Decrease in hepatic cytochrome *P*-450 by cobalt

Evidence for a role of cobalt protoporphyrin

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Exposure of cultured chick-embryo hepatocytes to increasing concentrations of CoCl_2 in the presence of allylisopropylacetamide results in formation of cobalt protoporphyrin, with a reciprocal decrease in haem and cytochrome *P*-450. Treatment of rats with CoCl_2 ($84 \mu\text{mol/kg}$) and 5-aminolaevulinate (0.2 mmol/kg) also results in formation of cobalt protoporphyrin and a decrease in cytochrome *P*-450 in the liver. Hepatic microsomal fractions from rats treated with phenobarbital, CoCl_2 and 5-aminolaevulinate were analysed by polyacrylamide-gel electrophoresis. Cobalt protoporphyrin was associated mainly with proteins of 50 000–53 000 mol.wt. The results suggest that the formation of cobalt protoporphyrin occurred at the expense of the synthesis of haem, leading to a decrease in cytochrome *P*-450. Furthermore, the cobalt protoporphyrin that was formed may itself have been incorporated into apocytochrome *P*-450.

Administration of CoCl_2 to rats results in decreased amounts of hepatic haem and of the hepatic haemoprotein cytochrome *P*-450 (Tephly *et al.*, 1973; Maines & Kappas, 1974; De Matteis & Gibbs, 1977). The decrease in hepatic haem has been proposed to result from (1) inhibition of 5-aminolaevulinate synthase, the first and rate-limiting enzyme in hepatic haem biosynthesis (Nakamura *et al.*, 1975; Maines *et al.*, 1976; De Matteis & Gibbs, 1977; Igarashi *et al.*, 1978; P. Sinclair *et al.*, 1979); (2) decreased formation of haem and formation of cobalt protoporphyrin owing to competition of cobalt with iron for ferrochelatase, the enzyme that inserts either Fe^{2+} or Co^{2+} into protoporphyrin (Igarashi *et al.*, 1978; P. Sinclair *et al.*, 1979; Watkins *et al.*, 1980) and (3) increases in the activity of haem oxygenase, the rate-limiting enzyme in the catabolism of haem (Maines & Kappas, 1974; De Matteis & Unseld, 1976).

In rats (P. Sinclair *et al.*, 1979) and cultured chick-embryo hepatocytes (Maines & Sinclair, 1977), small amounts of CoCl_2 decrease hepatic 5-aminolaevulinate synthase activity, whereas amounts approx. 10-fold greater are necessary to increase haem oxygenase (Maines & Sinclair, 1977; Woods & Carver, 1977; Sinclair *et al.*, 1978; Sassa *et al.*, 1980). These findings suggest that the two effects of cobalt are mediated by separate mechan-

isms. In rats, the decrease in 5-aminolaevulinate synthase coincides with the formation of cobalt protoporphyrin (P. Sinclair *et al.*, 1979). Cultured chick-embryo hepatocytes treated with an inducer of 5-aminolaevulinate synthase also synthesize cobalt protoporphyrin after exposure to CoCl_2 , with reciprocal decreases in 5-aminolaevulinate synthase and intracellular haem (Sinclair *et al.*, 1982). These results suggest that cobalt protoporphyrin, rather than the metallic ion, is responsible for the decrease in 5-aminolaevulinate synthase and that cobalt protoporphyrin acts like haem, the physiological regulator of this enzyme. Additional evidence supporting this possibility comes from the administration of cobalt protoporphyrin to either cultured chick-embryo hepatocytes (Sinclair *et al.*, 1982) or intact rats (Igarashi *et al.*, 1978). In both systems, cobalt protoporphyrin, like haem, decreased 5-aminolaevulinate synthase induced by allylisopropylacetamide. *In vitro*, cobalt causes some inhibition of 5-aminolaevulinate synthase activity, but not as marked as that observed after administration of the metal *in vivo* (Nakamura *et al.*, 1975).

