Formation of Cobalt Protoporphyrin in the Liver of Rats

A MECHANISM FOR THE INHIBITION OF LIVER HAEM BIOSYNTHESIS BY INORGANIC COBALT

By PETER SINCLAIR,* ANTHONY H. GIBBS,†

JACQUELINE F. SINCLAIR* and FRANCESCO DE MATTEIS†

†Biochemical Pharmacology Section, Toxicology Unit, Medical Research Council Laboratories,
Woodmansterne Road, Carshalton, Surrey SM5 4EF, U.K., and

*VA Center, Veterans Administration, White River Junction, VT 05001, U.S.A.

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1. Treatment of rats with small doses of CoCl_2 decreases liver 5-aminolaevulinate synthase (EC 2.3.1.37) activity and impairs incorporation of 5-amino ^{14}C laevulinate into liver haem. Salts of other metals (cadmium, nickel, manganese and zinc) are all relatively inactive. 2. The dose-response curves obtained for both these effects closely mirror the accumulation in the liver of a compound that is labelled by 5-amino ^{14}C laevulinate and is unextractable by acetone/HCl. 3. Incorporation of 5-amino ^{14}C laevulinate into unextractable compound is also obtained *in vitro* by incubating liver homogenates with label in the presence of cobalt: isotope-dilution experiments show that the radioactivity passes through pools of porphobilinogen and protoporphyrin, but not of haem. 4. The unextractable compound is not covalently bound to protein and possesses the same extraction and spectral properties as authentic cobalt protoporphyrin. 5. It is concluded (a) that cobalt protoporphyrin is readily formed not only *in vitro*, but also *in vivo*, and (b) that its formation accounts for the impaired incorporation of 5-aminolaevulinate into haem and may also be responsible for the action of cobalt on 5-aminolaevulinate synthase.

The administration of a single dose of CoCl_2 to rats is followed by a number of effects on the synthesis and degradation of hepatic haem and haemoproteins. These include: (1) rapid inhibition of the first and rate-limiting enzyme of the biosynthetic pathway, 5-aminolaevulinate synthase (EC 2.3.1.37), followed by recovery and stimulation of activity (Nakamura *et al.*, 1975; De Matteis & Gibbs, 1976, 1977; Maines *et al.*, 1976; Maines & Sinclair, 1977); (2) inhibition of the incorporation of 5-aminolaevulinate into haem and into microsomal haemoproteins and catalase (Tephly *et al.*, 1973; Yasukochi *et al.*, 1974; Nakamura *et al.*, 1975; De Matteis & Gibbs, 1976, 1977; Woods & Carver, 1977); (3) increased degradation of haem and haemoproteins accompanied by a stimulation of liver haem oxygenase activity (Maines & Kappas, 1974; De Matteis & Unseld, 1976); and (4) possible interference with the binding of haem to the apoprotein of cytochrome *P*-450 (Guzelian & Bissell, 1976). It is not yet clear by what mechanisms cobalt produces all these effects. It has been suggested that cobalt may act through the formation of cobalt protoporphyrin (De Matteis, 1975; De Matteis & Gibbs, 1976, 1977; Maines *et al.*, 1976; Maines & Sinclair, 1977), but

there are also proposals that the metal may produce these liver effects in a direct manner, specifically excluding formation of a metalloprophyrin (Maines & Kappas, 1977a,b).

In this paper we provide evidence for the formation of cobalt protoporphyrin in the liver of rats treated with CoCl_2 *in vivo* and also in liver homogenates incubated with cobalt *in vitro*. Evidence is presented which strongly suggests that formation of cobalt protoporphyrin and decreased incorporation of labelled 5-aminolaevulinate into liver haem are directly related. The possibility is also considered that cobalt protoporphyrin may be involved in the inhibition of 5-aminolaevulinate synthase observed in the liver of treated animals. Some of these findings have appeared as an abstract (Sinclair *et al.*, 1978).

Materials and Methods

Animals

Male rats (160–180g) of the Porton strain were first deprived of food overnight, then injected in the loose subcutaneous tissue of the neck with $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (4.2–250 μmol in 10 ml of 0.85% NaCl/kg body wt.) and kept unfed for the duration of the experiments. Other metal salts (doses indicated in the

Abbreviation used: SDS, sodium dodecyl sulphate.

Results section) were also injected subcutaneously. Some rats were given 3,5-diethoxycarbonyl-1,4-dihydrocollidine (200 mg/kg) dissolved in arachis oil (10 ml/kg) orally before being injected with CoCl_2 or other metal salts. 5-Amino[4- ^{14}C]laevulinate ($2\mu\text{Ci}$, 37 nmol/rat) was injected intraperitoneally. Animals were killed by decapitation and their livers quickly removed and homogenized (20%, w/v) in 0.25 M-sucrose/0.02 M-Tris/HCl, pH 7.4, in some cases after perfusion with ice-cold 0.85% NaCl through the vena cava.

Enzyme assays

5-Aminolaevulinate synthase was assayed by using total liver homogenate as the source of the enzyme by a modification (Abbritti & De Matteis, 1971) of the method of Marver *et al.* (1966a). The amount of 5-aminolaevulinate formed was assayed as described by De Matteis (1971). When low absorption values were obtained because of a low activity of the synthase, the spectrum of the coloured Ehrlich salt was determined and the ratio of the two peaks, at 525 and at 552 nm, was checked (Sinclair & Granick, 1977).

