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FORMATION OF COBALT PROTOPORPHYRIN BY CHICKEN HEPATOCYTES IN CULTURE

RELATIONSHIP TO DECREASE OF 5-AMINOLAEVULINATE SYNTHASE CAUSED BY COBALT

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Abstract—Cobalt protoporphyrin generated from 5-amino[4-^{14}C]laevulinate by homogenates or primary cultures of chick embryo liver exposed to $CoCl_2$ was found to be radioactivity unextractable by acid/acetone, when extra protein was added. The activity of ferrochelatase was required for formation of cobalt protoporphyrin since inhibition of ferrochelatase with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (in the presence of cycloheximide) inhibited formation of cobalt protoporphyrin and resulted in accumulation of protoporphyrin. Cobalt protoporphyrin was detected spectrophotometrically in hepatocyte cultures exposed to the combination of 2-allyl-2-isopropylacetamide and $CoCl_2$: (1) as the pyridine haemochrome of the protein pellet remaining after acid–acetone extraction of the cells, or (2) as the material extracted from the protein pellet with acetic acid–pyridine–chloroform. The amount of cobalt protoporphyrin increased with increasing $CoCl_2$ concentration as cellular haem declined. The decrease in haem was about equal to the amount of cobalt protoporphyrin that accumulated. 2-Allyl-2-isopropylacetamide and polychlorinated biphenyls were both powerful inducers of 5-aminolaevulinate synthase. The former led to protoporphyrin accumulation, whereas with the latter, uroporphyrin accumulated, probably due to a concomitant decrease in activity of uroporphyrinogen decarboxylase. The decrease in activity of 5-aminolaevulinate synthase produced by administration of $CoCl_2$ was greater after treatment with 2-allyl-2-isopropylacetamide than after treatment with allylisopropylacetamide and 3,4,3′,4′-tetrachlorobiphenyl. We conclude: (a) that cobalt protoporphyrin is readily formed in cultured hepatocytes, and (b) that its formation accounts for the action of cobalt on 5-aminolaevulinate synthase.

We have shown previously that, when rats are treated with $CoCl_2$, cobalt protoporphyrin is formed in the liver, and we have presented evidence to suggest a causal relationship between formation of cobalt protoporphyrin and inhibition of liver 5-aminolaevulinate synthase [1, 2]. Igarishi *et al.* [3] and Watkins *et al.* [4] have also identified cobalt protoporphyrin in rat liver after cobalt treatment; in addition, Igarishi *et al.* [3] have shown that administered cobalt protoporphyrin caused a decline in liver 5-aminolaevulinate synthase in rats *in vivo*.

However, Guzelian and Bissell [5] were unable to demonstrate formation of cobalt protoporphyrin in cultured hepatocytes from rat liver despite observing a decrease in cytochrome P-450 after $CoCl_2$ treatment. Maines and Sinclair [6] investigated the effect of $CoCl_2$ on cultures of chick embryo hepatocytes in which 5-aminolaevulinate synthase is strongly inducible by various drugs. Induction of 5-aminolaevulinate synthase in these cultures was readily inhibited by low concentrations of $CoCl_2$ but it was not possible to conclude whether this sensitivity was due to a direct action of cobalt or to formation of cobalt protoporphyrin. Maines and Kappas have, in several papers, suggested that cobalt and many other metals regulate 5-aminolaevulinate synthase by direct action, specifically excluding a role of the metalloporphyrins (for reviews, see Refs. 7–9). Thus, although it is clear that treatment with $CoCl_2$ causes a decrease in liver 5-aminolaevulinate synthase both in the intact rodent and in cultured hepatocytes, the mechanism is still controversial and, until quite recently [10], evidence that cobalt protoporphyrin could be formed by isolated hepatocytes was lacking.

In the present work, we show that the formation of cobalt protoporphyrin can be readily demonstrated in cultured chick embryo hepatocytes treated with $CoCl_2$, provided that our previously established technique [2] for detecting cobalt protoporphyrin is suitably modified. Further evidence is also presented that formation of cobalt protoporphyrin is important for the decrease in activity of 5-aminolaevulinate synthase caused by cobalt.

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Animals. Fertilized chicken eggs (White Leghorn) were obtained from Treslow Farms, MD, and were incubated in a Petersime incubator at 90% humidity and 37°. Where indicated, cycloheximide and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) (dissolved in saline and dimethylsulphoxide, respectively) were injected with a 1.5 inch, 21 gauge needle into the fluid surrounding the embryo at days 17 18 of age.

