect of cobalt protoporphyrin in the liver and, in particular, whether or not it alters drug metabolism activities other than those dependent on cytochrome P-450. The present study describes the effect of cobalt protoporphyrin on both phase I and phase II enzymes of drug metabolism and on the levels of the cosubstrates for the phase II enzymes.

* Current address: Lilly Research Laboratories, MC931 Bldg., 98-C-4 Lab., Lilly Corp. Center, Indianapolis, IN 46285.

† Correspondence: David J. Jollow, Ph.D., Department of Pharmacology, Medical University of South Carolina, 171 Ashley Ave., Charleston, SC 29425.

MATERIALS AND METHODS

Animals

Male golden Syrian hamsters (80–120 g; Charles Rivers, Lakeview, NJ) and male Sprague–Dawley

rats (100–150 g; CAMM, Wayne, NJ) were used throughout these studies. Animals were allowed Wayne Lab Blox (Allied Mills, Inc., Chicago, IL) and water *ad lib.*

Treatment regimens

The animals received cobalt protoporphyrin (Porphyrin Products, Logan, UT) (hamsters, 0–125 μmol/kg, s.c.; rats, 0–90 μmol/kg, s.c.) 72 hr (or as otherwise noted) prior to experimentation. Solutions of cobalt protoporphyrin were prepared by dissolving the compound in one-tenth volume of 0.1 M NaOH, adjusting the pH to 7.4, and bringing to final volume with normal saline. The cobalt protoporphyrin solution was administered (0.5 ml/100 g body weight) immediately after preparation. Control animals received an equivalent volume of saline.

Effect of cobalt protoporphyrin on hepatic microsomal cytochrome P-450 enzyme activities

Following a 72-hr pretreatment with cobalt protoporphyrin, animals were killed, their livers were removed, and the microsomal fraction was prepared according to the method of Potter *et al.* [16]. Cytochrome P-450 content was measured by the method of Omura and Sato [17]. Protein concentrations were determined by the procedure of Lowry *et al.* [18], using bovine serum albumin as the standard. Ethylmorphine and aminopyrine *N*-demethylase activities were determined by monitoring formaldehyde production according to the method of Nash [19], as described by Mazel [20]; aniline hydroxylase activity was quantitated by *p*-aminophenol [20]; and ethoxyresorufin and 7-ethoxycoumarin deethylase activities were determined fluorometrically as described by Prough *et al.* [21].

Polyacrylamide gel electrophoresis studies

To determine the hepatic cytochrome P-450 apoprotein profile, microsomal preparations were diluted to a concentration of 1 mg protein/ml with 0.125 M Tris–acetate buffer (pH 7.4) containing 20% glycerol (v/v), 1% sodium dodecyl sulfate (SDS) (w/v), and 5% β-mercaptoethanol (v/v). The samples were heated in a boiling water bath for 2 min, and aliquots of microsomal protein (20 μg) were applied for electrophoresis. Polyacrylamide slab gel electrophoresis (PAGE) was carried out at 4° in the presence of SDS utilizing a discontinuous buffer system [22] containing a 3% stacking gel followed by a 7.5 to 12.5% polyacrylamide gradient separating gel. The gels were stained overnight with Coomassie blue G-250 to visualize the proteins. Densitometric scans were performed at 635 nm utilizing a Hoefer densitometer (Hoefer Scientific Instruments, San Francisco, CA) which was interfaced to an Apple IIe Computer (Apple Computer Inc., Cupertino, CA) using a chromochart software driven Adlab analog/digital converter (Interactive Microwave Inc., State College, PA).

To determine the hepatic cytochrome P-450 hemoprotein profile, a similar set of PAGE studies were carried out in which SDS was omitted from the separating gel, stacking gel, and the electrophoresis sample and tank bubbers. Lithium dodecyl sulfate (LDS) was substituted for SDS in the sample and upper tank buffers, and electrophoresis (80 μg microsomal protein) was carried out at 4° in the dark under non-reducing conditions to increase heme retention. Hemoproteins were stained for peroxidase activity with 3,3′,5,5′-tetramethylbenzidine (Sigma Chemical Co., St Louis, MO) prior to densitometric scanning [23].

Effect of cobalt protoporphyrin on non-cytochrome P-450-dependent enzyme activities

Flavoprotein mixed-function oxidase activity was measured by the procedure of Ziegler and Pettit [24] using dimethylaniline as substrate. UDP-Glucuronyltransferase activity was measured in hamster microsomes using [^{3}H]acetaminophen as substrate [25], and in rat microsomes using *p*-nitrophenol as the substrate [26]. To measure phenolsulfotransferase activity, animals were killed, and their livers were excised and homogenized in 4 vol. of 0.15 M KCl. The homogenates were centrifuged at 178,000 *g* for 30 min at 4°. Aliquots of the 178,000 *g* supernatant fraction containing 2.5 mg protein/ml were incubated with β-mercaptoethanol (5 mM) and various concentrations of acetaminophen containing [^{3}H]acetaminophen (20 μCi/tube). The reaction mixture was preincubated for 5 min, and the reaction was initiated with the addition of 3′-phosphoadenosine 5′-phosphosulfate (PAPS; 0.2 mM) in a final volume of 0.25 ml. The reaction was allowed to proceed for 10 min and terminated by freezing in an acetone/dry ice bath. The untreated [^{3}H]acetaminophen was removed by extraction with ethyl acetate. The [^{3}H]acetaminophen sulfate was isolated via thin-layer chromatography and quantitated via liquid scintillation spectroscopy [25].

Glutathione transferase activity was measured in the 9000 *g* supernatant fraction with 1-chloro-2,4-dinitrobenzene as substrate [27]. The GSH conjugate formed was measured spectrophotometrically as described by Habig *et al.* [28].

Determination of hepatic levels of glutathione, UDP-glucuronic acid, glycogen, and blood glucose

To determine the effect of cobalt protoporphyrin on hepatic glutathione, UDP-glucuronic acid (UDPGA), glycogen, and blood glucose, control and cobalt protoporphyrin-pretreated animals were killed, blood was collected, and livers were excised and quickly frozen in liquid nitrogen. For the GSH studies, a similar group of animals received $CoCl_2 \cdot 6H_2O$ (60 mg/kg, s.c.) 48 hr prior to being killed. GSH levels were estimated by the method of Tietze [29]. Hepatic levels of UDPGA were measured using [^{3}H]diethylstilbestrol (DES; Amersham Corp., Arlington Heights, IL) as the substrate and guinea pig microsomes as the source of glucuronyltransferase [30]. Hepatic glycogen was determined as the difference between total hepatic non-anthrone positive sugars [31] and total hepatic glucose. Liver and serum glucose levels were measured by the glucose oxidase method using a kit from the Sigma Chemical Co.