Analytical methods

Proteins were determined by Aldridge's (1962) modification of the method of Robinson & Hogden (1940). Liver tissue was prepared for determination of cobalt content by a wet-ashing procedure (Thompson & Blanchflower, 1971) and cobalt was measured by atomic absorption spectrophotometry by using a Perkin-Elmer instrument, model 306, calibrated with an appropriate standard. Haem was measured as the pyridine haemochrome (Paul *et al.*, 1953). Porphobilinogen was measured by the method of Mauzerall & Granick (1956), and protoporphyrin in 1.37 M-HCl as described by Falk (1964).

Polyacrylamide-slab-gel electrophoresis

Slab-gel electrophoresis was performed by the methods of Studier (1973), with the following modifications. The separating gel contained 150 mM-Tris/HCl, pH 8.8, 2 mM-EDTA, 0.1% SDS and 15% acrylamide. The electrode buffer contained 50 mM-Tris/HCl, pH 8.4, 2 mM-EDTA, 0.1% SDS and 0.38 M-glycine. Gels were pre-electrophoresed for 30 min at 20 mA before application of the samples: 20 μl of sample was applied to each well and the gel run at 200 V until the dye front (Bromophenol Blue) was 1 cm from the bottom of the gel. One section of the gel was removed and stained to locate the proteins. Gels were first fixed for 60 min in propan-2-ol/acetic acid/water (5:2:13, by vol.), then stained for 60 min in the same solvent containing 0.05% Coomassie Blue R-250, and destained overnight in propan-2-ol/acetic acid/water (1:1:8, by vol.).

Preparation of radioactive samples for counting

The remaining unstained section of the gel was sliced vertically into the individual samples and the strips were then cut horizontally into 1 cm pieces: each of these was then placed in a scintillation vial containing 0.1 ml of water and 0.9 ml of Soluene. All vials were incubated at 50°C for 5 h and 10 ml of Insta-gel was then added.

Haemin was prepared for radioactivity counting by one of two different techniques. The first technique involved extraction of haemin from liver homogenate after addition of carrier haem as horse erythrocyte lysate, and crystallization of haemin by the method of Labbe & Nishida (1957); a known amount of haem was then applied in pyridine solution to a strip of blotting paper and its radioactivity determined by liquid-scintillation counting after combustion in an Inter technique Oxymat apparatus and after trapping the resulting $^{14}\text{CO}_2$ in an appropriate scintillant (De Matteis *et al.*, 1977).

The second technique, which provided in addition a purified protoporphyrin fraction and the extracted protein pellet, involved extraction of the liver homogenate with 15 vol. of a mixture (Morell *et al.*, 1961) of acetone, conc. HCl and water (20:1:4, by vol.) followed by two further extractions of the pellet obtained by centrifugation. The haemin was separated from porphyrins and transferred to diethyl ether by adding to the acid/acetone extract 2 vol. of peroxide-free diethyl ether and 1.5 vol. of 2 M-HCl. The ether phase was further washed with 2 M-HCl and then evaporated to dryness in a scintillation vial, after which 10 ml of Insta-gel was added: recovery of authentic haemin by this method was usually 65–75%.

Protoporphyrin was extracted back into ether from the pooled 2 M-HCl washings after adjustment of the pH to 4.5; the ether was then washed with 0.36% (w/v) HCl to remove any coporphyrin and then evaporated to dryness in scintillation vials in a 40°C shaking water bath, and 10 ml of Insta-gel added for determination of radioactivity. The efficiency of recovery of protoporphyrin throughout this procedure was not determined.

The pellet remaining after acid/acetone extraction was suspended in 1 ml of 2% (w/v) SDS in 0.1 M-Tris/HCl, pH 7.4, and NaOH was added dropwise until the pH was 8 and the pellet dissolved. After evaporation of any remaining acetone the solution was transferred to a scintillation vial by using two successive 5 ml portions of Insta-gel.

Total radioactivity of the liver homogenate was determined by taking 0.2 ml into a scintillation vial, adjusting to a volume of 1 ml with water and adding 10 ml of Insta-gel. All determinations of radioactivity were carried out in a Philips liquid-scintillation analyser and quenching was automatically corrected for by programmed quench curves.

Sources of special chemicals

Haemin and protoporphyrin IX dimethyl ester were purchased from Sigma (London) Chemical Co., Poole, Dorset BH17 7NH, U.K. The ester was hydrolysed as described by Falk (1964). Methaemalbumin was prepared as described by Tenhunen *et al.* (1968), by using human crystallized albumin obtained from Sigma. 5-Aminolaevulinate was obtained from Aldrich Chemical Co., Gillingham, Dorset SP8 4JL, U.K., and 5-amino[4- ^{14}C]laevulinate (53 mCi/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K.; porphobilinogen was a gift from Professor C. Rimington, F.R.S., University College Hospital Medical School, London W.C.1, U.K. Cobalt protoporphyrin was synthesized from protoporphyrin dimethyl ester as described by Falk (1964); a sample was also obtained from Dr. T. Yonetani. Insta-gel and Soluene were obtained from Packard Instruments, Caversham, Berks. RG4 7AA, U.K.