Unless otherwise indicated, hepatocytes were prepared from the liver of 16-day embryos and cultured as described in detail previously [11].

Separation of radioactive haem, protoporphyrin and protein-bound material. This was achieved as described previously for rat liver [2] by extracting haem and porphyrins from the chicken liver homogenates or cultured chicken hepatocytes with acetone/conc. HCl/water (final proportions 50:2.5:10, by vol.), except that: (1) carrier haem and protoporphyrin were routinely added to the samples prior to extraction; (2) excess protein was added to facilitate the retention of cobalt protoporphyrin by the protein pellet (see later); (3) the protein pellet remaining after acid/acetone extraction was dissolved in 2% (w/v) sodium dodecylsulphate without addition of Tris, since in the determination of Co protoporphyrin the presence of Tris appeared to retard the rate of reduction of the metalloporphyrin by dithionite; and, finally, (4) Aquasol, rather than Instagel, was used as the scintillation fluid. Radioactivity was determined in a Searle Mk III liquid scintillation analyzer. Quenching was corrected by use of an external standard and previously determined quench curves.

Estimation of cobalt protoporphyrin. Use was made of the characteristic Soret spectrum of the pyridine haemochrome [2]. The extinction coefficient ($\Delta\Sigma mM = 129\ mM^{-1}\ cm^{-1}$) for the difference in absorbance between 427 and 460 nm of the reduced versus oxidized difference spectrum was obtained using a solution of authentic cobalt protoporphyrin, the concentration of which was determined using the extinction coefficient of the oxidized Soret peak ($\Sigma mM = 152$, B. Burnham, personal communication, based on mol. wt = 812 for the complex, Co-protoporphyrin(pyridine)$_2$Cl.

Extraction of Cobalt protoporphyrin. A modification of the method of Igarishi *et al.* [3] was used as follows: to 0.5 ml of liver homogenate or cultured cell suspension, 12 mg egg albumin in 0.1 ml saline was added. This was extracted with 3.5 ml acetone/conc. HCl (50:2.5, v/v). To the pellet was added 0.5 ml acetic acid/pyridine (10:1, v/v) and, after Vortex mixing, 2 ml chloroform was added and the mixture was vortexed vigorously. A 10-min centrifugation (2000 *g*) produced a well-formed, cohesive pellicle of protein at the top of the tube. The spectrum of the clear solution against solvent mixture was scanned from 390 to 600 nm with the Aminco DW2 spectrophotometer.

Estimation of porphyrins. Porphyrins, extracted with perchloric acid/methanol, were assayed using a Hitachi 512 spectrofluorometer equipped with a Hamamatsu 928 red-sensitive photomultiplier, as described [12, 13].

Other assays. The activity of 5-aminolaevulinate synthase was determined in homogenates of cultured cells, essentially as described [14]. Proteins were determined by the method of Lowry *et al.* [15] using bovine serum albumin as the standard.

Sources of special chemicals. Williams E medium and foetal bovine serum were from Flow Laboratories, McLean, VA, and the 5-aminolaevulinate, porcine insulin and 3,5,3′-triiodothyronine from the Sigma Chemical Co., St. Louis, MO. Egg albumin was from the Fisher Chemical Co., Pittsburgh, PA, and 5-amino-[4-^{14}C]laevulinate (51.5 mCi/mmole) from the New England Nuclear Corp., Boston, MA. 3,5-Diethoxycarbonyl-1,4-dihydrocollidine was from Eastman Kodak, Rochester, NY, and was recrystallized from ethanol. 3,4,3′,4′-Tetrachlorobiphenyl was from the RFR Corp., Hope, RI, Aquasol from Amersham, Arlington Heights, IL, and cobalt protoporphyrin from Porphyrin Products, Logan, UT. 2-Allyl-2-isopropylacetamide and Arachlor 1254 were gifts from Hoffmann-La Roche, Nutley, NJ, and from Dr. A. Alvares, Uniformed Services University, Bethesda, MD, respectively.

