Comparative Studies on the Inducing Effects of Cobalt Chloride and Co-Protoporphyrin on Hepatic Ornithine Decarboxylase and Heme Oxygenase in Rats

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Co-protoporphyrin, like Co^{2+} , produced a significant and persistent induction of hepatic ornithine decarboxylase (ODC) as well as its known inducing effect on heme oxygenase and the decreasing effects on drug-metabolizing enzymes. The induction of ODC and heme oxygenase by Co-protoporphyrin occurred dose-dependently with the lowest effective dose of $6.25 \mu\text{mol/kg}$. Although Co-protoporphyrin produced similar effects on ODC and heme oxygenase to Co^{2+} , there were differences in the mode of ODC induction. In particular, pretreatment with diethyl maleate failed to augment the induction of ODC by Co-protoporphyrin. Moreover, multiple administrations of Co^{2+} , but not Co-protoporphyrin, caused super-additive induction of ODC to about 1000-fold over the controls. This super-additive induction of ODC by Co^{2+} was dependent on the doses and time intervals between two administrations. In parallel with a large induction of ODC evoked by two administrations of Co^{2+} , hepatic putrescine content was increased markedly, while spermine content was decreased as compared to the control levels. Pretreatment with Co^{2+} led to super-additive induction of ODC by subsequent administration of the metal ion itself or diethyl maleate, but not by other ODC inducers, such as Co-protoporphyrin and thioacetamide, and not by subsequent partial hepatectomy. Under these experimental conditions, the magnitudes of heme oxygenase induction were similar. ODC induced by two doses of Co^{2+} was insensitive to exogenous putrescine, but sensitive to α -difluoromethylornithine and 1,3-diaminopropane. These findings add new insight into the effects of Co^{2+} and Co-protoporphyrin on hepatic polyamine metabolism; and the results suggest that the metal ion could cause extensive derangement of the ODC regulatory system in a manner different from the metalloporphyrin.

Keywords — cobalt chloride; cobalt-protoporphyrin; ornithine decarboxylase induction; heme oxygenase induction; polyamine

Introduction

Cobalt chloride (Co^{2+}) has been shown to produce various biochemical changes in the liver, particularly decreases in cytochrome P-450 content and drug metabolizing enzymes,¹⁾ interference with heme metabolism,²⁾ the induction of heme oxygenase and the depression of δ -aminolevulinic acid (ALA) synthetase.^{3,4)} In addition, we have recently shown that Co^{2+} has an ability to induce hepatic ornithine decarboxylase (ODC),⁵⁾ which is a rate-limiting enzyme of polyamine synthesis and is involved in cell growth as well as responses to various hormonal and toxic stimuli.⁶⁻⁹⁾ Interestingly, evidence has appeared that the effects of Co-protoporphyrin on hepatic ALA synthetase,¹⁰⁾ heme oxygenase and drug metabolizing enzymes¹¹⁾ are closely

mimicked by those produced by Co^{2+} , and that the metalloporphyrin has more potent action than the metal ion. Since the formation of Co-protoporphyrin *in vivo* has been detected in the liver of rats treated with Co^{2+} ,^{10,11)} the metal ion might act *via* the formation of metalloporphyrin.

Taking these facts into consideration, the present investigation has compared the effects of Co^{2+} and Co-protoporphyrin on hepatic ODC and heme oxygenase activities.

Materials and Methods

Chemicals — Co-protoporphyrin was purchased from Porphyrin Products Co. (Logan, UT., U.S.A.). [$1\text{-}^{14}\text{C}$]Ornithine (55 mCi/mmol) was obtained from Japan Isotope Association (Tokyo, Japan). Nicotinamide adenine dinu-

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cleotide phosphate (NADP), glucose-6-phosphate (G-6-P) and G-6-P dehydrogenase were obtained from Kyowa Hakko Co. (Tokyo, Japan). Hemin, putrescine, spermidine, spermine and 1,3-diaminopropane (DAP) were purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A.). α -Difluoromethylornithine (DFMO) was a gift from Dr. W. J. Hudak, Merrel Dow Pharmaceuticals (Cincinnati, OH., U.S.A.). All other chemicals were of the highest grade available commercially.