Results*Conversion of 5-amino[^{14}C]laevulinate in vivo and in vitro into a compound that cannot be extracted into acid/acetone*

In agreement with previous findings (De Matteis & Gibbs, 1976, 1977) 5-amino[^{14}C]laevulinate given *in vivo* to rats previously injected with CoCl_2 was converted into material that cannot be extracted by acid/acetone (henceforth called unextractable material): 1 h after injection of the label in cobalt-treated rats

there was an increase in the radioactivity recovered in the protein pellet (33% of the total liver radioactivity as compared with 6% in control rats), with a corresponding decrease in the radioactivity present in the extract. The extraction mixture used in the present experiments [containing 16% (v/v) water] was found, however, to be much more effective in extracting haem radioactivity from control liver homogenates than the extraction mixture previously used, in which water was not added. For this reason in all the experiments described below liver homogenates were extracted with acetone/HCl in the presence of water. Extraction with pyridine/chloroform (1:2, v/v) (Caughey & York, 1962), ethyl acetate/acetic acid (3:1, v/v) (Falk, 1964) or ethyl methyl ketone at pH 3 and 4°C (Dickinson, 1976) all failed to extract any more radioactivity from the liver homogenates of rats treated with CoCl_2 than did the acetone/HCl procedure.

Incorporation of radioactive 5-aminolaevulinate into unextractable material could also be demonstrated *in vitro* on incubation of normal liver homogenates with CoCl_2 (Table 1). Under these conditions CoCl_2 caused a concentration-dependent increase in the radioactivity recovered in the pellet, with a corresponding decrease in the radioactivity recovered in the extracts: about half the maximum effect was seen at a concentration of CoCl_2 as low as $10\text{ }\mu\text{M}$ (Table 1). CoCl_2 injected *in vivo* apparently remained available in the liver homogenate for the reaction that produced the unextractable material, and only a small additional effect was seen after addition of more CoCl_2 to the

Table 1. *Incorporation in vitro of 5-amino[4- ^{14}C]laevulinate into unextractable material by liver homogenates incubated with CoCl_2*

Rats were starved overnight and some were also treated with CoCl_2 ($250\text{ }\mu\text{mol/kg}$) and killed 1 h after CoCl_2 injection. Their livers were homogenized (20%, w/v) in 0.225 M-sucrose/0.05 M-Tris/HCl, pH 7.4, and 2.5 ml of the homogenate was incubated for 1 h at 37°C with shaking in the presence of 0.5 μCi of 5-amino[4- ^{14}C]laevulinate, with the addition (where indicated) of CoCl_2 , in a total volume of 2.75 ml. The reaction was stopped by addition of 15 ml of acetone, the pellet was then extracted with acetone/conc. HCl/water as described in the Materials and Methods section and the radioactivity recovered in this extract and in the solubilized pellet determined. Results given are those of an individual observation or averages of two observations with individual results in parentheses.

Treatment of rat <i>in vivo</i>	CoCl_2 added <i>in vitro</i> (final concn.)	Radioactivity recovered in (% of total radioactivity added)			
		Acid/acetone extract (a)	Protein pellet (b)	Total (a + b)	b/(a + b)
None	None	66.6 (64.4, 68.8)	8.2 (7.6, 8.9)	74.8	0.11
None	$10\text{ }\mu\text{M}$	52.6 (52.0, 53.2)	19.3 (18.6, 20.0)	71.9	0.27
None	$100\text{ }\mu\text{M}$	45.9 (44.5, 47.3)	20.7 (17.8, 23.5)	66.6	0.31
None	1 mM	41.2 (40.1, 42.3)	27.9 (25.1, 30.6)	69.1	0.40
CoCl_2	None	47.7 (48.0, 47.3)	27.8 (26.1, 29.5)	75.5	0.37
CoCl_2	1 mM	40.0	28.7	68.7	0.42

homogenates of liver from rats treated *in vivo* with CoCl_2 (Table 1).

Characterization of unextractable material labelled with radioactive 5-aminolaevulinate

An isotope-dilution experiment was carried out *in vitro* under the conditions described above to determine whether the radioactivity from 5-amino[^{14}C]laevulinate that was incorporated into unextractable material could be diluted by intermediates of the pathway of haem synthesis. Considerable dilution of the radioactivity recovered after treatment with CoCl_2 in unextractable material was obtained not only with 5-aminolaevulinate, but also with porphobilinogen and protoporphyrin, whereas haemin was completely ineffective (Table 2). This suggested that radioactivity from 5-aminolaevulinate proceeded along the biosynthetic pathway of haem and branched off for incorporation into unextractable material before the stage of haem, probably at the stage of protoporphyrin.

When liver homogenates were incubated with cobalt and protoporphyrin together the protein pellets, even after extensive washings with acetone/HCl/

water, exhibited a deep-purple colour, suggesting that cobalt could stimulate the uptake of protoporphyrin itself in an insoluble form, most probably as cobalt protoporphyrin. This finding is compatible with the previous observation (De Matteis & Gibbs, 1972) that formation of metalloprotoporphyrin by liver homogenates is markedly stimulated by increasing the concentration of protoporphyrin. It might also provide an explanation for the present finding (Table 2) that protoporphyrin was appreciably less effective than porphobilinogen in diluting the radioactivity recovered in the pellet, since with protoporphyrin a marked fall in specific radioactivity produced by isotope dilution would be compensated by formation of an increased quantity of labelled cobalt protoporphyrin.