RESULTS

Detection of cobalt protoporphyrin in chicken embryo liver homogenates and in cultured hepatocytes. In previous work [2], use was made of the relative insolubility of cobalt protoporphyrin in acid/acetone (as compared with haem) to detect and identify the cobalt protoporphyrin in rat liver homogenate. It was shown that $CoCl_2$ could increase the incorporation of [^{14}C]-5-aminolaevulinate into a compound which remained bound onto the protein pellet after extraction and which exhibited the same solubility and spectral characteristics as authentic cobalt protoporphyrin. Preliminary experiments with cultures of chicken embryo hepatocytes incubated with [^{14}C]-5-aminolaevulinate and $CoCl_2$ failed to detect any increase caused by $CoCl_2$ in the amount of radioactively labelled material that was not extractable by acid/acetone. On account of the small amount of cellular protein present in these experiments, we suspected that the cobalt protoporphyrin formed in the culture may have been lost to the supernatant fraction during the extraction.

Table 1 shows the results of an experiment in which egg albumin was added to a chick embryo liver homogenate previously incubated with 5-amino[4-^{14}C]laevulinate with and without $CoCl_2$. Addition of albumin before extraction caused some increase in protein-bound radioactivity in the absence of $CoCl_2$, probably due to haem. However, a greater increase in protein-bound radioactivity occurred when albumin was added to $CoCl_2$-treated cells, suggesting that more cobalt protoporphyrin could then be recovered bound to the extracted protein pellet. The ability of egg albumin to improve recovery of cobalt protoporphyrin added exogenously to 10% (w/v) chick embryo liver homogenates was determined. Co protoporphyrin bound to pellet after acid/acetone extraction was determined by the pyridine haemochrome method as described in Materials and Methods. After a single extraction with acid/acetone, the recovery of added cobalt pro-

Table 1. Effect of added protein on the recovery of unextractable labelled material from liver homogenates incubated with $CoCl_2$ and labelled 5-aminolaevulinate*

Addition before incubation	Addition before extraction	Radioactivity recovered in protein pellet (dpm × 10^{-3})	Increase in pellet radioactivity due to $CoCl_2$ (dpm × 10^{-3})
		17.4 (16.6, 18.2)	
$CoCl_2$		27.6 (26.7, 28.5)	10.2
	Albumin	27.2 (26.2, 28.2)	
$CoCl_2$	Albumin	50.0 (49.8, 50.3)	22.8

* Livers from 18-day-old chick embryos were homogenized (10%, w/v) in 0.225 M sucrose/0.05 M Tris–HCl, pH 7.4, and 0.5 ml of the homogenate was incubated for 1 hr at 37° with shaking in the presence of 0.1 μCi of 5-amino[4-^{14}C]laevulinate, with the addition (where indicated) of $CoCl_2$ to a final concentration of 1 mM. After cooling in ice, the homogenates were diluted 1:1 with sucrose/Tris and to 0.5 ml portions was added 6 mg egg albumin in 50 μl saline, where indicated. The homogenate was extracted three times with acetone/conc. HCl/water and the radioactivity in the pellet was determined as described in Materials and Methods. Results given are averages of two observations with individual results in parentheses.

toporphyrin was increased 1.3-fold by the presence of egg albumin. After two extractions with acid–acetone, the recovery of Co protoporphyrin was increased 1.7-fold by the presence of the albumin. Retention of cobalt protoporphyrin in the protein pellet could also be improved by using either a more concentrated chick homogenate (10–20%, w/v) or a rat liver homogenate.

Similar findings were obtained with cultured chick embryo hepatocytes. Here again, when no egg albumin was added prior to extraction, the effects caused by $CoCl_2$ treatment on the radioactivity of both the unextractible liver fraction and the haem fraction were either small (Table 2) or absent. However, addition of albumin produced a 2- to 5-fold increase in unextractable radioactivity in the presence of $CoCl_2$ with an approximate and equal percentage decrease in the radioactivity of the haem fraction (Table 2).

To increase protoporphyrin production and, therefore, the yield of cobalt protoporphyrin, hepatocyte cultures were pretreated, not only with $CoCl_2$, but also with 2-allyl-2-isopropylacetamide, an inducer of 5-aminolaevulinate synthase and of protoporphyrin synthesis [12]. The pyridine haemochrome of the extracted protein pellet from cobalt-treated hepatocytes was then prepared and compared with that of control hepatocytes. In the absence of $CoCl_2$ treatment, the peak of reduced cytochrome *c* (at 417 nm) was observed; in contrast, in cells exposed

Table 2. Conversion of 5-amino[4-^{14}C]laevulinate to cobalt-protoporphyrin by hepatocytes in culture*