Animals and Treatment — Male Wistar rats, weighing 150–200 g, were used in this study. The animals were housed at room temperature ($22 \pm 2^\circ \text{C}$) under a 12-h light/dark cycle. CoCl_2 was dissolved in 0.9% NaCl. Co-protoporphyrin was dissolved in a small volume of 0.1 N NaOH solution, adjusted to pH 7.4 by 1 N HCl, and brought to an appropriate volume by adding 0.9% NaCl. The rats were injected subcutaneously with Co^{2+} or Co-protoporphyrin at the appropriate doses, and killed at the times indicated. The rats were fasted for 48 h before being killed. The rats pretreated with Co^{2+} by a single administration were injected with the metal ions ($125 \mu\text{mol/kg}$, s.c.), Co-protoporphyrin ($25 \mu\text{mol/kg}$, s.c.), diethyl maleate (0.3 ml/kg , i.p.) or thioacetamide (75 mg/kg , i.p.), or were subjected to two-thirds partial hepatectomy under ether anaesthesia as described by Higgins and Anderson,¹²⁾ and killed at appropriate times thereafter. In some experiments, the rats were injected repetitively with Co^{2+} , and then intraperitoneally administered DFMO (500 mg/kg) at 4 h, or DAP (2 mmol/kg) or putrescine (2 mmol/kg) at 4 and 1 h before being killed.

Tissue Preparation — Livers were perfused *in situ* with 0.9% NaCl, excised, and homogenized (1:6, w/v) in 0.05 M sodium/potassium phosphate buffer (pH 7.2) containing 1 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and $40 \mu\text{M}$ pyridoxal phosphate. The homogenates were centrifuged at $9000 \times g$ for 20 min, and the resulting supernatant fractions were again centrifuged at $105000 \times g$ for 60 min. The final supernatant fractions were used for the determination of ODC activity. The supernatant fractions from

the control rats were saved and used as the source of biliverdin reductase for the determination of heme oxygenase activity. The microsomal pellets were washed once, resuspended in buffer, and used for the determinations of heme oxygenase and aminopyrine demethylase activities, and cytochrome P-450 content. A portion of liver was homogenized in 10 volumes of 0.4 N HClO_4 . The homogenate was centrifuged at $2000 \times g$ for 10 min to precipitate protein. The resulting supernatant fraction was used for the assay of polyamine contents.

Assays — Hepatic ODC activity was determined by the method of Russell and Snyder¹³⁾ by measuring the $^{14}\text{CO}_2$ liberated from [1- ^{14}C]-ornithine as described previously.^{5,14)} Microsomal heme oxygenase activity was assayed by the method of Tenhunen *et al.*,¹⁵⁾ by using $105000 \times g$ supernatant fraction obtained from the control rats as the source of biliverdin reductase, as described previously.¹⁶⁾ Cytochrome P-450 content was determined by the method of Omura and Sato.¹⁷⁾ Aminopyrine demethylase activity was measured by the method of Cochin and Axelrod.¹⁸⁾ Hepatic polyamine content was determined by using gas chromatograph equipped with a flame-thermoionic detector according to the method of Yamamoto *et al.*,¹⁹⁾ as described previously.²⁰⁾ Protein concentrations were determined by the method of Lowry *et al.*²¹⁾ for the microsomal fraction and by that of Gornall *et al.*²²⁾ for the supernatant fraction.

Statistical Analysis — The results were subjected to statistical analysis according to the Student's *t* test.

Results

Time Course of the Effect of Co-Protoporphyrin on Hepatic ODC, Heme Oxygenase and Drug-Metabolizing Enzymes

In addition to Co^{2+} as reported previously,⁵⁾ Co-protoporphyrin was also able to increase hepatic ODC activity (Fig. 1). The maximum increase in ODC activity was seen at 12 h, and the increased enzyme activity was sustained for up to 3 d after administration. The ODC activity maximally increased to about 40 times of the

Fig. 1. Time Course of the Effect of Co-Protoporphyrin on Hepatic ODC, Heme Oxygenase, and Drug-Metabolizing Enzymes

Rats were injected subcutaneously with Co-protoporphyrin (50 $\mu\text{mol/kg}$) and killed at the times indicated. Each time point represents the mean for three to four rats. Control values at each time point were within 10% deviation from the 0-time values. The Co-protoporphyrin-mediated changes in ODC activity (from 6 to 72 h), heme oxygenase activity (from 6 to 168 h), aminopyrine demethylase activity and cytochrome P-450 content (from 12 to 168 h) were statistically significant ($p < 0.05$ or 0.01) with respect to the controls.