The possibility was considered that most of the unextractable material seen after treatment with CoCl_2 was the cobalt chelate of protoporphyrin (cobalt protoporphyrin) formed at the expense of the iron chelate, i.e. protohaem. Direct evidence for this was sought by separating the acid/acetone-soluble radioactivity into purified haem and protoporphyrin fractions and by comparing the radioactivity recovered in these two fractions with that recovered in the extracted protein pellet after incubation of the liver homogenates with or without cobalt and protoporphyrin (Table 3).

In the absence of added protoporphyrin over 50% of the radioactivity was recovered in the purified haem fraction of control samples; CoCl_2 did not affect significantly the total recovery of radioactivity, but markedly increased the radioactivity of the protein pellet with a corresponding decrease in the radioactivity recovered in the haem fraction. When excess protoporphyrin was added to the liver homogenates, most of the radioactivity recovered at the end of the incubation was in the protoporphyrin fraction, indicating that the added protoporphyrin had effectively diluted out the intermediary pool of radioactivity. Under these conditions CoCl_2 again caused accumulation of radioactivity in the protein pellet, but this time there was a corresponding decrease in the radioactivity of the protoporphyrin fraction (Table 3). These observations are compatible with the unextractable material being cobalt protoporphyrin and also with its production from protoporphyrin at the expense of haem.

Additional evidence in favour of cobalt protoporphyrin formation was provided by a comparison of the solubility properties and spectral characteristics of the unextractable material with those of authentic cobalt protoporphyrin. When the authentic compound was added to liver homogenates it was found to become very tightly bound to the protein pellet, from which neither extensive extraction with acetone/HCl/water nor treatment with a methylating mixture

Table 2. *Effect of intermediates of the haem biosynthetic pathway on the incorporation of 5-amino[4- ^{14}C]laevulinate into unextractable material by liver homogenates incubated with CoCl_2*

Two rats were starved overnight, their livers were homogenized (20%, w/v) in 0.225 M-sucrose/0.05 M-Tris/HCl, pH 7.4, and the homogenates pooled. A 0.5 ml portion of the homogenate was incubated at 37°C with shaking in the presence of 5-amino[^{12}C]laevulinate (2 mM), porphobilinogen (0.6 mM), protoporphyrin (0.25 mM) or haemin (0.25 mM as methaemalbumin) and, where indicated, CoCl_2 (1 mM). After 5 min, 0.1 μCi (1.89 nmol) of 5-amino[4- ^{14}C]laevulinate was added to each tube and the incubation continued for 90 min in a total volume of 0.56 ml. The reaction was stopped by the addition of acetone/conc. HCl/water and the radioactivity recovered in the extensively extracted pellets determined as indicated in the Materials and Methods section. The numbers in parentheses are the values for duplicate tubes.

Additions		Radioactivity recovered in pellet (% of added radioactivity)
None	—	8.75 (7.5, 10.0)
None	CoCl_2	29.2 (28.6, 29.9)
5-Amino[^{12}C]laevulinate	—	1.3 (1.3, 1.3)
5-Amino[^{12}C]laevulinate	CoCl_2	2.0 (1.9, 2.1)
Porphobilinogen	—	1.3 (1.2, 1.4)
Porphobilinogen	CoCl_2	2.1 (1.9, 2.3)
Protoporphyrin	—	3.4 (3.4, 3.5)
Protoporphyrin	CoCl_2	11.6 (11.6, 11.7)
Haemin	—	7.6 (7.0, 8.2)
Haemin	CoCl_2	28.2 (28.2, 28.2)

Table 3. *Effect of protoporphyrin and CoCl₂ on the incorporation in vitro of 5-amino[4-¹⁴C]laevulinate into unextractable material, haem and protoporphyrin by liver homogenates*

Liver homogenates prepared from starved rats were incubated in the presence of CoCl₂ (1 mM) and/or protoporphyrin (0.18 mM) and the reaction was stopped as indicated in the legend to Table 2. At the end of the incubation protoporphyrin was added to those samples incubated in its absence and the radioactivity present in the acid/acetone extract was fractionated into purified haem and protoporphyrin fractions, and the radioactivity of these and of the extensively extracted protein pellets was then determined. Results are averages of two observations with individual observations in parentheses or averages $\pm$ S.E.M. of either three or four observations. No correction was applied for loss of haem and protoporphyrin encountered during the extraction procedure.

Additions	Radioactivity (% of added radioactivity) recovered in:		
	Haem fraction	Protoporphyrin fraction	Pellet after extraction
(A) None	54.0 (53.0, 54.3)	9.1 (8.4, 9.9)	5.3 (5.0, 5.7)
(B) CoCl ₂	7.0 (7.0, 7.0)	4.1 (4.0, 4.3)	53.4 (52.6, 54.3)
Decreased radioactivity caused by cobalt (A - B)	47.0	—	—
Increased radioactivity caused by cobalt (B - A)	—	—	48.0
(C) Protoporphyrin	1.0 $\pm$ 0.25 (3)	35.0 $\pm$ 4.2 (3)	2.95 $\pm$ 0.29 (4)
(D) Protoporphyrin + CoCl ₂	4.2 $\pm$ 0.64 (3)	24.2 $\pm$ 2.8 (3)	15.7 $\pm$ 2.61 (4)
Decreased radioactivity caused by cobalt (C - D)	—	10.8	—
Increased radioactivity caused by cobalt (D - C)	—	—	12.75