In the present paper we show that, in both the intact rat and the chick cell culture, small amounts of CoCl_2 caused a decrease in hepatic cytochrome *P*-450 concomitant with the formation of cobalt

protoporphyrin. Furthermore, cobalt protoporphyrin was mainly associated with microsomal proteins of 50 000–53 000 mol.wt. We suggest that the cobalt protoporphyrin synthesized by the hepatocytes is incorporated into apocytochrome *P*-450 in place of haem, the physiological prosthetic group. This possibility represents an additional means by which cobalt administration causes a decrease in hepatic cytochrome *P*-450.

Methods

Chemicals

Williams E medium was purchased from Flow Laboratories, McLean, VA, U.S.A.; trypsin (3× crystallized), glutamine and penicillin–streptomycin were from GIBCO, Grand Island, NY, U.S.A.; Varidase was from Lederle, Pearl River, NY, U.S.A.; 5-aminolaevulinic acid, 3,3',5-tri-iodothyronine, bovine pancreatic insulin and Coomassie Brilliant Blue R were from Sigma, St. Louis, MO, U.S.A.; dexamethasone phosphate was from Merck and Co., West Point, PA, U.S.A.; egg albumin was from Fisher Chemical Co., Pittsburgh, PA, U.S.A.; 3,3',5,5'-tetramethylbenzidine was from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; phenobarbital was from Eli Lilly and Co., Indianapolis, IN, U.S.A.; lithium dodecyl sulphate was from BDH Chemicals, Poole, Dorset, U.K.; acrylamide, *NN'*-methylenebisacrylamide and *NNN'*-tetramethylethylenediamine were from Eastman Kodak Co., Rochester, NY, U.S.A.; cobalt protoporphyrin and protoporphyrin were from Porphyrin Products, Logan, UT, U.S.A. Allylisopropylacetamide was a gift from Hoffmann–LaRoche, Nutley, NJ, U.S.A.

Primary cultures of hepatocytes

Primary cultures of chick-embryo hepatocytes were prepared as described previously (J. Sinclair *et al.*, 1979), with the following modifications: livers were digested with 0.066% Gibco trypsin for 30 min and a pinch of solid Varidase was added 11 min after addition of trypsin (Griener *et al.*, 1978). Cells were plated in Williams E medium containing insulin, dexamethasone and tri-iodothyronine as described by J. Sinclair *et al.* (1979). After 24 h, the medium was changed to Williams E containing dexamethasone and tri-iodothyronine, but no insulin. After the final change of medium, cells were exposed to allylisopropylacetamide (20 µg/ml) and different concentrations of CoCl₂ for 20 h. At the end of the drug treatment, cells from individual 10 cm plates (approx. 5 mg of protein/plate) were harvested in sodium phosphate buffer (0.1 M, pH 7.4, 0.5 ml), homogenized in a Potter–Elvehjem homogenizer (600 rev./min, 11 strokes) and portions were removed for separate measurement of haem, cobalt

protoporphyrin and cytochrome *P*-450, as described below.

Treatment of rats

Male Sprague–Dawley rats (approx. 200 g) were starved for 48 h before being killed, and treated as follows: phenobarbital, dissolved in 0.15 M NaCl, was administered in three injections (intraperitoneally), 80 mg/kg body wt. at 48 h and 24 h before death and 40 mg/kg 10 h before death; CoCl₂, 6H₂O, dissolved in 0.9% NaCl, was administered at a dose of 84 µmol/kg by subcutaneous injection and 5-aminolaevulinic acid (dissolved in 0.9% NaCl) was injected intraperitoneally at a dose of 0.2 mmol/kg. CoCl₂ and 5-aminolaevulinic acid were given 40 h, 24 h, 10 h and 3 h before death. Controls were injected with 0.9% NaCl at all time points. Altogether, there were four groups of rats, four rats per group, that received the following treatment: (1) 0.9% NaCl control, (2) phenobarbital alone, (3) CoCl₂ and 5-aminolaevulinate, (4) phenobarbital, CoCl₂ and 5-aminolaevulinate.

Preparation of rat hepatic microsomal fraction

This was done from rat liver homogenate by the calcium precipitation method, as described by J. Sinclair *et al.* (1981).