control at 12 h after the administration of Co-protoporphyrin was comparable to that produced by Co^{2+} at a dose of 250 $\mu\text{mol/kg}$, as described previously.⁵⁾ The persistent increase in microsomal heme oxygenase and the decreases in cytochrome P-450 content and aminopyrine demethylase activity evoked by Co-protopor-

phyrin were consistent with those reported by Drummond and Kappas.¹⁰⁾

Since Co-protoporphyrin was able to produce similar effects on hepatic ODC, heme oxygenase and drug metabolizing enzymes to those evoked by Co^{2+} , we chose the former two enzymes as parameters for comparing the inducing effects

TABLE I. Dose-Response Effects of Co-Protoporphyrin on Hepatic ODC and Heme Oxygenase Activities

Treatment	ODC activity (pmol/mg protein/30 min)	Heme oxygenase activity (nmol/mg protein/h)
Control	3.90 ± 0.97	1.94 ± 0.14
Co-protoporphyrin ($\mu\text{mol/kg}$)		
6.25	$65.7 \pm 19.3^a)$	$4.34 \pm 0.63^a)$
12.5	$101 \pm 26.5^a)$	$7.01 \pm 1.27^a)$
25	$109 \pm 15.4^a)$	$11.7 \pm 1.09^b)$
50	$127 \pm 29.4^a)$	$15.3 \pm 2.13^b)$
100	$243 \pm 64.5^a)$	$15.2 \pm 0.49^b)$
250	$230 \pm 35.2^a)$	$17.2 \pm 1.50^b)$

Rats were injected subcutaneously with Co-protoporphyrin at the doses indicated, and killed 12 h later for the determination of ODC and heme oxygenase activities. Values are the mean $\pm$ S.E. for three rats.

a) $p < 0.01$, b) $p < 0.001$, different from controls.

TABLE II. Influence of Pretreatment with Diethyl Maleate on the Inductive Effect of Co-Protoporphyrin on Hepatic ODC and Heme Oxygenase

Treatment	ODC activity (pmol/mg protein/30 min)	Heme oxygenase activity (nmol/mg protein/h)
Control	2.72 ± 0.71	1.73 ± 0.19
Diethyl maleate	48.4 ± 5.9 ^{a)}	6.52 ± 1.13 ^{a)}
Co-protoporphyrin	115 ± 37.7 ^{a)}	8.34 ± 0.59 ^{a)}
Diethyl maleate + Co-protoporphyrin	190 ± 23.3 ^{b)}	12.4 ± 0.78 ^{b)}

One group of rats was injected intraperitoneally with diethyl maleate (0.6 ml/kg) 1 h before the injection of Co-protoporphyrin (25 μ mol/kg). Other groups were injected with diethyl maleate or Co-protoporphyrin at the above dosages. The animals were killed 12 h after administration of Co-protoporphyrin for the determinations of hepatic ODC and heme oxygenase activities. Values are the mean $\pm$ S.E. for three rats. ^{a)} $p < 0.05$, different from control; ^{b)} $p < 0.05$, different from either diethyl maleate or Co-protoporphyrin.

of the metal ion and metalloporphyrin. Co-protoporphyrin induced ODC and heme oxygenase in a dose-dependent manner (Table I). No further increase of ODC activity, however, could be seen by the administration of Co-protoporphyrin at a dose of 250 μ mol/kg. This

could be due, at least partly, to the restricted solubility and absorption of Co-protoporphyrin. The lowest dose of 6.25 μ mol/kg of Co-protoporphyrin was still effective to induce both ODC and heme oxygenase. From these results, it seems that Co-protoporphyrin is a more

Fig. 2. Effects of Multiple Administrations of CoCl₂ and Co-Protoporphyrin on Hepatic ODC and Heme Oxygenase Induction

Rats were injected repeatedly with CoCl₂ (125 μ mol/kg, s.c.) or Co-protoporphyrin (50 μ mol/kg, s.c.) at the times indicated by arrows. Black and white arrows indicate the time points where the rats received Co²⁺ injection at every 6 h and where the animals received the metal ion or Co-protoporphyrin injection at every 12 h, respectively. CoCl₂ single injection (○—○); multiple injections (●—●, every 6 h; ○—○, every 12 h). Co-protoporphyrin multiple injections (□, every 12 h). Each value represents the mean of three to four rats. The increased enzyme activities are significantly ($p < 0.05$ or 0.01) different from the controls.