Addition to medium	Addition to cell suspension	Radioactivity recovered in: (% of total cellular radioactivity) Protein pellet (a)	Haem (b)	(a + b)
		4.7 (4.60, 4.70)	62.5 (62.0, 63.0)	67.2
$CoCl_2$		7.8 (7.4, 8.2)	57.5 (56.0, 59.0)	65.3
	Albumin	6.8 (6.6, 6.9)	58.0 (57.0, 59.0)	64.8
$CoCl_2$	Albumin	18.2 (17.8, 18.5)	47.5 (47.0, 48.0)	65.7

* Hepatocytes were cultured in 10 cm dishes for 24 hr in Williams E medium containing insulin, dexamethasone and triiodothyronine. After changing to Williams E medium containing the hormones, the cells were incubated for another 16 hr, and $CoCl_2$ was then added to a final concentration of 30 μM. After a further 2 hr of incubation, 0.75 μCi 5-amino[4-^{14}C]-laevulinate was added and 2 hr later the cells were rinsed with cold saline and, then, harvested into 0.5 ml saline. Where indicated, 12 mg egg albumin was added to each sample before two extractions with 3.5 ml acetone/conc. HCl/water. Haem and protein-bound material were separated as described in Materials and Methods. The results represent data from duplicate dishes. Total dpm per 0.5 ml cell suspension were 30,280.

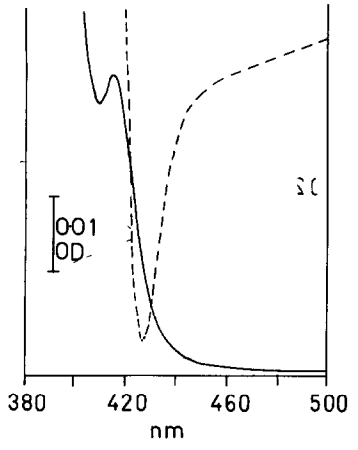

Fig. 1. Pyridine haemochrome absorption spectra of solubilized protein pellets from cultured hepatocytes incubated in the presence or absence of $CoCl_2$. Hepatocytes were cultured as described in the legend to Table 2 except that the medium included 3% (v/v) foetal bovine serum during the first 24 hr of culture and included dexamethasone and triiodothyronine, but not insulin, during day 2 of culture. 2-Allyl-2-isopropylacetamide (50 μg/ml) was then added to all dishes and to some of them $CoCl_2$ (15 μM) was also added. After a further 14 hr of culture, the dishes were rinsed with saline, and the cells from each dish were homogenized in 0.5 ml saline containing 12 mg egg albumin and then extracted with acetone/conc. HCl/water as described in Materials and Methods. The final protein pellet was dissolved in 2% sodium dodecylsulphate at pH 8.0. Pyridine haemochromes were prepared in a total volume of 1.75 ml and the difference (reduced minus oxidized) spectra were recorded as described previously [2]. Key: (—) cells treated with drug only, and (- - -) cells treated with drug and $CoCl_2$.

to $CoCl_2$ a trough at 427 nm was observed (Fig. 1) due to the presence of cobalt protoporphyrin [2].

Cobalt protoporphyrin remaining in the pellet after acid/acetone extraction was further identified by chloroform extraction of cells cultured as in Fig. 1 using the modification of the procedure of Igarishi *et al.* [3] described in Materials and Methods. The spectrum of the extracted material had peaks at 423, 539 and 568 nm, whereas those of the commercial cobalt protoporphyrin were at 423, 536 and 569 nm. These spectra were very similar to that of the material Igarishi *et al.* [3] extracted from liver of rats treated with $CoCl_2$ and allylisopropylacetamide. The advantage of the modified Igarishi procedure is that cobalt protoporphyrin is separated from cytochrome *c* making the detection potentially more sensitive. The modified Igarishi method takes about the same time as the pyridine haemochrome method. The disadvantage of the Igarishi method is that an absolute spectrum is measured and, thus, any porphyrin or other chloroform-extractable material remaining in the pellet after acid/acetone extraction can potentially distort the spectrum and affect quantitation by this method. We adopted acid/acetone as the haem extraction solvent rather than ethyl acetate/acetic acid which was used in the original procedure [3] because acid/acetone gave a finer suspension of cellular material that was more readily sedimented.