Haem and cobalt protoporphyrin determinations

Haem was extracted from both microsomal fractions and cell homogenates as described previously (P. Sinclair *et al.*, 1979) and its concentration determined from the reduced-minus-oxidized spectrum of the pyridine haemochrome (Falk, 1964). Cobalt protoporphyrin was measured in homogenates of cultured hepatocytes and in microsomal fraction from rat liver as follows: at the acid pH utilized during extraction of haem, most of the cobalt protoporphyrin remains associated with protein (P. Sinclair *et al.*, 1979). Addition of egg albumin (dissolved in 0.9% NaCl) before the acid–acetone extraction increases the amount of cobalt protoporphyrin retained in the protein pellet (Sinclair *et al.*, 1982). Therefore, for analysis of cobalt protoporphyrin, egg albumin was routinely added to tissue material (approx. 5 mg of egg albumin/mg of protein) before extraction with acetone/HCl/water (20:1:4, by vol.). The protein pellet, after extraction, was resuspended in 0.85 ml of 2% sodium dodecyl sulphate; 0.2 ml of pyridine and 0.4 ml of 1 M NaOH were added and the reduced-versus-oxidized difference spectrum was recorded with an Aminco DW2 spectrophotometer. The reduced-versus-oxidized difference spectrum of the pyridine haemochrome of cobalt protoporphyrin has a trough at 427 nm (P. Sinclair *et al.*, 1979). The concentration of cobalt protoporphyrin was calculated by using a ΔA_{mM} of 129 mM⁻¹·cm⁻¹ for the

difference in absorbance between the oxidized and reduced forms (Sinclair *et al.*, 1982).

Cytochrome *c* or residual haem present in the pellet after acid extraction of homogenates of cultured hepatocytes does not interfere with the cobalt protoporphyrin measurement. Evidence for this is the finding that after treatment of the cells with $1\mu\text{M}$ - CoCl_2 for 20h, which produces cobalt protoporphyrin concentrations at the limit of detection (Sinclair *et al.*, 1982), no peak or shoulder was observed at 424–420 nm in the reduced-versus-oxidized spectrum of the pyridine haemochrome, prepared from the acid–acetone-extracted pellet. In rat liver homogenates, however, under some conditions, residual haem did interfere with the determination of cobalt protoporphyrin (see the Results section).

Lithium dodecyl sulphate/polyacrylamide-gel electrophoresis of rat liver microsomal fraction

Microsomal proteins were analysed by the system of polyacrylamide-gel electrophoresis, in which lithium dodecyl sulphate is used instead of the sodium salt (J. Sinclair *et al.*, 1981). We have found that this procedure yields good resolution of microsomal protein with retention of approximately half of the haem on proteins of 50 000, 48 000 and 45 000 mol.wts. Each sample was applied to five separate tracks. After electrophoresis, one track was stained to locate haem and protein (Ryan *et al.*, 1978). The remaining four tracks were sliced into $0.5\text{ cm} \times 1\text{ cm} \times 0.25\text{ cm}$ pieces and analysed in duplicate (two adjacent tracks per sample) for the presence of cobalt protoporphyrin and haem by the following technique: two pieces were homogenized in 1.6 ml of a solution containing 20% (v/v) glycerol and 2% (w/v) sodium dodecyl sulphate, then 0.2 ml of pyridine and 0.1 ml of 1 M NaOH were added, and the reduced-versus-oxidized difference spectrum was recorded. We tested the recovery of cobalt protoporphyrin as follows: a 40 nM solution of cobalt protoporphyrin was made in 20% glycerol/2% sodium dodecyl sulphate from a stock solution of $625\mu\text{M}$ in 50% ethanol/0.02 M- NaOH . The reduced-versus-oxidized spectrum of the pyridine haemochrome was analysed (1) directly, (2) after homogenization (Potter–Elvehjem, 600 rev./min, 11 strokes) and (3) after homogenization with polyacrylamide-gel slices ($1\text{ cm} \times 1.0\text{ cm} \times 0.25\text{ cm}$). No cobalt protoporphyrin was lost under conditions (1) and (2); however, for unknown reasons, 30–40% was lost under condition (3). In addition, under condition (3), the position of the trough (at 427 nm) shifts to 432 nm.