Effect of cobalt protoporphyrin on hepatic cytochrome P-450 reductase and cytochrome b_5 content

Cytochrome P-450 reductase activity was quantitated using horse heart cytochrome *c* as the electron acceptor [32]. The cytochrome b_5 content was measured from the oxidized versus reduced difference spectrum using sodium dithionite as the reducing agent [20].

In vivo *studies*

Antipyrine clearance. Antipyrine (100 mg/kg, i.p.; Eastman Kodak Co., Rochester, NY) was administered, and blood samples (75 μl) were collected from the orbital sinus into a heparinized glass capillary tube at various times after drug dosage. Plasma was prepared and extracted twice with dichloromethane/pentane (1:1). Antipyrine in the dichloromethane/heptane extract was quantitated by HPLC using a modification of the procedure of Danhof *et al.* [33]. Briefly, the HPLC system used consisted of a model 6000 pressure pump, a model U6K injector, and a model 400 UV detector with a 254 nm cell (Waters Associates, Milford, MA). The column, 25 cm × 10 mm i.d., was packed with 5 μm Spherisorb ODS (Altech Associates, Inc., Deerfield, IL). The solvent system consisted of 40% methanol in 50 mM NaH_2PO_4 buffer containing 0.4% tetrahydrofuran. Chromatography was performed at a flow rate of 1.5 ml/min. The internal standard used was *o*-acetanisidide (2 μg/ml; Pfaltz & Bauer, Inc., Stamford, CT). The area under the concentration–time curve (AUC) for antipyrine in plasma was calculated by the trapezoidal method and extrapolated to infinity. The intraperitoneal clearance (Cl_{ip}) was calculated from the relationship, $Cl_{ip} = Dose_{ip}/AUC_{ip}$.

Acetaminophen studies. For *in vivo* metabolism studies, hamsters received acetaminophen (25 mg/kg i.p.; Eastman-Organic Chemicals, Rochester, NJ) containing [^{3}H]acetaminophen (200 μCi/kg; Amersham Corp.) dissolved in normal saline. Each animal was placed in a separate metabolic cage and urine was collected for 24 hr over dry ice. Serial blood samples (75 μl) were taken from the orbital sinus for a time equivalent to at least four half-lives of the parent compound. Blood acetaminophen and urinary metabolites were quantitated as described by Price and Jollow [34]. The elimination rate constant (β) was obtained from linear regression analysis of the blood concentration–time curve, and the half-life ($T_{\frac{1}{2}}$) was determined as equal to $0.693/\beta$.

Statistical analysis

Data were analyzed using a one-way analysis of variance or Student's paired *t*-test, and significance was determined at the level of $P < 0.05$.

RESULTS

Effect of cobalt protoporphyrin pretreatment on hepatic cytochrome P-450

Administration of a single dose of cobalt protoporphyrin (90 μmol/kg) to rats resulted in a rapid and dramatic fall in hepatic P-450 (Fig. 1A). In agreement with Drummond and Kappas [14], the nadir was reached within 1 day and cytochrome P-450 levels remained low (>80% depletion) through out the experimental time period (10 days). To assess the dose–response relationship, cytochrome P-450 levels were measured 72 hr after treatment with various doses of cobalt protoporphyrin. The decrease in cytochrome P-450 levels was dose dependent (Fig. 1B). The ED_{50} in the rat was approximately 25 μmol/kg.

In the hamster, cobalt protoporphyrin administration caused a similar marked depletion of hepatic cytochrome P-450 (Fig. 2A). The nadir was reached within 48 hr and was long-lasting (>10 days). The ED_{50} in the hamster was approximately 15 μmol/kg

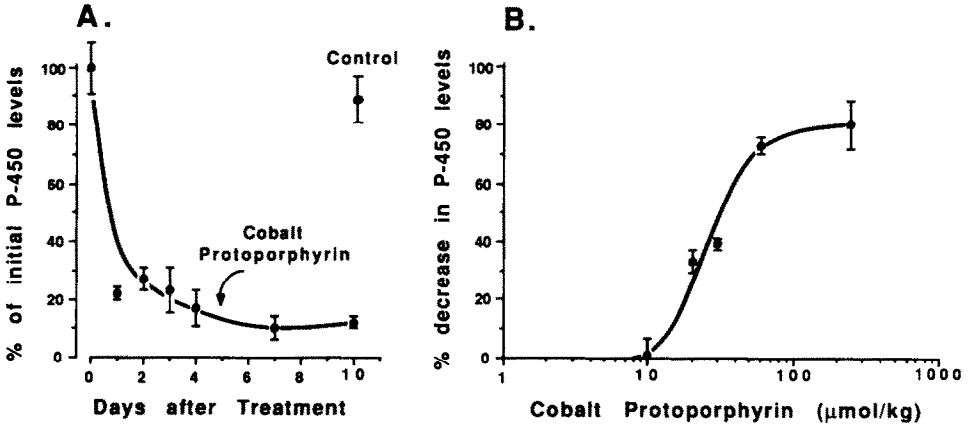

Fig. 1. Effect of administration of a single dose of cobalt protoporphyrin on hepatic microsomal cytochrome P-450 levels in rats. (A) Time-dependency of cytochrome P-450 depletion. Rats were killed at the indicated times after cobalt protoporphyrin (90 μmol/kg), and hepatic microsomal fraction was prepared and assayed for cytochrome P-450 content. (B) Dose–response study. Rats received the indicated doses of cobalt protoporphyrin 72 hr prior to being killed. Hepatic microsomal cytochrome P-450 levels were determined from the oxidized versus reduced monoxide difference spectra. Values are means ± SE, N = 4–6.