Fig. 1. *Pyridine haemochrome absorption spectra (reduced minus oxidized) of solubilized protein pellets from liver homogenates incubated with protoporphyrin and CoCl₂ or with preformed cobalt protoporphyrin*

Rat liver homogenate [20% (w/v) in 0.25 M-sucrose/20 mM-Tris/HCl, pH 7.4; 0.5 ml] was incubated at 37°C for 30 min in the dark with either protoporphyrin alone (1), with protoporphyrin and CoCl₂ (2) or with pre-formed cobalt protoporphyrin (3). The final concentration of protoporphyrin was 95 μ M, that of cobalt protoporphyrin 88 μ M and that of CoCl₂ 1 mM. After incubation the protein pellet was extensively extracted with acetone/conc. HCl/water (20:1:4, by vol.) and finally dissolved in 1.7 ml of 1% SDS at pH 8. Pyridine (0.4 ml) and 1 M-NaOH (0.2 ml) were added just before the difference spectrum (dithionite-reduced minus oxidized) was recorded by using either the DW2 Aminco or the 356 Perkin-Elmer spectrophotometer. The spectrum of cytochrome *c* (4) was similarly determined for the sake of comparison. Note that the scale of the ordinates has been expanded on the right-hand side of the Figure.

of methanol/ H_2SO_4 (followed by chloroform extraction) could release it. On incubation at 37°C in the presence of Co^{2+} ions, rat liver homogenate could convert protoporphyrin into a compound which strongly resembled authentic cobalt protoporphyrin not only in solubility characteristics but also in spectral properties (Fig. 1). Accumulation of this compound could not be detected on incubation of liver homogenate with cobalt and protoporphyrin at 4°C , whereas on incubation of homogenate with preformed cobalt protoporphyrin similar amounts of pigments could be detected in association with the washed protein pellet, whether the incubation had been carried out at 37°C (Fig. 1) or at 4°C . This suggests that it is the formation of cobalt protoporphyrin, rather than its association with the protein, that is temperature-dependent.

The spectrum detected in the protein pellet after incubation without CoCl_2 was very similar to that of cytochrome *c* (Fig. 1). It is therefore possible that most of the haem-type absorption (and of the radioactivity from 5-amino[^{14}C]laevulinate; Tables 1–4) seen in washed pellets of control homogenates is due to cytochrome *c*. Since the haem moiety of this cytochrome is covalently bound to protein, it would be expected to be left behind on extraction with acid/acetone in the protein pellet.

Fig. 1 shows that distinguishing features of the cobalt chelate of protoporphyrin (compared with the iron chelate) are: (1) a slightly longer wavelength for the α maximum; (2) a considerably shorter wavelength for the Soret peak; and (3) the presence of a trough at 424–428 nm. This confirms previous findings (R. J. Porra, personal communication).

In contrast with cytochrome *c*, most of the unextractable material formed on incubation with CoCl_2 was not irreversibly (or covalently) bound to proteins. When the solubilized labelled protein pellet (obtained by acetone treatment of the homogenate) was subjected to SDS/polyacrylamide-gel electrophoresis, over 87% of the radioactivity from CoCl_2 -treated homogenates was recovered as low-molecular-weight compounds (fractions 5–10; Fig. 2) migrating faster than any stainable proteins. However, when the same pellets were also extracted with acetone/HCl/water before solubilization and electrophoresis, as much as 41% of the radioactivity was found in association with material with a molecular weight estimated to be greater than 4000. This suggests that acid treatment may contribute to the binding of the unextractable material to protein.

The protein pellet of control homogenates was also obtained by precipitation with excess acetone, solubilized and subjected to gel electrophoresis: most of the radioactivity was recovered in the rapidly migrating or low-molecular-weight fractions. Nevertheless 27% of the radioactivity was found in the intermediary

Fig. 2. *Electrophoretic profile of radioactivity of liver homogenates after incubation with 5-amino[^{14}C]laevulinate and cobalt with and without extraction with acid/acetone* Rat liver homogenate [20% (w/v) in 0.225 M-sucrose/0.05 M-Tris/HCl, pH 7.4; 0.5 ml] was incubated with 0.5 μCi of 5-amino[^{14}C]laevulinate with and without 1 mM- CoCl_2 for 90 min at 37°C . The reaction was stopped by chilling (0°C), and after washing with 3×10 ml of cold acetone/water (4:1, v/v) the pellet was divided into two equal portions. One was resuspended in 50 mM-Tris/HCl buffer, pH 6.8, containing 1% SDS, 1% mercaptoethanol and 2.0 mM-EDTA. The remaining portion was extracted three times with acetone/HCl as described in the Materials and Methods section before centrifuging and resuspending the pellet in the above buffer. All samples were placed in a boiling-water bath for 5 min, frozen-thawed and reboiled for 2 min before application to the gel. Total radioactivity (d.p.m.) applied per sample: control, unextracted (□), 6420; cobalt-treated, unextracted (○), 5144; cobalt-treated, extracted with acetone/HCl (●), 4732. Some 85–90% of the applied radioactivity was recovered in the gel slices. Control samples extracted with acetone/HCl had too little radioactivity to analyse by electrophoresis. The arrow indicates the boundary of stainable proteins.

fractions (nos. 4–7, Fig. 2), immediately after the stainable protein boundary; whether this represents haem bound to undetected low-molecular-weight proteins or haem in a higher state of aggregation (Brown & King, 1976) we do not know.