Cytochrome P-450 and protein

The concentration of cytochrome P-450 was determined in rat liver microsomal fraction by the

method of Omura & Sato (1964) and in rat liver homogenates as previously described (Bonkowsky *et al.*, 1981), with an Aminco DW2 spectrophotometer. Cell homogenates of the cultured hepatocytes were diluted in a buffer containing the non-ionic detergent Emulgen, and cytochrome P-450 was measured in the 8700 g supernatant, as previously described (J. Sinclair *et al.*, 1979). Proteins were determined by the method of Lowry *et al.* (1951).

Results

Formation of cobalt protoporphyrin, haem and cytochrome P-450

(a) *Cultured hepatocytes.* In the experiment of Fig. 1, cultured chick-embryo hepatocytes were treated simultaneously with allylisopropylacetamide, an inducer of cytochrome P-450 (Krupa *et al.*, 1974) and increasing concentrations of CoCl_2 . The amount of cobalt protoporphyrin increased, with a reciprocal decrease in both haem and cytochrome P-450. No cobalt protoporphyrin was detected if the inducer was omitted (results not shown).

(b) *Rats.* We next investigated whether the reciprocal relationship between cobalt protoporphyrin and cytochrome P-450 could also be demonstrated in rats treated with CoCl_2 . The design of the experiment, presented in the Methods section, was

Fig. 1. Effect of increasing concentrations of CoCl_2 on amounts of haem, cytochrome P-450 and cobalt protoporphyrin in cultured hepatocytes

Cells were exposed to allylisopropylacetamide ($20\mu\text{g/ml}$) alone or with increasing amounts of CoCl_2 . After 20h exposure, the cells were harvested, homogenized, and separate portions of the same cell homogenate analysed for cytochrome P-450 (●) haem (Δ) and cobalt protoporphyrin (○) as described in the Methods section. All values are pmol/mg of protein. The bars indicate s.e.m. ($n = 3$).

based on the following rationale: (1) phenobarbital was administered to induce the apoprotein of cytochrome *P*-450, (2) CoCl_2 was administered in repeated low doses to provide a substrate for continual cobalt protoporphyrin formation, and (3) 5-aminolaevulinate was administered to bypass any decrease in the activity of 5-aminolaevulinate synthase resulting from cobalt administration (P. Sinclair *et al.*, 1979). Analyses of liver homogenates from rats treated with this regime showed that CoCl_2 caused a decrease in both control and phenobarbital-induced cytochrome *P*-450 (Table 1). In addition, cobalt protoporphyrin was detected in microsomal preparations from these homogenates (Table 2). The data presented in Table 2 were obtained with microsomal fractions from rats given 5-aminolaevulinate in addition to CoCl_2 . In microsomal fractions and liver homogenates from rats given CoCl_2 but not 5-aminolaevulinate, cobalt protoporphyrin was detectable. However, this cobalt protoporphyrin could not be quantified accurately because only small amounts were present relative to unextractable haem.

Analysis of rat hepatic microsomal fraction for association of cobalt protoporphyrin with proteins

Hepatic microsomal proteins from the experiment presented in Table 2 were analysed by polyacrylamide-gel electrophoresis to determine if

cobalt protoporphyrin was associated with any particular proteins.

The distribution of cobalt protoporphyrin on the gel is shown in Fig. 2, which includes a photograph of a separate track stained for proteins. In rats treated with phenobarbital, CoCl_2 and 5-aminolaevulinate, most of the cobalt protoporphyrin was associated with microsomal proteins of 50 000–53 000 mol.wt. (Fig. 2). Separate analyses of hepatic microsomal fractions from two such rats showed that the amount of cobalt protoporphyrin associated with these proteins was 30% and 35% of that applied to the gel. However, the proportion of cobalt protoporphyrin associated with these proteins may actually be greater, because of: (1) the loss of cobalt protoporphyrin when homogenized with polyacrylamide slices (see the Methods section) and (2) the presence of haem in these slices, which interferes with the spectral measurement of cobalt protoporphyrin.

Fig. 3 shows the reduced-versus-oxidized difference spectrum of the pyridine haemochrome of cobalt protoporphyrin in the gel slice containing proteins of 50 000–53 000 mol.wt. The spectrum in the Soret region was the same as for the pyridine haemochrome of authentic cobalt protoporphyrin homogenized in the presence of polyacrylamide.