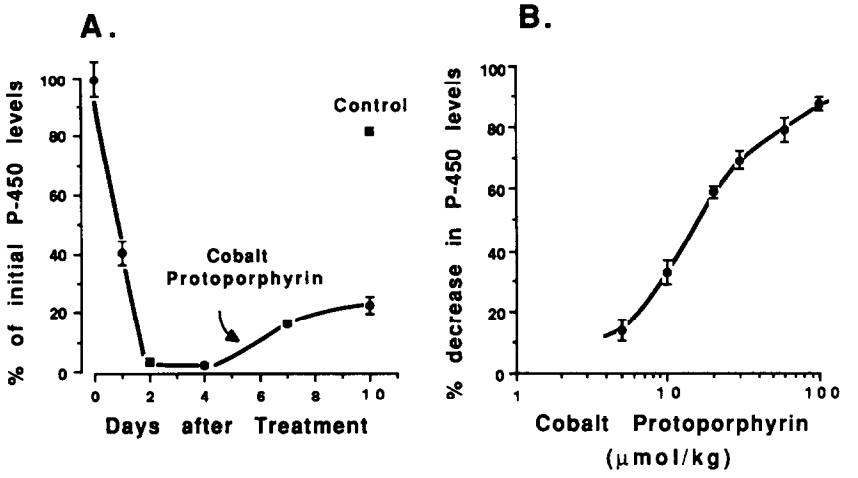

Fig. 2. Effect of administration of a single dose of cobalt protoporphyrin on hepatic microsomal cytochrome P-450 levels in hamsters. (A) Time dependency of cytochrome P-450 depletion. Hamsters were killed at the indicated times after cobalt protoporphyrin (125 μmol/kg), the hepatic microsomal fraction was prepared, and cytochrome P-450 levels were determined by carbon monoxide difference spectroscopy. (B) Dose–response study. The animals received the indicated doses of cobalt protoporphyrin 72 hr prior to being killed. Hepatic cytochrome P-450 levels were determined from the oxidized versus reduced carbon monoxide difference spectra. Values are means ± SE, N = 4–6.

(Fig. 2B). All subsequent studies were carried out 72 hr after cobalt protoporphyrin administration, since at this time hepatic cytochrome P-450 depletion was maximal. Typical cytochrome P-450 concentrations in the liver of rats 72 hr after cobalt protoporphyrin or saline were: control, 0.903 ± 0.083; cobalt protoporphyrin, 0.149 ± 0.051 nmol cytochrome P-450/mg microsomal protein ($P < 0.05$, N = 6, mean ± SE). In hamsters, the values were: control, 1.025 ± 0.103; cobalt protoporphyrin, 0.053 ± 0.007 nmol cytochrome P-450/mg microsomal protein ($P < 0.05$, N = 6, mean ± SE).

Effect of cobalt protoporphyrin pretreatment on hepatic cytochrome P-450 dependent microsomal metabolism

Pretreatment of hamsters with cobalt protoporphyrin resulted in marked decreases in the V_{max}

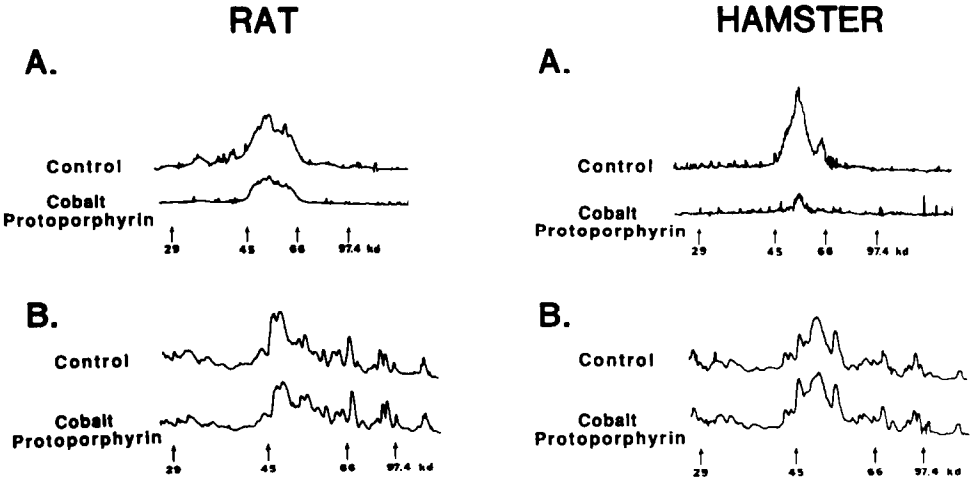

Fig. 3. Effect of cobalt protoporphyrin pretreatment on hepatic cytochrome P-450 hemeprotein and apoprotein content of hepatic microsomes. Animals were pretreated with either cobalt protoporphyrin (left panel, rats, 90 μmol/kg; right panel, hamsters, 125 μmol/kg) or saline 72 hr prior to removal of livers for preparation of microsomes. Densitometric scans of hepatic microsomal membrane preparations were performed on freshly PAGE separated and stained preparations. (A) Heme stain. PAGE (80 μg protein/well) was carried out in the presence of LDS under non-reducing conditions at 4° in the dark. Hemeproteins were stained for peroxidase activity with 3,3′, 5,5′-tetramethylbenzidine. (B) Coomassie blue stain. PAGE (20 μg protein/well) was carried out under reducing conditions in the presence of SDS.

values for the microsomal metabolism of 7-ethoxycoumarin (*ca.* 68%), aminopyrine (*ca.* 69%) and ethylmorphine (*ca.* 85%) (Table 1). The K_m values for these activities did not change significantly. 7-Ethoxyresorufin *O*-deethylase activity was measurable in liver microsomes from normal hamsters, permitting the determination of the kinetic parameters (Table 1). However, in microsomes from cobalt protoporphyrin-treated hamsters, this activity was suppressed markedly (>95%) and was too low to permit determination of K_m and V_{max} values.

The aniline hydroxylase activity of the microsomes from the liver of cobalt protoporphyrin pretreated hamsters showed a decrease in both K_m and V_{max} values as compared with controls; the decrease was approximately 2-fold in both parameters and hence the apparent first-order rate constant for the enzyme-catalyzed reaction (V_{max}/K_m) was largely unchanged.

Rats showed a generally similar response to cobalt protoporphyrin. The V_{max} values determined for the microsomal metabolism of aniline, aminopyrine, ethylmorphine, and 7-ethoxyresorufin were decreased by 63, 61, 68 and 43% respectively (Table 1). The K_m values were not altered by cobalt protoporphyrin pretreatment. Activity toward 7-ethoxycoumarin was not detectable in cobalt protoporphyrin pretreated rats.