Possible relevance of formation of cobalt protoporphyrin to the inhibition of liver haem biosynthesis caused by cobalt treatment in vivo

When starved rats were treated with increasing

Fig. 3. Effect of a single dose of CoCl_2 on the 5-aminolaevulinate synthase activity and on the cobalt content of rat liver homogenate (a) and on the recovery of radioactivity from 5-amino ^{14}C laevulinate in the liver haem (b) and in washed protein pellets of the liver homogenate (c).

Rats were starved overnight then injected subcutaneously with NaCl (controls) or with increasing doses of CoCl_2 and killed 1 h later. In Experiment (a) one group of rats (open symbols) were also given a single dose of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (200 mg/kg body wt.) orally, 5 h before CoCl_2 (●, ○, 5-aminolaevulinate synthase activity; ■, □, liver cobalt concentration). Control enzyme activities (nmol of product/min per g wet wt. of liver, average $\pm$ S.E.M. of five observations) obtained in animals given NaCl instead of CoCl_2 were as follows: rat, pretreated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine, 16.0 ± 2.0 ; rats without such pretreatment, 2.6 ± 0.2 . In experiments involving measurement of radioactivity rats were given 5-amino ^{14}C laevulinate ($2 \mu\text{Ci/rat}$) intraperitoneally 0.5 h after CoCl_2 and were killed after a further 0.5 h. Note that in (b) the radioactivity recovered in haem was determined both by recrystallization (●) and by solvent extraction (○). Results given are means $\pm$ S.E.M. of the numbers of observations in parentheses or averages of at least two observations.

CoCl_2 -treated rats whether the liver haem was isolated by recrystallization or by solvent extraction followed by purification (Fig. 3b), and this was accompanied by a corresponding increase in the radioactivity recovered in extensively extracted protein pellets (Fig. 3c). Cobalt-treated rats also showed a marked decrease of activity of liver 5-aminolaevulinate synthase: most of the loss of activity was observed already with $12.5 \mu\text{mol/kg}$, a dose that produced a concentration of cobalt in the liver of less than $4 \mu\text{g/g}$ wet wt. of tissue (Fig. 3a). Both decrease of 5-aminolaevulinate synthase activity and impaired incorporation of 5-amino ^{14}C laevulinate into haem was therefore seen with very low doses of CoCl_2 , producing very low liver concentrations of metal, and the dose-response curves obtained for both the above responses closely mirrored the accumulation of unextractable radioactivity.

When rats were pretreated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine to induce their liver 5-aminolaevulinate synthase and were then given CoCl_2 , their induced enzyme activity was also very susceptible to inhibition by cobalt. Although the percentage loss of activity seen in these animals was only slightly greater than in control uninduced rats (Fig. 3a), the inhibition caused by cobalt (in terms of total units of enzyme activity lost) was considerably greater in induced rats. For example, with a dose of $12.5 \mu\text{mol}$ of CoCl_2/kg body wt. the total enzyme activity lost (calculated as the difference between the activities of cobalt-treated rats and corresponding controls given no cobalt) was 7.7 nmol of 5-aminolaevulinate formed/min per g of liver in induced rats and 0.61 nmol/min per g of liver in uninduced rats. An attempt was made to demonstrate accumulation of cobalt protoporphyrin in the livers of rats pretreated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine: although an increased incorporation of labelled 5-aminolaevulinate *in vivo* into unextractable material could also be demonstrated in the liver of these rats, a precise dose-response curve could not be obtained, as the dilution of the label by the enlarged pools of 5-aminolaevulinate and protoporphyrin present in these porphyrin livers made the interpretation of the results very difficult.

A marked loss of induced liver 5-aminolaevulinate synthase activity was also seen when rats were first treated with 2-allyl-2-isopropylacetamide (400 mg/kg) and after 5 h were given CoCl_2 ($250 \mu\text{mol/kg}$) and killed 30 min or 1 h later. Enzyme activities in liver homogenates, expressed as nmol of product/min per g wet wt. of liver (average $\pm$ S.E.M. of four observations) were as follows: rats killed 5 h after 2-allyl-2-isopropylacetamide, 6.33 ± 0.43 ; rats killed 30 min after CoCl_2 , 2.87 ± 0.12 ; rats killed 1 h after CoCl_2 , 1.92 ± 0.15 . The rate of loss of activity caused by cobalt in these rats was considerably greater than that previ-

doses of CoCl_2 and killed 1 h later, they showed a significant decrease in their incorporation of 5-amino ^{14}C laevulinate into liver haem, starting from doses of CoCl_2 as low as $4\text{--}12 \mu\text{mol/kg}$ body wt. A similar percentage decrease in radioactivity was found in

ously reported after puromycin (45% of initial activity lost within 1 h; Marver *et al.*, 1966b), suggesting that cobalt cannot merely act by blocking the formation of the enzyme. Inhibition of the activity of the enzyme must also be involved. Similar findings were obtained when the enzyme was induced with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (De Matteis & Gibbs, 1977; see also Table 4).