When cultured hepatocytes were exposed to 2-allyl-2-isopropylacetamide and at the same time to increasing concentrations of $CoCl_2$, increasing amounts of cobalt protoporphyrin could be detected, a small amount being detectable at concentrations of $CoCl_2$ as low as 0.5 μM (Fig. 2). A cobalt-dependent decrease in porphyrins and haem concentration was also observed. The decrease in haem was approximately equivalent to the increase in cobalt protoporphyrin. Also, the decrease in porphyrin accumulation caused by $CoCl_2$ was about 20-fold greater than the increase in cobalt protoporphyrin. This suggests that cobalt protoporphyrin formation cannot alone account for the decrease in both porphyrins and haem, for example by more protoporphyrin being drawn into the formation of cobalt protoporphyrin at the expense of haem synthesis. The most likely explanation for the marked inhibition of porphyrin accumulation is offered by the finding that $CoCl_2$ decreased 5-aminolaevulinate synthase (and therefore porphyrin formation) in the cultured hepatocytes together with the increased formation of cobalt protoporphyrin (see Table 4).

Evidence that ferrochelatase activity is required for formation of cobalt protoporphyrin. Hepatic ferrochelatase in chick embryos can be inhibited almost completely by low concentrations of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) without an increase in 5-aminolaevulinate synthase [16, 17]. Cycloheximide does not prevent the inhibition of the chelatase but completely abolishes the secondary increase in 5-aminolaevulinate synthase [18]. We injected both cycloheximide and DDC into

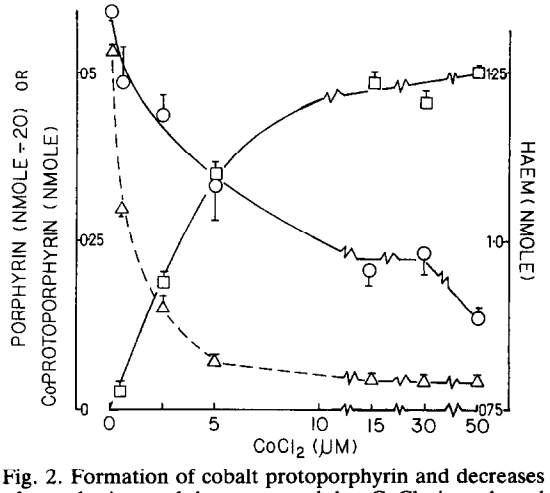

Fig. 2. Formation of cobalt protoporphyrin and decreases of porphyrins and haem caused by $CoCl_2$ in cultured hepatocytes. Chicken embryo hepatocytes were treated with 2-allyl-2-isopropylacetamide and with $CoCl_2$ as indicated in the legend to Fig. 1, except that different concentrations of $CoCl_2$ were studied. Porphyrin values (△) refer to total porphyrins recovered from both cells and medium; haem (○) was extracted from cells with acetone/conc. HCl/water and was determined as described in Materials and Methods. Cobalt protoporphyrin (□) was determined from the pyridine haemochrome absorption spectrum (reduced minus oxidized) of the extracted protein pellet (see Fig. 1). Values given are means and ranges of results per dish obtained with duplicate dishes.

chick embryos to ensure that changes in pool size of either 5-aminolaevulinate or protoporphyrin after DDC treatment would not significantly affect the results of the isotopic experiment. In parallel experiments (results not shown), we confirmed that, under these conditions, cycloheximide could prevent the rise in liver porphyrins caused by DDC and other porphyrinogenic compounds.

When liver homogenates were prepared from embryos treated with cycloheximide alone (controls), the bulk of the radioactivity recovered after incubation with 5-amino[4-^{14}C]laevulinate was in the haem fraction(Table 3). Addition of $CoCl_2$ to the incubation mixture shifted the accumulated label from haem to cobalt protoporphyrin. In contrast, with liver homogenates prepared from embryos treated with DDC, the conversion of 5-aminolaevulinate to haem was inhibited and the radioactivity accumulated in the protoporphyrin fraction. This is to be expected from a marked inhibition of the ferrochelatase. When $CoCl_2$ was added to liver homogenates from DDC-treated chick embryos, the loss of radioactivity from the haem fraction and the increase in the radioactivity of the protein pellet (as compared with values seen without cobalt) were much less marked than with control homogenates (Table 3).