Polyacrylamide gel electrophoresis studies

The effect of cobalt protoporphyrin pretreatment on the hepatic cytochrome P-450 isozyme profile was determined with the use of PAGE, utilizing both heme and protein staining procedures. Following solubilization and separation of microsomal proteins by LDS–PAGE, the hepatic hemoproteins were subjected to heme staining to detect intact hemoproteins. In normal animals, there was extensive heme staining in the 45–60 kD region of the gel. In both hamsters and rats, cobalt protoporphyrin caused a marked reduction in heme staining in this region (Fig. 3A) consistent with decreases in total cytochrome P-450 content of these preparations.

In marked contrast to the results obtained with the heme stain, staining of protein of the SDS–PAGE with Coomassie blue revealed that cobalt protoporphyrin pretreatment had no discernible effect on the (apo)protein profile of the cytochrome P-450 region of the gel, for either the hamster or the rat (Figs. 3B and 4).

Effect of cobalt protoporphyrin on non-cytochrome P-450 dependent drug-metabolizing activities

The hepatic microsomal flavoprotein mixed-function oxidase is shown to catalyze the oxygenation of a wide variety of diverse types of nucleophilic organic sulfur and nitrogen compounds and hence, like the hepatic cytochrome P-450 monooxygenase system, is responsible for phase I oxidation [35]. The effect of cobalt protoporphyrin pretreatment on the flavoprotein mixed-function oxidase is shown in Table 2. Microsomes from hamster liver showed a modest, though statistically significant, increase (1.5-fold) in the activity of this enzyme toward dimethylaniline. In normal rats, the microsomal flavoprotein mixed-function oxidase activity was significantly lower and more variable between animals than was seen in

Table 1. Effect of cobalt protoporphyrin pretreatment on cytochrome P-450-dependent mixed-function oxidase activities

Treatment	Ethylmorphine N-demethylation		Aminopyrine N-demethylation		Aniline hydroxylation		7-Ethoxyresorufin O-deethylation		7-Ethoxycoumarin O-deethylation	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Hamster										
Control	0.30 ± 0.03	4.90 ± 0.27	0.27 ± 0.01	5.74 ± 0.34	0.13 ± 0.01	0.34 ± 0.04	0.004 ± 0.001	0.102 ± 0.005	0.026 ± 0.002	1.148 ± 0.168
Cobalt protoporphyrin	0.21 ± 0.04	0.98* ± 0.15	0.29 ± 0.02	1.79* ± 0.16	0.07* ± 0.02	0.19* ± 0.01	ND	ND	0.029 ± 0.017	0.385* ± 0.036
Rat										
Control	0.62 ± 0.06	4.54 ± 0.50	0.37 ± 0.01	4.33 ± 0.21	0.062 ± 0.008	0.157 ± 0.004	0.009 ± 0.001	0.040 ± 0.002	0.080 ± 0.028	0.096 ± 0.023
Cobalt protoporphyrin	0.37 ± 0.02	1.47* ± 0.23	0.33 ± 0.04	1.68* ± 0.15	0.064 ± 0.005	0.058 ± 0.007*	0.008 ± 0.001	0.023 ± 0.002*	ND	ND

Animals received either cobalt protoporphyrin (hamster, 125 μmol/kg; rat, 90 μmol/kg) or saline 72 hr prior to being killed. Microsomal fractions were isolated, and activities were determined as described under Materials and Methods.

ND = not determinable due to loss of microsomal enzyme activity. Units of K_m are mM and of V_{max} are nmol product formed/mg microsomal protein·min. Values are means ± SE, N = 4.

* Significantly different from control animals, $P < 0.05$.

Table 2. Effect of cobalt protoporphyrin pretreatment on non-cytochrome-P-450-dependent drug-metabolizing enzyme activities

Treatment	Flavoprotein oxidase* nmol product / mg protein · min	UDP-Glucuronyl-transferase† μmol product / mg protein · min	Sulfo-transferase‡ μmol product / mg protein · min	GSH-transferase§ nmol product / mg protein · min
Hamster				
Control	1.19 ± 0.10	71.5 ± 17.8	5.63 ± 0.54	5.36 ± 0.74
Cobalt protoporphyrin	1.82 ± 0.12‖	32.5 ± 3.8	6.79 ± 1.47	5.31 ± 0.78
Rat				
Control	0.153 ± 0.056	1.00 ± 0.10	6.89 ± 0.64	14.04 ± 3.91
Cobalt protoporphyrin	0.040 ± 0.019	1.20 ± 0.10	8.47 ± 0.72	20.46 ± 4.28

Animals received either cobalt protoporphyrin (hamster, 125 μmol/kg; rat, 90 μmol/kg) or saline 72 hr prior to being killed. Preparation of hepatic enzyme and assay of enzymatic activities were carried out as described under Materials and Methods. Values are means ± SE, N = 4.

* *N,N*-Dimethylaniline (3 mM).

† Hamster, acetaminophen (V_{max}); rat, *p*-nitrophenol (0.5 mM).

‡ Acetaminophen (V_{max}).

§ 1-Chloro-2,4-dinitrobenzene (1 mM).

‖ Significantly different from control animals, $P < 0.05$.

hamster microsomes. Although the activity appeared to be depressed in cobalt-treated rats, the difference was not statistically significant.

The UDP-glucuronyltransferase activity of rat liver microsomes was measured using a standard concentration (0.5 mM) of *p*-nitrophenol, as described by Bock *et al.* [26] (Table 2). Pretreatment of the rats with cobalt protoporphyrin did not alter *p*-nitrophenol glucuronyltransferase activity of the liver microsomal preparations. In contrast to the situation in the rat, *p*-nitrophenol glucuronyltransferase activity could not be detected in microsomal preparations of hamster liver. UPD-Glucuronyltransferase activity in the hamster liver was assessed using [^{3}H]acetaminophen as substrate [25]. Lineweaver–Burk analysis revealed no statistically significant difference in the V_{max} (Table 2) between microsomal preparations from normal and cobalt protoporphyrin pretreated animals.

The phenolsulfotransferase activities of rat and hamster liver were measured using [^{3}H]acetaminophen. Lineweaver–Burk analysis of the data indicated that pretreatment with cobalt protoporphyrin did not alter the V_{max} (Table 2) or K_m (rat: normal, 0.166 ± 0.029 mM vs cobalt protoporphyrin, 0.197 ± 0.018 mM (NS, N = 6, mean ± SE); hamster: normal, 0.105 ± 0.034 mM vs cobalt protoporphyrin, 0.233 ± 0.122 mM (NS, N = 6, mean ± SE) of the reaction in either rats or hamsters.