potent inducer of ODC as well as heme oxygenase than Co^{2+} , because the 125 $\mu\text{mol/kg}$ dose of metal ion was required for the induction of these enzymes as reported previously.⁵⁾

Effect of Pretreatment with Diethyl Maleate on the Induction of ODC and Heme Oxygenase by Co-Protoporphyrin

To examine whether both Co-protoporphyrin and Co^{2+} produce the increases in ODC and heme oxygenase in a similar manner, experiments were carried out by employing diethyl maleate, a depletor of hepatic glutathione (GSH), which augmented the Co^{2+} -mediated induction of ODC and induced heme oxygenase additively with the metal ion.⁵⁾ Pretreatment of animals with diethyl maleate did not augment the Co-protoporphyrin-mediated induction of ODC, but rather both agents additively increased both ODC and heme oxygenase activities (Table II). The results thus suggest that both Co^{2+} and Co-protoporphyrin could produce the induction of ODC, but not heme oxygenase, in somewhat different manner.

Effects of Multiple Administrations of Co^{2+} and Co-Protoporphyrin on Hepatic ODC and Heme Oxygenase Induction

A more pronounced difference in the effects of Co^{2+} and Co-protoporphyrin on ODC induc-

tion was seen following their multiple administrations (Fig. 2). The multiple administrations of Co^{2+} to rats resulted in profound increases in both ODC and heme oxygenase activities. Specifically, the magnitude of increase in ODC activity following the two or three administrations of Co^{2+} (125 $\mu\text{mol/kg}$, s.c.) was far greater than that after the corresponding single dose. The maximal increase of ODC activity elicited by the two or three doses of Co^{2+} was several hundred-fold over the control. Contrary to such a super-additive induction of ODC evoked by Co^{2+} , multiple administrations of Co-protoporphyrin did not accelerate ODC synthesis, and rather had the opposite effect. The findings thus suggest that Co^{2+} and Co-protoporphyrin could evoke ODC induction in separate ways, or that the metalloprophyrin, when administered repeatedly, has less access to the ODC induction site(s) than the metal ion. This super-additive induction of ODC by repetitive administrations of Co^{2+} was also seen in other animal species. Namely, when mice were repetitively injected with Co^{2+} at the dose of 125 $\mu\text{mol/kg}$, hepatic ODC activity was increased to 1960 ± 150 pmol/mg protein/30 min as compared to 60 ± 15 pmol/mg protein/30 min produced by a single administration.

TABLE III. Effects of the Different Repeated Dosages of CoCl_2 on the Induction of ODC and Heme Oxygenase

Treatment		ODC activity (pmol/mg protein/30 min)		Heme oxygenase activity (nmol/mg protein/h)
Control		3.74 ±	0.67	1.94 ± 0.25
CoCl_2	($\mu\text{mol/kg}$)			
First	Second			
0	62.5	4.74 ±	1.64	3.20 ± 0.17
	125	41.44 ±	4.68 ^{a)}	6.34 ± 0.89 ^{b)}
	250	177 ±	86 ^{b)}	13.97 ± 0.81 ^{b)}
125	62.5	57.62 ±	9.42 ^{a)}	12.87 ± 0.65 ^{b)}
	125	976 ±	168 ^{b,c)}	20.60 ± 0.86 ^{b)}
	250	2832 ±	655 ^{b,c)}	22.05 ± 3.40 ^{b)}
250	62.5	1303 ±	121 ^{b,c)}	18.79 ± 4.20 ^{b)}
	125	3401 ±	432 ^{b,c)}	24.81 ± 4.36 ^{b)}
	250	4147 ±	311 ^{b,c)}	24.97 ± 2.65 ^{b)}

Rats were pretreated subcutaneously with CoCl_2 (125 or 250 $\mu\text{mol/kg}$), and again injected 12 h later with a second dose of the metal ion. The animals were killed 12h after the second dosage of CoCl_2 for the determinations of hepatic ODC and heme oxygenase activities. Values are the mean ± S.E. for three rats. ^{a)} $p < 0.05$, ^{b)} $p < 0.01$, different from control; ^{c)} $p < 0.01$, different from single dosage.