Requirement for protoporphyrin synthesis and cobalt protoporphyrin formation for the effect of $CoCl_2$ on 5-aminolaevulinate synthase. Many chlorinated aromatic hydrocarbons cause accumulation of uroporphyrin in the chick hepatocyte culture [19, 20], probably due to inhibition of uroporphyrinogen decarboxylase. This contrasts with the effects of drugs like 2-allyl-2-isopropylacetamide, where marked accumulation of protoporphyrin is observed. We now find that the two types of porphyria (characterized by accumulation of protoporphyrin or uroporphyrin, respectively) appear to differ in their sensitivity to $CoCl_2$. The accumulation of protoporphyrin caused by 2-allyl-2-isopropylacetamide was very sensitive to inhibition by $CoCl_2$, whether cobalt was given with the drug (Fig. 2) or a few hours later (Fig. 3A), whereas, the accumulation of uroporphyrin caused by the polychlorinated biphenyl mixture, Arachlor 1254, was relatively resistant under both

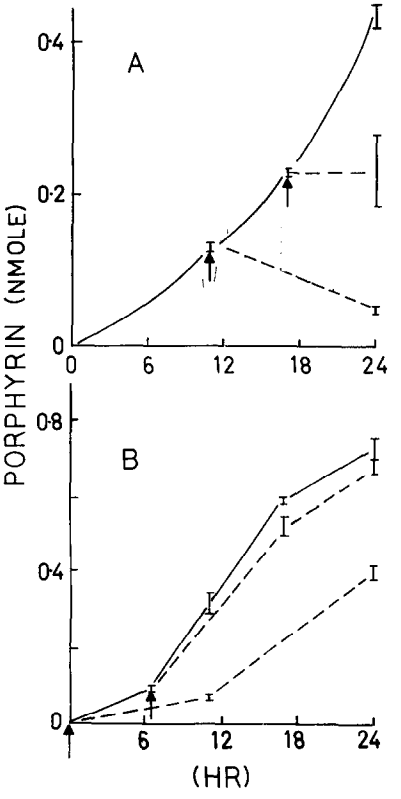

Fig. 3. Effect of cobalt on the accumulation of porphyrins, caused in cultured hepatocytes by treatment with either 2-allyl-2-isopropylacetamide (A) or polychlorinated biphenyls (B). Cells were cultured in 3.5 cm dishes for 24 hr in Williams E medium containing foetal bovine serum. The cells were then rinsed and incubated in the presence of either 2-allyl-2-isopropylacetamide (50 μg/ml) or Arachlor 1254 (2 μg/ml) in Williams E medium containing insulin. $CoCl_2$ (15 μM) was added where indicated by an arrow. Cellular porphyrins, estimated as described in Materials and Methods, are shown thus: (—) in the absence of $CoCl_2$; (- - -) in the presence of $CoCl_2$. Parallel dishes, to which $CoCl_2$ had not been added, were incubated with 25 μg/ml 5-aminolaevulinate for 6-hr periods and the porphyrins thus generated, determined as described elsewhere [12], were found to be at all time points 90–100% protoporphyrin in the cells incubated with 2-allyl-2-isopropylacetamide, and 90–100% uroporphyrin in those treated with Arachlor.

Table 3. Effect of 3,5-diethoxycarbonyl-1,4-dihydrocollidine treatment of chick embryos on the incorporation of 5-amino[4-^{14}C]laevulinate into Co-protoporphyrin by liver homogenates*

Embryo treatment	Addition to homogenate	Radioactivity recovered in: (% of total radioactivity added)			
		Haem	Protein pellet	Protoporphyrin	Total
		44.3 ± 0.9	8.7 ± 0.7	5.3 ± 0.3	58.0 ± 0.6
	$CoCl_2$	9.3 ± 0.3	31.3 ±0.7	5.3 ± 0.3	45.5 ± 1.2
DDC		6.7 ± 0.3	6.0 ± 0.6	24.7 ± 0.9	37.3 ± 0.7
DDC	$CoCl_2$	3.0 ± 0	9.7 ± 0.3	21.3 ± 1.3	34.0 ± 1.5

* Chick embryos (18-day) were injected with 10 μg cycloheximide in 0.1 ml saline, and 1 hr later, where indicated, with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC, 0.15 mg) in 0.1 ml dimethylsulphoxide or with solvent alone. Seven hours later liver homogenates were prepared (20%, w/v) in sucrose/Tris–HCl and 0.5 ml samples were incubated with 0.1 μCi of 5-amino[4-^{14}C]laevulinate with and without 1 mM $CoCl_2$ for 1 hr at 37°. After cooling in ice, egg albumin (9 mg) was added to each tube. Radioactivity in haem, protein pellet and protoporphyrin was determined as described in Materials and Methods. The results given are the averages ± S.E.M. of three determinations.

dosing time schedules (Fig. 3B). Both 2-allyl-2-isopropylacetamide and polychlorinated biphenyls are powerful inducers of 5-aminolaevulinate synthase and the stimulation of this enzyme contributes substantially to the accumulation of porphyrins. We wondered whether the activity of 5-aminolaevulinate synthase might show different sensitivity to $CoCl_2$, depending on the drug used to induce the enzyme. Perhaps, when uroporphyrin is the main porphyrin accumulating, the formation of protoporphyrin is limiting (because of the block at uroporphyrinogen decarboxylase) and cobalt protoporphyrin is then formed in amounts too small to inhibit 5-aminolaevulinate synthase.