Glutathione-*S*-transferase activities of both rat and hamster liver were measured using a standard concentration (1 mM) of 1-chloro-2,4-dinitrobenzene (Table 2). Cobalt protoporphyrin had no effect on this glutathione-*S*-transferase activity in the liver of either rats or hamsters.

Effect of cobalt protoporphyrin pretreatment on hepatic levels of glutathione, UDPGA, glycogen, and on blood glucose

Administration of cobalt chloride to rats, mice, and hamsters is known to cause an increase in hepatic glutathione levels [11–13]. To determine whether cobalt protoporphyrin has a similar effect, hepatic glutathione levels were measured 72 hr after pre-

Table 3. Effect of cobalt protoporphyrin pretreatment on hepatic glutathione, UDPGA, and glycogen and on blood glucose

Treatment	Hepatic GSH (mM)	Hepatic UDPGA (nmol/g liver)	Hepatic glycogen (mg/g liver)	Blood glucose (mg/dl)
Hamster				
Control	7.10 ± 0.21	210.1 ± 2.5	60.7 ± 4.0	90.8 ± 10.5
Cobalt protoporphyrin	7.11 ± 0.30	221.9 ± 10.1	42.6 ± 4.7*	124.6 ± 15.9
Rat				
Control	5.38 ± 0.23	274.8 ± 16.2	49.1 ± 2.7	137.7 ± 2.7
Cobalt protoporphyrin	4.75 ± 0.35	177.0 ± 11.7*	12.5 ± 2.9*	86.4 ± 11.2*

Animals were pretreated with either cobalt protoporphyrin (hamster, 125 μmol/kg; rat, 90 μmol/kg) or saline 72 hr prior to being killed. Hepatic GSH, UDPGA and glycogen, and blood glucose levels were determined as described under Materials and Methods. Values are means ± SE, N = 4.

* Significantly different from control animals, $P < 0.05$.

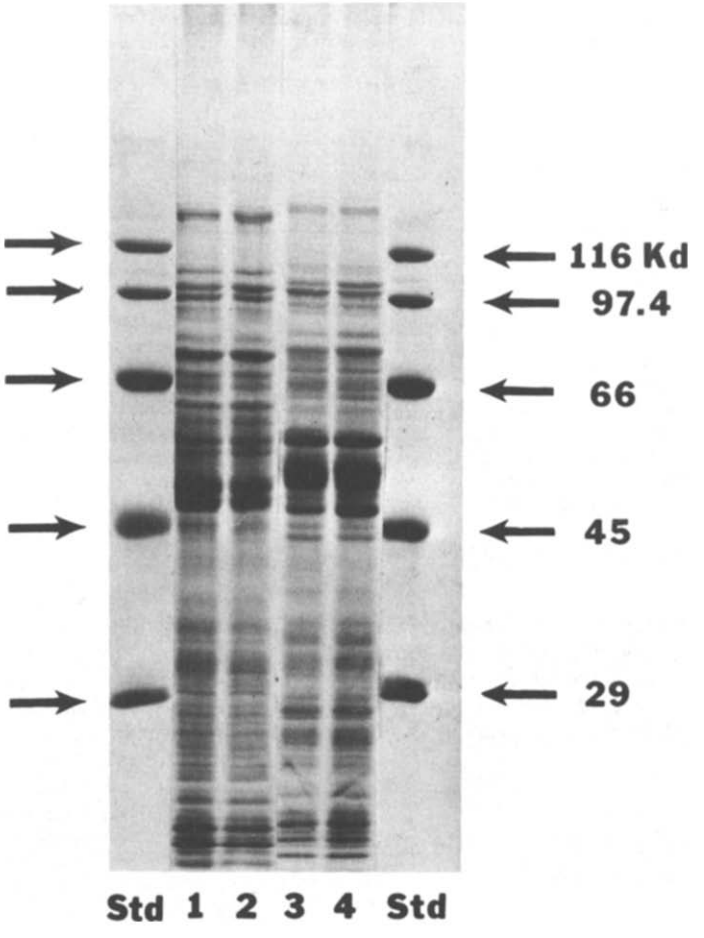

Fig. 4. Effect of cobalt protoporphyrin on hepatic cytochrome P-450 apoprotein profile. Animals were pretreated with either cobalt protoporphyrin (rats, 90 μmol/kg s.c.; hamsters, 125 μmol/kg s.c.) or saline 72 hr prior to being killed, removal of liver, and preparation of hepatic microsomal fractions. Microsomal protein (20 μg; lane 1, control rat; lane 2, cobalt protoporphyrin rat; lane 3, control hamster; lane 4, cobalt protoporphyrin hamster) was separated by SDS–PAGE, and the apoprotein profile was visualized by staining with Coomassie blue.

treatment with cobalt protoporphyrin. Glutathione levels in livers of cobalt protoporphyrin treated hamsters and rats were not different from their saline-treated controls (Table 3). As a positive control, a third group of hamsters received $CoCl_2 \cdot 6H_2O$ (60 mg/kg, s.c). As expected, glutathione hepatic levels were elevated markedly in this group (12.78 ± 0.75 mM; $P < 0.05$, $N = 4$, mean ± SE) as compared to either control or cobalt protoporphyrin treated hamsters (Table 3).

Determination of the effect of cobalt protoporphyrin pretreatment on hepatic levels of UDPGA indicated that cobalt protoporphyrin had no effect on basal hepatic UDPGA levels in hamsters (Table 3). However, in the rat, basal levels were decreased modestly (*ca.* 35%) (Table 3). It has been suggested that hepatic glycogen is the major source of glucose units for UDPGA synthesis and hence that levels of hepatic glycogen, in part, may regulate UDPGA availability [36]. Examination of the effect of cobalt protoporphyrin on hepatic glycogen stores indicated that levels of hepatic glycogen were reduced modestly, but significantly (*ca.* 30%), in hamsters, and reduced dramatically in the rat (*ca.* 75%) (Table 3). Blood glucose levels were not altered in the cobalt protoporphyrin treated hamster, but were decreased modestly (*ca.* 35%) into the low normal range in the rat (Table 3).

Effect of cobalt protoporphyrin pretreatment on cytochrome P-450 reductase and cytochrome b_5

The effect of cobalt protoporphyrin on the activity of cytochrome P-450 reductase in the hamster and rat is shown in Table 4. Cobalt protoporphyrin pretreatment caused a modest, though statistically significant, decrease in cytochrome P-450 reductase activity in both the hamster (*ca.* 25%) and the rat (*ca.* 44%).