Comparison of the acute effects of cobalt with those of other metals

A number of other metals have been shown, like cobalt, to stimulate liver haem oxygenase and to depress liver cytochrome P-450 (Maines & Kappas,

1976, 1977c; Tephly *et al.*, 1978). Experiments were therefore carried out to ascertain whether, when given at doses known to result in stimulation of haem oxygenase, these metals could also inhibit the activity of induced liver 5-aminolaevulinate synthetase or the incorporation of 5-amino[4-¹⁴C]laevulinate into liver haem *in vivo*. Under the conditions of these experiments (Tables 4 and 5) cobalt was found to be the only metal capable of producing both these effects. A low dose of Ni²⁺ (25 µmol/kg) stimulated, rather than decreased, 5-aminolaevulinate synthase activity, and Zn²⁺ increased the uptake of labelled 5-aminolaevulinate into liver haem, a finding probably explained by its ability to stimulate 5-aminolaevulinate dehydratase (Abdulla & Haeger-Aronsen, 1971), a zinc-containing enzyme.

Since Zn²⁺ is known to be a good substrate for the assay of the porphyrin-metal chelatase enzyme *in vitro* (Mazanowska *et al.*, 1966; Jones & Jones, 1969) it was considered possible that some of the radioactivity recovered as recrystallized haemin from the liver of rats treated with zinc acetate might in fact be represented by zinc protoporphyrin. This possibility was examined by treating an ether solution of radioactive haemin with aq. 10% (w/v) HCl, a treatment known to dissociate zinc from protoporphyrin and to extract the resulting free porphyrin: no evidence could be obtained that zinc protoporphyrin had been produced *in vivo* under the conditions of these experiments (results not shown).

Discussion

The findings presented in this paper strongly suggest that cobalt protoporphyrin is readily formed in the liver of rats given CoCl₂ *in vivo* and also when liver homogenates are incubated *in vitro* in the presence of CoCl₂. This conclusion is based on the following lines of evidence. (1) In agreement with previous findings (De Matteis & Gibbs, 1977), when cobalt-treated rats were given 5-amino[¹⁴C]laevulinate, there was a

Table 4. *Effect of treatment with several metals on the activity of induced hepatic 5-aminolaevulinate synthase*

Starved rats were treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (200 mg/kg) orally and 5 h later they were injected subcutaneously with one of different metal salts dissolved in 0.9% NaCl, and were killed after 1 h. Results given are averages ± S.E.M. of the numbers of observations in parentheses or averages with individual results in parentheses, and are all expressed as percentages of the corresponding control values, 18.9 ± 1.68 nmol of 5-aminolaevulinate/min per g wet wt. of liver. *P < 0.001, when compared with corresponding (control) values for just 0.9% NaCl.

Treatment	Dose (µmol/kg body wt.)	5-Aminolaevulinate synthase activity (% of control)
0.9% NaCl	(10 ml/kg; control)	100 ± 4.7 (7)
CdCl ₂	2.5	97.7 ± 14.5 (5)
CoCl ₂	25	44.3 ± 7.1 (3)*
	62.5	29.5 ± 2.2 (3)*
	250	14.6 (13.5, 15.7)
MnCl ₂	250	95.9 ± 10.3 (7)
NiCl ₂	25	135.3 ± 19.1 (3)
	250	104.9 ± 7.6 (4)
	500	99.1 ± 9.8 (3)
Zinc acetate	250	93.3 ± 13.3 (6)

Table 5. *Effect of treatment with several metals on the recovery of radioactivity from injected 5-amino[4-¹⁴C]laevulinate in the total liver homogenate and in the haem isolated in a crystalline form from the liver homogenate*

Rats were starved for 24 h, then injected with a solution of one of several metal salts; 1 h later they were given 2 µCi of 5-amino[4-¹⁴C]laevulinate intraperitoneally and killed after 30 min. Results are given as averages with individual observations in parentheses.

Treatment	Dose (µmol/kg body wt.)	Percentage of injected radioactivity recovered in:	
		Total liver homogenate	Total haem of liver
0.9% NaCl	(10 ml/kg; Control)	19.6 (18.6, 20.6)	16.55 (16.4, 16.7)
CdCl ₂	2.5	19.1 (17.8, 20.3)	15.6 (15.4, 15.8)
CoCl ₂	250	19.0 (18.6, 19.5)	2.75 (2.7, 2.8)
MnCl ₂	250	21.3 (19.5, 23.1)	18.4 (16.8, 20.0)
NiCl ₂	250	21.95 (20.9, 23.0)	18.3 (17.7, 18.9)
Zinc Acetate	250	24.65 (24.3, 25.0)	22.4 (21.1, 23.7)

decreased incorporation of the label into liver haem with corresponding accumulation of radioactivity in a pool that would not be extracted by acid/acetone. (2) Unextractable material was also formed *in vitro* in presence of CoCl_2 (Table 1) and this was shown to possess the same spectral and solubility characteristics as authentic cobalt protoporphyrin (Fig. 1). (3) Isotopic-dilution experiments indicated that this unextractable material was derived mainly, if not entirely, from protoporphyrin (Table 2), just as is cobalt protoporphyrin formed by isolated mitochondria under the influence of their chelatase enzyme (Jones & Jones, 1969).