TABLE IV. Effects of Time Interval between the First and Second Doses of CoCl₂ on the Induction of ODC and Heme Oxygenase

Treatment	ODC activity (pmol/mg protein/30 min)	Heme oxygenase activity (nmol/mg protein/h)
Control	5.90 ± 0.76	2.02 ± 0.31
CoCl ₂ (μmol/kg, 12 h)		
125	36.4 ± 11.5 ^{a)}	5.58 ± 1.93 ^{a)}
250	243 ± 57.1 ^{a)}	14.0 ± 0.83 ^{b)}
375	341 ± 28.7 ^{b)}	23.4 ± 2.89 ^{b)}
Time interval between the first and second doses (h)		
1	1374 ± 287 ^{b,c)}	24.9 ± 0.49 ^{b)}
3	3002 ± 342 ^{b,c)}	24.8 ± 0.60 ^{b)}
6	3093 ± 834 ^{b,c)}	24.1 ± 0.39 ^{b)}
12	3579 ± 296 ^{b,c)}	27.2 ± 0.42 ^{b)}
16	2896 ± 290 ^{b,c)}	25.7 ± 0.66 ^{b)}
24	252 ± 78.5 ^{a)}	19.6 ± 1.03 ^{b)}

Rats were injected subcutaneously with CoCl₂ (125 μmol/kg), and again injected after various time intervals with a second dose of CoCl₂ (250 μmol/kg). The animals were killed 12 h after the second dose of CoCl₂ for the determination of ODC and heme oxygenase activities. Values are the mean ± S.E. at least for three rats. ^{a)} $p < 0.05$, ^{b)} $p < 0.01$ compared with controls; ^{c)} $p < 0.01$ compared with 375 μmol/kg of CoCl₂.

TABLE V. Effects of Time Interval between the First and Second Doses of CoCl₂ on Hepatic Polyamine Contents

Treatment	Polyamine content (nmol/g wet weight liver)		
	Putrescine	Spermidine	Spermine
Control	8.07 ± 1.79	1204 ± 81.1	1059 ± 50.3
CoCl ₂ (μmol/kg, 12 h)			
125	16.9 ± 3.3	1009 ± 187	1070 ± 29.2
250	217 ± 34.1 ^{a)}	1230 ± 60.5	837 ± 11.5
375	431 ± 72.7 ^{a)}	1162 ± 46.4	678 ± 11.5 ^{a)}
Time interval between the first and second doses (h)			
1	846 ± 59.0 ^{a,b)}	1150 ± 74.4	570 ± 26.5 ^{a)}
3	987 ± 71.2 ^{a,b)}	1173 ± 50.8	639 ± 50.2 ^{a)}
6	1521 ± 31.2 ^{a,b)}	1210 ± 130	634 ± 26.5 ^{a)}
12	1230 ± 195 ^{a,b)}	1346 ± 51.1	668 ± 22.5 ^{a)}
16	973 ± 99.9 ^{a,b)}	1515 ± 41.8	845 ± 31.8
24	108 ± 5.3 ^{a)}	1574 ± 29.9	808 ± 142

Rats were treated with CoCl₂ and killed as described in detail in Table IV. ^{a)} $p < 0.01$ compared with controls; ^{b)} $p < 0.01$, compared with 375 μmol/kg of CoCl₂.

Dose- and Time-Dependent Effects of Two Administrations of Co²⁺ on Hepatic ODC and Heme Oxygenase Induction

Because the multiple administrations of Co²⁺ caused super-additive induction of ODC, dose-response and time interval studies were carried out in order to define further this phenomenon. The super-additive induction of ODC by Co²⁺

was shown to be dose-dependent when examined by changing the first and second administrations (Table III). The maximally increased ODC activity obtained by doses of 250 μmol/kg was about 1000 times the control levels. The induction of heme oxygenase was also significantly augmented with maximum values of approximately 25 nmol/mg protein/h under the experi-

mental conditions.

Next, a time interval study was done by setting the first and second dosages of Co^{2+} at 125 and 250 $\mu\text{mol/kg}$, respectively. The super-additive induction of ODC by Co^{2+} occurred when the time intervals between the first and second administration were from 1 to 16 h (Table IV). No super-additive induction of ODC could be seen with a 24-h time interval. Again, the magnitude of heme oxygenase induction by Co^{2+} plateaued at a highly increased level of 25 nmol/mg protein/h. Based on these finding, it seems clear that Co^{2+} treatment increases the sensitivity of hepatic ODC synthesis to the subsequent administration of the metal ion.