The following experiment was carried out to test this hypothesis. Chick hepatocytes in culture were exposed to 2-allyl-2-isopropylacetamide and, at the same time, to either a low (10 ng/ml) or a high concentration (250 ng/ml) of 3,4,3′,4′-tetrachlorobiphenyl. Sixteen hours later, when induction of 5-aminolaevulinate synthase and accumulation of porphyrins had already taken place, $CoCl_2$ was added and the culture continued for 6 hr to investigate the inhibition of 5-aminolaevulinate synthase and the formation of cobalt protoporphyrin. At the low concentration of tetrachlorobiphenyl, cobalt protoporphyrin was formed and 5-aminolaevulinate synthase was decreased by $CoCl_2$. In contrast, at 250 ng/ml tetrachlorobiphenyl, almost all accumulating porphyrin was of the uro-type and no cobalt porphyrin could be detected. Under this condition, there was no decrease in 5-aminolaevulinate synthase caused by $CoCl_2$. However, if cobalt protoporphyrin or haemin was added, the enzyme could be inhibited (Table 4, experiment 2). These results are consistent with our proposal that inhibition of 5-aminolaevulinate synthase by $CoCl_2$ occurs via formation of cobalt protoporphyrin and that the action of cobalt protoporphyrin may be analogous to that of haemin. For comparison, the effect of 6 hr of treatment with cycloheximide on enzyme levels is also shown in Table 4, experiment 1; at 10 ng/ml tetrachlorobiphenyl, cycloheximide caused a 74% decrease in enzyme activity and at 250 ng/ml there was a 70% decrease, indicating that the half-lives of the enzyme in each case were about the same.

DISCUSSION

The findings presented in this paper show that cobalt protoporphyrin is formed both in liver homogenates and in cultured hepatocytes obtained from chick embryos. As in our previous work with rats [2], we find that $CoCl_2$ stimulated the formation of a compound which can be labelled with [^{14}C]-5-aminolaevulinate and, which, like authentic cobalt

Table 4. Formation of cobalt protoporphyrin and decrease of 5-aminolaevulinate synthase by $CoCl_2$ in hepatocyte culture*

Treatment	5-Aminolaevulinate synthase† (nmoles/hr/mg protein)	Porphyrin composition (% protoporphyrin)	Cobalt protoporphyrin (pmoles/mg protein)
Expt. 1			
AIA + TCB (10)	5.1 ± 1.2	55	
+ cycloheximide	1.3 ± 0.2‡§	30	
+ $CoCl_2$ (2.5)	2.8 ± 0.6‡§	40	11.5 (9.7, 13.3)
+ $CoCl_2$ (15)	2.4 ± 0.3‡§	35	78 (72, 83)
AIA + TCB (250)	34.8 ± 2.5§	5	
+ cycloheximide	10.5 ± 2.5‡	15	
+ $CoCl_2$ (2.5)	38.6 ± 3.1§	0	Not detectable
+ $CoCl_2$ (15)	34.7 ± 3.7§	0	Not detectable
Expt. 2			
AIA + TCB (250)	29.40 + 3.50§	0	
+ cycloheximide	6.30 ± 0.40‡	0	
+ $CoCl_2$ (15)	24.60 ± 2.90§	0	Not detectable
+ haem	5.30 ± 0.30‡	0	
+ Co protoporphyrin	2.80 ± 0.30‡	0	468