The liver of rats treated with cobalt and 5-aminolaevulinate in the absence of phenobarbital

Table 1. *Effect of CoCl_2 , phenobarbital, and 5-aminolaevulinate on cytochrome *P*-450 in rat liver homogenates*
Treatment of the rats and the measurement of cytochrome *P*-450 are described in the Methods section. Each value represents the mean $\pm$ S.E.M. for four animals. Means were compared by analysis of variance procedures and the *F* statistics (Dixon & Massey, 1968). **P* < 0.03 with respect to appropriate treatment without the metal.

Treatment	Cytochrome <i>P</i> -450 (pmol/mg of liver-homogenate protein)
Saline	217 $\pm$ 3
CoCl_2 + 5-aminolaevulinate	187 $\pm$ 6*
Phenobarbital	679 $\pm$ 30
Phenobarbital + CoCl_2	509 $\pm$ 48*
Phenobarbital + 5-aminolaevulinate	653 $\pm$ 31
Phenobarbital + CoCl_2 + 5-aminolaevulinate	477 $\pm$ 18*

Table 2. *Cytochrome *P*-450 and cobalt protoporphyrin in rat hepatic microsomal fraction*
The methods for treatment of the rats, preparation of the microsomal fraction, and analyses of cytochrome *P*-450 and cobalt protoporphyrin are given in the Methods section. Each value represents the mean $\pm$ S.E.M., *n* = 3.

Treatment	Content (nmol/mg of microsomal protein)	
	Cytochrome <i>P</i> -450	Cobalt protoporphyrin
Saline	0.89 $\pm$ 0.01	—
CoCl_2 + 5-aminolaevulinate	0.63 $\pm$ 0.04	0.18 $\pm$ 0.02
Phenobarbital	2.80 $\pm$ 0.07	—
Phenobarbital + CoCl_2 + 5-aminolaevulinate	2.10 $\pm$ 0.12	0.48 $\pm$ 0.04

Fig. 2. Association of cobalt protoporphyrin with hepatic microsomal proteins

Rats were treated with phenobarbital, CoCl_2 and 5-aminolaevulinate as described in the Methods section. Microsomal proteins were electrophoresed on lithium dodecyl sulphate/polyacrylamide slab gels 0.25 cm thick; 200 μg of microsomal protein from each sample was applied to each of three adjacent tracks, and the amount of cobalt protoporphyrin in the sample applied was 82 pmol/track. Two adjacent tracks were sliced into 0.5 cm $\times$ 1 cm pieces and analysed for the presence of cobalt protoporphyrin (see the Methods section). The remaining track was left unsliced and stained for protein (J. Sinclair *et al.*, 1981). A photograph of the gel stained for protein is presented at the bottom of the Figure. The amount of cobalt protoporphyrin associated with each protein fraction is plotted above. Pure cobalt protoporphyrin, applied to a separate track, migrated as one band at the dye front (results not shown). The solid arrows mark the positions of the proteins of 68 000, 60 000, 50 000, 40 000 and 36 000 as indicated. The dashed arrow indicates the protein (and dye) front.

Fig. 3. Reduced-minus-oxidized difference spectrum of the pyridine haemochrome of cobalt protoporphyrin associated with the proteins of 50 000–53 000 mol.wt.

Two 0.5 cm $\times$ 1 cm $\times$ 0.25 cm gel slices (from the gel presented in Fig. 2), containing the proteins of 50 000–53 000 mol.wt., were homogenized in 20% glycerol/2% sodium dodecyl sulphate, and the reduced-minus-oxidized spectrum of the pyridine haemochrome was recorded as described in the Methods section.

dimethyl sulphoxide) and 100 μM - CoCl_2 for 30 min at 37°C. Microsomal preparations from these homogenates contained the following amounts of cobalt protoporphyrin, in nmol/mg of microsomal protein: saline-treated rats, 0.8; phenobarbital-treated rats, 0.54. When these microsomal fractions were subjected to lithium dodecyl sulphate/polyacrylamide-gel electrophoresis, no cobalt protoporphyrin was detected associated with the proteins of 50 000–53 000 mol.wt.