To examine the specificity of cobalt protoporphyrin-dependent heme depletion for cytochrome P-450 as compared with the other major

Table 4. Effect of cobalt protoporphyrin pretreatment on hepatic microsomal cytochrome P-450 reductase activity and cytochrome b_5 levels

Treatment	Cytochrome P-450 reductase (nmol cyto. *c* reduced / mg protein · min)	Cytochrome b_5 (nmol / mg protein)
Hamster		
Control	174.0 ± 16.2	0.315 ± 0.023
Cobalt protoporphyrin	129.9 ± 3.6*	0.312 ± 0.046
Rat		
Control	137.2 ± 3.7*	0.402 ± 0.046
Cobalt protoporphyrin	76.8 ± 2.2*	0.235 ± 0.015*

Animals received either cobalt protoporphyrin (hamster, 125 μmol/kg; rat, 90 μmol/kg) or saline 72 hr prior to being killed. Microsomal fractions were isolated, and cytochrome P-450 reductase activity and cytochrome b_5 levels were determined as described under Materials and Methods. Values are means ± SE, N = 4.

* Significantly different from control animals, $P < 0.05$.

hepatic microsomal hemoprotein, the effect of cobalt protoporphyrin on hepatic microsomal cytochrome b_5 content was examined. Cobalt protoporphyrin did not alter cytochrome b_5 levels in the hamster but caused a significant decrease (*ca.* 40%) in the rat (Table 4).

In vivo *studies*

Antipyrine clearance. It is well known that antipyrine is rapidly and completely absorbed following oral (or i.p.) administration; that it is distributed in total body water with negligible binding to tissue or plasma proteins; that it is almost completely metabolized by the liver via a cytochrome P-450-dependent pathway with a low extraction ratio; and that it has negligible renal clearance [37]. Antipyrine has thus been widely used experimentally *in vivo* as an indicator of hepatic cytochrome P-450 drug-metabolizing

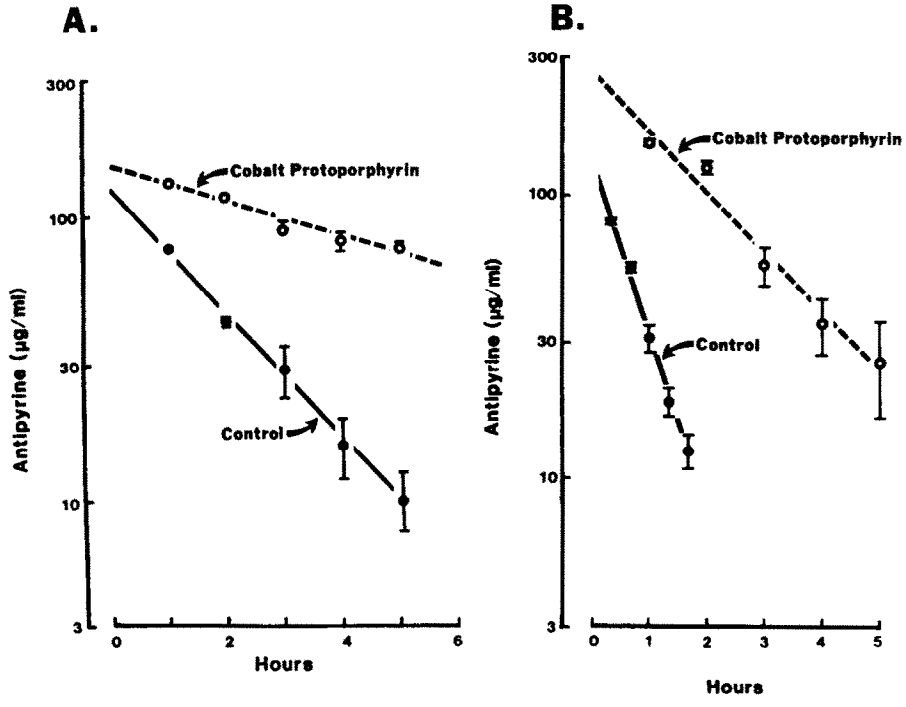

Fig. 5. Effect of cobalt protoporphyrin pretreatment on the systematic clearance of antipyrine. Animals received either cobalt protoporphyrin (○) (A, hamsters, 125 μmol/kg; B, rats, 90 μmol/kg) or saline (●) 72 hr prior to administration of antipyrine (100 mg/kg i.p.) Plasma was collected at various times after administration of drug, and the antipyrine was extracted with dichloromethane:pentane (1:1, v/v) and quantitated by HPLC as described under Materials and Methods. Values are means ± SE, N = 4.

Table 5. Effect of cobalt protoporphyrin pretreatment on the elimination of acetaminophen and on the apparent rate constants (K') for formation of acetaminophen metabolites in hamsters

Treatment	β (hr^{-1})	Apparent rate constants for metabolic formation				K_E (hr^{-1})
		K'_G	K'_S (hr^{-1})	K'_{MA}	$K'_{MTAG+SOX}$	
Control	1.798 ± 0.065	0.773 ± 0.143	0.444 ± 0.161	0.298 ± 0.072	0.119 ± 0.017	0.021 ± 0.005
Cobalt protoporphyrin	1.302 ± 0.102*	0.721 ± 0.040	0.344 ± 0.057	0.132 ± 0.011*	0.033 ± 0.004*	0.013 ± 0.004

Hamsters received either cobalt protoporphyrin (125 μmol/kg) or saline 72 hr prior to administration of 25 mg/kg [^{3}H]acetaminophen (i.p.) and placed in individual metabolic cages. Serial blood samples and total urinary collections were obtained and used to calculate the kinetic parameters as previously described [34]. The apparent rate constant for formation of each metabolite was calculated as $\beta \times$ urinary metabolite fraction [25, 34]. Values are means ± SE, N = 4, of acetaminophen-glucuronide (K'_G); acetaminophen sulfate (K'_S); acetaminophen mercapturate (K'_{MA}); and the methylthio derivatives, methylthioacetaminophen glucuronide and methylthioacetaminophen sulfoxide ($K'_{MTAG+SOX}$). K_E = renal elimination rate constant.