* Hepatocytes were cultured for 16 hr in 10 cm dishes as described in the legend to Table 2. After changing to Williams E medium containing dexamethasone and triiodothyronine, but not insulin, 2-allyl-2-isopropylacetamide (AIA) (100 μg/ml) was added as well as 3,4,3′,4′-tetrachlorobiphenyl (TCB) to the indicated concentration (ng/ml) in ethanol (2 μl/ml). After 16 hr, the following were added to the indicated concentrations: cycloheximide in saline (1 μg/ml), $CoCl_2$ in saline to indicated concentrations (μM), haemin or Co protoporphyrin (2.5 μg/ml) in ethanol/0.05 N NaOH [1:1 (v/v), 2 μl/ml]. After 6 hr further incubation, cells were rinsed with saline and homogenized in 0.6 ml of solution used for the 5-aminolaevulinate synthase homogenate assay [14]. Co protoporphyrin in a portion of the homogenate was assayed as described in the legend to Fig. 1. Porphyrins were measured in medium and cell homogenate and the percentage that was protoporphyrin was determined by the spectrofluorometric procedure of Grandchamp *et al.* [13]. Data were analyzed by analysis-of-variance procedures and the comparisons among means were made using the q statistic [21].

† Mean ± S.D.

‡ Significantly different from AIA + TCB, alone ($P < 0.05$).

§ Not significantly different from each other ($P > 0.05$).

protoporphyrin, can be recovered bound to the protein pellet after extraction with acid/acetone. We have developed improved conditions for this radiolabelled technique of detection of cobalt protoporphyrin and have shown that retention of cobalt protoporphyrin by the protein pellet depends on the presence of sufficient protein. With isolated hepatocytes, additional protein must be added, and egg albumin is particularly suitable.

Further evidence that the unextractable labelled material is, in fact, cobalt protoporphyrin was provided by the following observations: (1) its formation was totally dependent on ferrochelatase activity (Table 2); and (2) when protoporphyrin synthesis was stimulated by addition to the hepatocyte cultures of either 2-allyl-2-isopropylacetamide (Fig. 1) or of excess 5-aminolaevulinate (results not shown), the material recovered bound to the protein pellet exhibited the spectral characteristics of authentic cobalt protoporphyrin.

Watkins *et al.* [4] have developed an electron paramagnetic resonance (EPR) method for detection of cobalt protoporphyrin and have used it to demonstrate formation of cobalt protoporphyrin in the liver of rats treated with $CoCl_2$. We estimate that our spectral method is as sensitive as the EPR method for small amounts of protein such as those used in tissue culture. Figure 2 shows that we could detect as little as 25 pmoles of cobalt protoporphyrin in the cells obtained from a 10 cm culture dish containing approximately 3 mg of protein.

In rat hepatocyte cultures exposed to $CoCl_2$, Guzelian and Bissell [5] were unable to detect cobalt protoporphyrin or to find any inhibition by $CoCl_2$ of haem synthesis, using either 5-amino[4-^{14}C]laevulinate or ^{59}Fe as radioactive precursors. It is probable that their spectral techniques were insufficiently sensitive to detect cobalt protoprophyrin. Furthermore, they exposed cells to label for 24 hr, and this may explain why no inhibition of haem synthesis was seen. We have found that, whereas inhibition of haem synthesis by $CoCl_2$ was demonstrable after 2 hr exposure to labelled 5-aminolaevulinate (Table 3), none could be demonstrated when labelling continued for 24 hr. We do not know the reason for this finding.

Maines and Sinclair [6] have shown that both the accumulation of porphyrins and the stimulation of 5-aminolaevulinate synthase caused by 2-allyl-2-isopropylacetamide in chicken hepatocyte cultures could be inhibited by very small concentrations of $CoCl_2$.

We have confirmed this and also found that cobalt protoporphyrin could be detected after treatment of cells with similar concentrations of $CoCl_2$ and that formation of cobalt protoporphyrin progressively increased when more $CoCl_2$ was added to the culture medium (Fig. 2). In contrast to the sensitivity of porphyrin accumulation increased by 2-allyl-2-isopropylacetamide, the accumulation of uroporphyrin and the stimulation of 5-aminolaevulinate synthase caused by treatment of hepatocyte cultures with polychlorinated biphenyls were relatively resistant to addition of $CoCl_2$ (Fig. 3) and, under these conditions, little or no formation of cobalt protoporphyrin could be demonstrated (Table 4). These findings provide additional support for the view that cobalt protoporphyrin, rather than free cobalt [7, 8], may be involved in the decrease of 5-aminolaevulinate synthase caused by cobalt. The action of cobalt protoporphyrin to decrease 5-aminolaevulinate synthase may be analogous to the repression of synthesis of this enzyme caused by haem; this suggestion has been discussed eslewhere [9, 22].

While this paper was in preparation, a report appeared showing that rat hepatocytes in suspension incubated with 5-aminolaevulinate and 0.5 mM $CoCl_2$ form extractable cobalt protoporphyrin [10].

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