Discussion

Administration of CoCl_2 to rats and cultured rat hepatocytes results in a decrease in hepatic cytochrome P-450 (Tephly *et al.*, 1973; De Matteis & Unseld, 1976; Guzelian & Bissell, 1976; Maines & Kappas, 1976). The following causes have been proposed for this decrease by CoCl_2 : (1) inhibition of haem synthesis owing to decreased activity of 5-aminolaevulinate synthase (De Matteis & Gibbs, 1977; Igarashi *et al.*, 1978; P. Sinclair *et al.*, 1979), (2) increased haem catabolism owing to enhanced haem oxygenase activity (Maines & Kappas, 1974; De Matteis & Unseld, 1976), and (3) inhibition by cobalt of the association of haem with apocytochrome P-450 (Guzelian & Bissell, 1976). Our results suggest an additional explanation for the

also contained cobalt protoporphyrin (Table 2). However, after electrophoresis of microsomal fractions isolated from such rats, no cobalt protoporphyrin was detected associated with the proteins of 50 000–53 000 mol.wt. (results not shown).

In microsomal fractions isolated from rats treated with phenobarbital, CoCl_2 and 5-aminolaevulinate, the association of cobalt protoporphyrin with the proteins of 50 000–53 000 mol.wt. may have been non-specific. To investigate this possibility, cobalt protoporphyrin was generated *in vitro* in 20% (w/v) liver homogenates (in 20 mM-Tris/HCl/0.25 M-sucrose, pH 7.4) from rats treated with 0.9% NaCl or phenobarbital by incubation of these homogenates with 125 μM -protoporphyrin (dissolved in

cobalt-mediated decrease in cytochrome *P*-450, namely that cobalt protoporphyrin is formed at the expense of haem (iron protoporphyrin) making less haem available for incorporation into apocytochrome *P*-450. It is not unexpected that cobalt protoporphyrin would be formed, since Co^{2+} is as good a substrate for ferrochelatase as is Fe^{2+} . In rat liver mitochondria, ferrochelatase has a similar V_{max} and K_m for both Fe^{2+} and Co^{2+} when protoporphyrin is used as the porphyrin substrate for the enzyme (Labbe & Hubbard, 1961), under conditions in which the iron is maintained in the Fe^{2+} form. Under non-reducing conditions, Fe^{2+} is readily oxidized to Fe^{3+} , which is not a substrate for ferrochelatase (Jones & Jones, 1969). In contrast with Fe^{2+} , Co^{2+} is not as readily oxidized. Thus, depending on the environment of the enzyme, Co^{2+} may be a better substrate for ferrochelatase than Fe^{2+} .

We have found that cobalt protoporphyrin is formed at the expense of haem by primary cultures of chick-embryo hepatocytes treated with allylison-propylacetamide (Sinclair *et al.*, 1982). Here we showed that the formation of cobalt protoporphyrin occurs with a reciprocal decrease in cytochrome *P*-450 (Fig. 1). A decrease in cytochrome *P*-450 was detectable with as little as $1\mu\text{M}$ - CoCl_2 (Fig. 1), a concentration previously reported not to induce haem oxygenase (Maines & Sinclair, 1977). Therefore, we conclude that the decrease in cytochrome *P*-450 at 1 – $15\mu\text{M}$ - CoCl_2 may be due to the formation of cobalt protoporphyrin at the expense of haem, and not to induction of haem oxygenase. The similarity between the amount of cytochrome *P*-450 and the amount of haem lost after CoCl_2 treatment indicates that, under these conditions, changes in total haem reflect changes in cytochrome *P*-450.

Guzelian & Bissell (1976) were not able to detect cobalt protoporphyrin in cultured rat hepatocytes after treatment with cobalt and 5-aminolaevulinate, even though a decrease in cytochrome *P*-450 was observed. They concluded that cobalt prevented the association of haem with apocytochrome *P*-450 because haem accumulated, but not cytochrome *P*-450 or cobalt protoporphyrin. However, cobalt protoporphyrin may have been synthesized by these cells but not detected, owing to their method of analysis.