* Significantly different from control animals, $P < 0.05$.

capacity (i.e. hepatic cytochrome P-450-dependent intrinsic clearance capacity). We examined the clearance of antipyrine *in vivo* to determine the effect of cobalt protoporphyrin on the hepatic cytochrome P-450-dependent clearance capacity in the intact animal (Fig. 5). The clearance of antipyrine was delayed 85% in the hamster (control, 22.6 ± 1.3 $ml \cdot min^{-1} \cdot kg^{-1}$; cobalt protoporphyrin, 3.3 ± 0.2 $ml \cdot min^{-1} \cdot kg^{-1}$) and 77% in the rat (control, 6.9 ± 0.5; cobalt protoporphyrin, 1.6 ± 0.1) following cobalt protoporphyrin pretreatment. These data indicate that hepatic cytochrome P-450-dependent intrinsic clearance capacity *in vivo* parallels cytochrome P-450 content as measured *in vitro*.

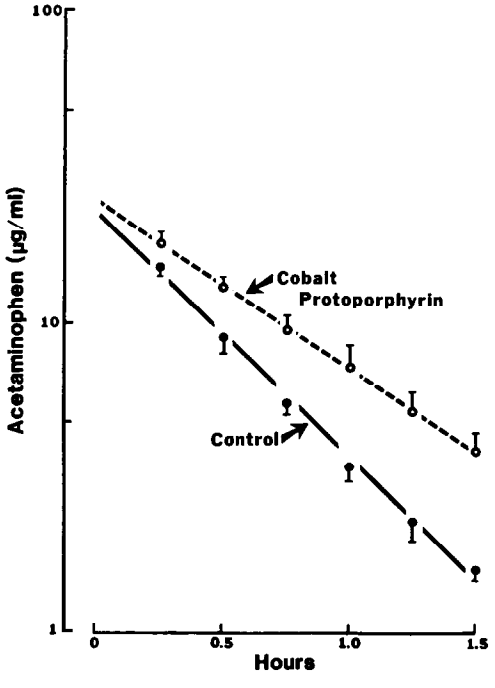

Fig. 6. Effect of cobalt protoporphyrin on the blood half-life of acetaminophen in the hamster. Animals received either cobalt protoporphyrin (○) (90 μmol/kg s.c.) or saline (●) 72 hr prior to experimentation. Concentrations of acetaminophen were determined at various times after administration of [^{3}H]acetaminophen (25 mg/kg i.p.) as previously described [34]. Values are means ± SE, N = 4.

Acetaminophen metabolism. Acetaminophen is cleared predominantly by phase II conjugative pathways in humans and animals [38]. The activity of the various pathways *in vivo* can be examined by fractional metabolism analysis [39] in which the apparent rate constant (k') for formation of a metabolite is estimated by the product of the overall elimination rate constant (β) of the parent compound and the urinary metabolite fraction. Experimentally, a low dose of acetaminophen (25 mg/kg) was used to test the specificity of cobalt protoporphyrin for the cytochrome P-450-dependent metabolic pathway versus the two major routes of elimination, glucuronidation and sulfation. In hamsters given acetaminophen (25 mg/kg), cobalt protoporphyrin pretreatment resulted in a marked slowing of drug elimination as indicated by an approximate 30% decrease in β (Table 5; Fig. 6). This decrease was due to a statistically significant decrease in the apparent rate constant for formation of the hepatic cytochrome P-450-dependent metabolites acetaminophen mercapturate and the methylthio-derivatives (methylthioacetaminophen glucuronide and methylthioacetaminophen sulfoxide). The glucuronidation and sulfation pathways were unaltered in the cobalt protoporphyrin pretreated animals.

DISCUSSION

The results of the present studies confirmed that cytochrome P-450 is decreased in rats by a single administration of cobalt protoporphyrin, in a dose- and time-dependent manner (Fig. 1) [14, 40], and extended the observation to the hamster (Fig. 2). The depletion in both species was extensive (>80%) and long-lasting (>10 days), and was accompanied by major decreases in the cytochrome P-450-dependent metabolism of xenobiotics, as measured in isolated liver microsomes (Table 1).

Of interest, Lineweaver–Burk analysis indicated that the pattern of inhibition of metabolism of all of

the type I substrates was non-competitive; that is, that the V_{max} values were decreased whereas the K_m values were unchanged. Non-competitive inhibition is consistent with decrease in the quantity of enzyme present in the microsomal membrane without change in the properties of the enzyme, especially the affinity of the enzyme for its substrate. Of particular interest, the decrease in cytochrome P-450 content and enzyme activity, as measured in the microsomal preparations *in vitro*, was matched by a decrease in the *in vivo* hepatic cytochrome P-450-dependent intrinsic clearance capacity, as determined by antipyrine clearance (Fig. 5). Collectively these data confirmed that cobalt protoporphyrin treatment of animals results in an extensive and long-lasting depletion of hepatic cytochrome P-450 and indicate that this depletion was reflected in loss of cytochrome P-450-dependent clearance of xenobiotic substances *in vivo*.

In contrast to cobalt chloride [11–13], cobalt protoporphyrin did not cause an increase in hepatic glutathione (Table 3). Further, cobalt protoporphyrin did not increase hepatic UDP-glucuronyltransferase or decrease hepatic phenolsulfotransferase activities, as measured *in vitro* in liver cell fractions (Table 2). The specificity of cobalt protoporphyrin for the cytochrome P-450-dependent pathway over these conjugative pathways was confirmed in hamsters given a low dose of acetaminophen (25 mg/kg) (Fig. 6, Table 5). At low dosage of drug, the apparent rate constants of formation of the metabolites (K′, Table 5) may be taken to approximate the enzymic first-order rate constants (V_{max}/K_m) for the pathways. If the K_m values are assumed not to alter in response to cobalt protoporphyrin, change in the apparent rate constants for metabolite formation may be taken to reflect the quantity of enzyme present in the liver. As shown in Table 5, cobalt protoporphyrin in pretreatment of the hamsters caused a marked diminution of the cytochrome P-450-dependent activities (mercapturate and methylthiometabolite formation) and no change in the conjugative pathways (glucuronide and sulfate ester formation). Since glutathione levels in the liver (Table 3) and glutathione-*S*-transferase activity (Table 2) were unaffected by cobalt protoporphyrin, the diminution in mercapturate formation was unlikely to be related to a decreased capacity for conjugation of the reactive metabolite of acetaminophen (NAPQI) with glutathione.