The present finding that formation of cobalt protoporphyrin is temperature-dependent suggests a requirement for the activity of the enzyme porphyrin-metal chelatase. In rats made porphyric by 3,5-diethoxycarbonyl-1,4-dihydrocollidine increased incorporation of 5-amino[^{14}C]laevulinate into unextractable material could still be demonstrated. We consider it likely that cobalt protoporphyrin is formed in greater amounts in the liver of rats induced with this drug than in the liver of uninduced rats, for the following reasons: (1) the amount of cobalt protoporphyrin formed is increased on increasing the concentration of protoporphyrin (Table 3), (2) the inhibition of the chelatase seen in these rats is not complete (De Matteis *et al.*, 1973), and (3) previous work in similarly induced rats (De Matteis & Gibbs, 1972) has shown that the formation of metalloporphyrins is in fact stimulated rather than decreased, as the inhibition of the chelatase is more than compensated by the accumulation of protoporphyrin, which drives the reaction.

Previous workers have failed to demonstrate or have discounted the occurrence of cobalt protoporphyrin *in vivo* either in liver cells in culture (Guzelian & Bissell, 1976), or in the intact liver (Nakamura *et al.* 1975; Maines & Kappas, 1977a) and kidney (Maines & Kappas, 1977d). It is apparent now that formation of cobalt protoporphyrin *in vivo* may only be detected by sensitive enough techniques like the radioactive one described in the present paper. Accumulation of acid/acetone-unextractable radioactivity could also be demonstrated in the kidneys of rats pretreated with CoCl_2 *in vivo* and in cultured hepatocytes of adult rat and embryonic chicken exposed to CoCl_2 *in vitro* (P. Sinclair, A. H. Gibbs, J. F. Sinclair & F. De Matteis, unpublished work), suggesting formation of cobalt protoporphyrin in all these tissues. Evidence in favour of formation of cobalt porphyrins *in vitro* has been provided by Hunter & Jackson (1975) and by Sone (1971) in erythrocytes and *Propionibacterium arabinosum* respectively. These authors have also drawn attention to the strong binding of cobalt porphyrins to proteins from which they are not readily extracted by acid/acetone.

This ability of cobalt protoporphyrin to become bound tightly to proteins from which it cannot be readily extracted by acid/acetone is not understood, since haem (iron protoporphyrin) is so readily extracted by this solvent mixture. We have shown that, in the absence of acidic treatment, the radioactive unextractable material was not tightly (or covalently) bound to proteins, but that on treatment with acid a considerable proportion of the radioactivity migrated on electrophoresis in association with high-molecular-weight material. This suggests that treatment with acid may be involved in the binding of cobalt protoporphyrin to protein: possibly the affinity of cobalt protoporphyrin for proteins may become greater on exposure to acid; or, alternatively, the proteins to which cobalt protoporphyrin is bound may become modified by acid treatment in such a way as to make the subsequent release of pigment more difficult.

The present work has provided strong evidence that formation of cobalt protoporphyrin is directly related to the inhibition of haem synthesis at the level of the chelatase. Very low doses of cobalt were found to inhibit incorporation of labelled 5-aminolaevulinate into haem *in vivo* (see also Woods & Carver, 1977), increasing at the same time the incorporation of the label into cobalt protoporphyrin, suggesting that cobalt treatment had caused diversion of protoporphyrin from formation of one metalloporphyrin to another, rather than inhibiting the overall activity of the porphyrin-metal chelatase enzyme. This may reflect the well-established fact that cobalt is a better substrate than iron for the chelatase reaction and also, possibly, interference by cobalt with the mobilization of iron in a form which can be utilized by the chelatase. Cobalt given *in vivo* was still available *in vitro* for incorporation by liver homogenates into protoporphyrin (Table 1); this observation may offer an explanation for the apparent inhibition of ferrochelatase assayed *in vitro*, after injection of CoCl_2 *in vivo* (Woods & Carver, 1977; Maines, 1977).

The present work has also suggested that accumulation of cobalt protoporphyrin may be responsible for inhibition of 5-aminolaevulinate synthase. The activity of 5-aminolaevulinate synthase was found to be very sensitive to cobalt administration and its inhibition paralleled the formation of cobalt protoporphyrin. A role of cobalt protoporphyrin in the inhibition of 5-aminolaevulinate synthase is supported by the following additional findings.

(1) Pretreatment of rats with either 3,5-diethoxycarbonyl-1,4-dihydrocollidine or 2-allyl-2-isopropylacetamide increased the total loss of liver 5-aminolaevulinate synthase activity caused by cobalt (see above and De Matteis & Gibbs, 1977), and under these conditions formation of cobalt protoporphyrin would also be expected to be greater than in control animals, as both these porphyrogenic agents cause

accumulation of protoporphyrin; (2) other metals which share with cobalt the ability to stimulate liver haem oxygenase failed to inhibit significantly 5-aminolaevulinate synthase and, after their administration *in vivo*, diversion of labelled 5-aminolaevulinate into a pool different from haem could not be demonstrated in the liver (Tables 4 and 5).

There is evidence from studies *in vivo* that cobalt protoporphyrin is not readily converted into bile pigments and, once formed, tends to persist in the liver (De Matteis & Gibbs, 1976, 1977). Thus cobalt protoporphyrin may, like haem, exert a feedback inhibition on 5-aminolaevulinate synthase, yet, because of its persistence and apparent lack of utilization for haemoprotein synthesis, be a more powerful inhibitor of the enzyme than haem.

Note Added in Proof (Received 11 December 1978)

Since this paper was accepted for publication Igarashi *et al.* (1978) have reported formation of cobalt protoporphyrin in the liver of rats given CoCl_2 .

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