More recently, by electron paramagnetic resonance and spectrophotometric analyses, cobalt protoporphyrin has been detected both in rat liver (Igarashi *et al.*, 1978; P. Sinclair *et al.*, 1979; Watkins *et al.*, 1980) and in suspensions of rat hepatocytes (Lodola, 1980) after administration of CoCl_2 . Using spectrophotometric techniques, we have also detected cobalt protoporphyrin in hepatic microsomal fractions after treatment of rats with CoCl_2 (Table 2). Furthermore, the formation of

cobalt protoporphyrin is associated with a decrease in cytochrome *P*-450.

In hepatic microsomal fractions from rats treated with phenobarbital, CoCl_2 and 5-aminolaevulinate, cobalt protoporphyrin was detected associated with proteins of 50000–53000 mol.wt. (Fig. 2). At least one species of cytochrome *P*-450 induced by phenobarbital is present in this region of the gel (Ryan *et al.*, 1978). Thus it is possible that the cobalt protoporphyrin present in this region is associated with an apocytochrome *P*-450 induced by phenobarbital. It is unlikely that cobalt protoporphyrin was merely sticking non-specifically to the major proteins on the gel. Microsomal preparations from liver homogenates of phenobarbital-treated rats in which cobalt protoporphyrin is generated *in vitro* contained similar amounts of cobalt protoporphyrin to those in microsomal preparations from rats treated with phenobarbital, CoCl_2 and 5-aminolaevulinate. Yet, after electrophoresis, no cobalt protoporphyrin was detected in the 50000–53000-mol.wt. region of the gel.

Although cobalt protoporphyrin was detected in microsomal fractions from rats given cobalt and 5-aminolaevulinate, but no phenobarbital, no association of this chelate with 50000–53000-mol.wt. proteins was detected. If, as in the samples from phenobarbital-treated rats, 30% of the cobalt protoporphyrin applied remained associated with the proteins of 50000–53000 mol.wt., the amount of cobalt protoporphyrin in this region would be approx. 24 pmol, which would be measurable. However, since no cobalt protoporphyrin was detected in this region of the gel, the results suggest that the species of apocytochrome *P*-450 induced by phenobarbital, which constitutes less than 10% of control cytochrome *P*-450 (Lu & West, 1980), may have a greater affinity for cobalt protoporphyrin than do control apocytochromes *P*-450. Another possible explanation is that the turnover of control cytochrome *P*-450 haem may be slower than that of the phenobarbital-induced species. Thus the amounts of cobalt protoporphyrin that would become associated with the apoprotein of cytochrome *P*-450 during the duration of the treatment would be lower in control than in phenobarbital-treated rats.

A precedent exists for the association of cobalt protoporphyrin with apohaemoproteins. Cobalt-protoporphyrin-containing myoglobins and haemoglobins have been prepared *in vitro* (Hoffman & Petering, 1970; Yonetani *et al.*, 1974). These artificial globins are capable of reversible oxygenation, though to a lesser extent than the haem complexes. Cytochrome *P*-450_{cam} containing cobalt protoporphyrin in place of haem has been prepared *in vitro* (Wagner *et al.*, 1981). This cobalt analogue had 2–3% of enzymic activity relative to

the native complex. In addition, the reduced form of the cobalt analogue did not bind CO. Isolated rabbit reticulocytes, on exposure to CoCl_2 , form cobalt protoporphyrin, which appears to be associated with haemoglobin (Hunter & Jackson, 1975). If, as we suggest, cobalt protoporphyrin is associated with an apocytochrome P-450 *in vivo*, then the complex appears to be inactive enzymically, since, in the cultured chick-embryo hepatocytes, a decrease in aminopyrine demethylase activity parallels the decrease in cytochrome P-450 after CoCl_2 treatment (Sinclair *et al.*, 1980). In addition, we have found that, in the reduced form, neither authentic cobalt protoporphyrin nor the form made *in vivo* binds CO (results not shown).

We conclude that, in hepatocytes exposed to CoCl_2 , cobalt protoporphyrin is made at the expense of iron protoporphyrin, making less haem available for incorporation into apocytochrome P-450. In addition, cobalt protoporphyrin may itself be incorporated into apocytochrome P-450, particularly the apocytocytomes induced by phenobarbital.

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