However, cobalt protoporphyrin is clearly not without non-heme related effects in the liver. Hepatic glycogen was depressed significantly in both rats and hamsters, and hepatic UDPGA and blood glucose were diminished in the rat (Table 3). This cluster of effects on glucose metabolism raises the possibility of an interaction between heme metabolism and glucose homeostasis. Additionally, cobalt protoporphyrin is known to suppress weight gain in rats and to cause reduction in serum testosterone, thyroxine and 3,4,5-triodiothyroxine levels [41]. While the mechanisms of these several effects are unknown, it is of interest that high carbohydrate diets are well known to suppress heme synthesis in acute intermittent porphyria patients [42]

Examination of the specificity of cobalt protoporphyrin for hepatic cytochrome P-450 versus other components of the monooxygenase system indicated that cobalt protoporphyrin pretreatment modestly decreased cytochrome P-450 reductase activity in hepatic microsomes from both species studied (Table 4). Suppression of cytochrome P-450 reductase in rats by cobalt protoporphyrin has been reported previously by Cheeseman *et al.* [43]. It is well known that the activity of cytochrome P-450 reductase greatly exceeds the overall activity of the various microsomal cytochrome P-450-dependent mixed-function oxidases (for example, compare the V_{max} values of Table 1 with reductase activities of Table 4). Thus, it seems unlikely that the modest decrease in cytochrome P-450 reductase activity observed (Table 4) would play a significant role in the depression of cytochrome P-450-dependent metabolism.

In agreement with earlier studies [14], we observed a decrease in the content of cytochrome b_5 in the livers of rats pretreated 72 hr earlier with cobalt protoporphyrin. In contrast, the level of cytochrome b_5 in hamster liver was unaffected, implying a slower turnover of hepatic cytochrome b_5 in this species. The physiological significance of a decrease of cytochrome b_5 in rat liver is unknown.

As pointed out by Drummond and Kappas [14], the ability of cobalt protoporphyrin to cause extensive depletion of hepatic cytochrome P-450, and to maintain that depletion on a long-term basis, offers a variety of experimental uses. In addition to its utility in evaluating the role of cytochrome P-450 in the metabolic activation of drugs and other xenobiotics to chemically reactive species [43], cobalt protoporphyrin has found use in assessment of the role of cytochrome P-450 in endogenous steroid homeostasis [15]. The present observations on the specificity of cobalt protoporphyrin strongly support its use for metabolic studies. While its biological effects are clearly not restricted entirely to modulation of cytochrome P-450 levels and activities, its specificity is markedly superior to that of cobalt chloride [11–13]. Of importance, cobalt protoporphyrin appears to be significantly less toxic to the laboratory animals than cobalt chloride. In agreement with Drummond and Kappas [14], we observed that the cobalt protoporphyrin treated animals did not gain weight at a rate similar to controls. However, the animals did not show the characteristic halt in feeding, the dramatic hair and weight loss, and the general wasting that is seen with cobalt chloride treated animals. Hamsters tolerated the metalloporphyrin better than the rats with a mortality rate during the 3-day treatment regimen of about 1–2% at 125 μmol/kg (with death usully occurring within the first 48 hr). In the rat, a mortality rate of 20% was observed in animals given 90 μmol/kg. We have not further evaluated the effect of cobalt protoporphyrin administration on the pathophysiology of these animals.

It is also of interest that the extent of reduction in cytochrome P-450-dependent microsomal metabolic activity (*ca.* 40–95%) did not always parallel the magnitude of cytochrome P-450 depletion (>80%). One possible explanation is that since the isozymes have been shown to turn over at different rates [44], depression of free cellular heme may result in a selective depletion of those isozymes which turn over

most rapidly, leaving the slower turning over cytochrome P-450 isozymic species relatively intact. However, this possibility was not supported by the PAGE studies. Staining of the LDS–PAGE of microsomal protein for heme (Fig. 3) indicated a generalized loss of heme protein in preparations derived from cobalt protoporphyrin treated rats and hamsters. The distribution of the residual heme in the heme-depleted preparations is not suggestive of a selective retention of specific isozymes of cytochrome P-450.

Kappas and Drummond [1] have reported that, 4 days after the administration of cobalt protoporphyrin to rats, there is a noticeable diminution of protein in the 50–55 kD region of SDS–PAGE of hepatic microsomes, as indicated by visual inspection of Coomassie blue stained gels. They suggested that this loss of protein reflected a decrease in the amounts of the apoproteins of the cytochrome P-450 isozymes present in microsomes. In the present studies, we observed little or no loss of protein in the 48–55 kD range in Coomassie blue stained SDS–PAGE of liver microsomal preparations of rats and hamsters, as judged visually (Fig. 4), or by densitometric scans (Fig. 3). The reason for this apparent stabilization is unknown, but may be related to association of the cobalt protoporphyrin with the cytochrome P-450 apoproteins as has been observed with *Pseudomonas putida* cytochrome P-450 apoprotein [45]. Of interest, our data raise the possibility that treatment of rats and hamsters with cobalt protoporphyrin dissociates the control of heme synthesis from that of the synthesis of the cytochrome P-450 apoproteins in the liver. It follows that cobalt protoporphyrin treated rodents may represent highly useful experimental models for the study of the control of cytochrome P-450 apoprotein synthesis and of the way this system may be influenced by exogenous heme.

In summary, cobalt protoporphyrin pretreatment dramatically reduced hepatic cytochrome P-450 levels resulting in decreased cytochrome P-450-dependent metabolic activity in both species studied, as determined both *in vitro* and *in vivo*. The specificity exhibited by cobalt protoporphyrin pretreatment for the hepatic cytochrome P-450 system relative to other phase I and phase II enzymes was notable both *in vitro* and *in vivo*. While cobalt protoporphyrin pretreatment modestly altered cytochrome b_5 in the rat, and cytochrome P-450 reductase in both the rat and the hamster, these effects appeared to be inconsequential relative to the effect on hepatic cytochrome P-450. Of particular interest, cobalt protoporphyrin pretreatment had no effect on hepatic UDP-glucuronyltransferase, phenolsulfotransferase, and glutathione-*S*-transferase determined *in vitro* in either species studied. This specificity was supported by *in vivo* metabolic studies on acetaminophen in the hamster. Cobalt protoporphyrin pretreatment reduced acetaminophen (25 mg/kg) metabolism via the cytochrome P-450-dependent pathway specifically; there was no effect on either acetaminophen sulfation or glucuronidation.

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