Effects of Two Administrations of Co^{2+} on Hepatic Polyamine Content

The changes in hepatic polyamine content were determined in the liver of rats employed in the time interval study (Table V). In parallel with the super-additive induction of ODC, hepatic putrescine content was markedly increased by two administrations of Co^{2+} , while hepatic spermine content, but not spermidine content, was reduced significantly as compared with the controls. The increased putrescine con-

tent in the liver reached levels similar to or greater than the spermidine and spermine contents. The maximal increase of putrescine content was seen in the case of a 6-h time interval between the first and second administrations. These results indicated that multiple administrations of Co^{2+} could produce remarkable changes in hepatic polyamine content as a consequence of ODC induction.

Effects of Pretreatment with Co^{2+} on ODC Induction by Other Inducers

Since pretreatment of rats with Co^{2+} resulted in a large increase in hepatic ODC activity by the subsequent dose of the metal ion, experiments were also carried out to examine whether this phenomenon could be produced with other ODC inducers (Table VI). Only Co^{2+} and diethyl maleate gave super-additive induction of ODC in the metal ion-pretreated rats. No augmentation or synergistic induction of ODC was seen in Co^{2+} -pretreated rats injected subsequently with Co-protoporphyrin or thioacetamide, or subjected to partial hepatectomy. Thus, the results suggest that the super-additive induction of ODC in the liver of Co^{2+} -pretreated rats is not a general occurrence on subsequent administration of other enzyme inducers. Rather, both

TABLE VI. Effect of Pretreatment with CoCl_2 on the Induction of ODC and Heme Oxygenase by Co-Protoporphyrin, Diethyl Maleate, Thioacetamide and Partial Hepatectomy

Treatment	ODC activity (pmol/mg protein/30 min)	Heme oxygenase activity (nmol/mg protein/h)
Control (subcutaneous)	2.43 ± 0.78	1.89 ± 0.14
CoCl_2 (12 h)	78.8 ± 22.5 <i>a)</i>	8.86 ± 2.23 <i>a)</i>
CoCl_2 (22 h)	17.4 ± 4.14 <i>a)</i>	5.87 ± 1.63 <i>a)</i>
Co-protoporphyrin (12 h)	42.1 ± 3.71 <i>a)</i>	8.95 ± 0.43 <i>a)</i>
CoCl_2 + Co-protoporphyrin	233 ± 10.6 <i>a,b)</i>	25.1 ± 0.43 <i>a,b)</i>
Control (intraperitoneal)	1.37 ± 0.52	1.32 ± 0.12
Diethyl maleate (6 h)	49.1 ± 5.32 <i>a)</i>	4.56 ± 0.74 <i>a)</i>
CoCl_2 + diethyl maleate	616 ± 98.1 <i>a,b)</i>	29.1 ± 1.92 <i>a,b)</i>
Thioacetamide (16 h)	394 ± 47.1 <i>a)</i>	1.83 ± 0.09 <i>a)</i>
CoCl_2 + thioacetamide	258 ± 30.5 <i>a)</i>	4.62 ± 0.52 <i>a)</i>
Control (subcutaneous)	2.96 ± 0.41	1.51 ± 0.02
Partial hepatectomy (12 h)	74.5 ± 11.7 <i>a)</i>	6.27 ± 0.65 <i>a)</i>
CoCl_2 + partial hepatectomy	142 ± 6.81 <i>a,b)</i>	16.5 ± 2.88 <i>a,b)</i>

Rats were pretreated with CoCl_2 (125 $\mu\text{mol/kg}$, s.c.) 6 h before the administration of Co-protoporphyrin (25 $\mu\text{mol/kg}$, s.c.), diethyl maleate (0.3 ml/kg, i.p.) or thioacetamide (75 mg/kg, i.p.), or before partial hepatectomy, and killed 18, 12, 22 and 18 h later, respectively, for the determinations of ODC and heme oxygenase activities. Values are the mean $\pm$ S.E. for three rats. *a)* $p < 0.05$, different from control; *b)* $p < 0.01$, different from CoCl_2 or other treatment alone.

TABLE VII. Effects of DFMO, DAP and Putrescine on CoCl_2 -Induced ODC Activity

Treatment	ODC activity (pmol/mg protein/30 min)		
Control	4.08 ±	1.44	
CoCl_2	3898 ±	189	a)
+DFMO	99.8 ±	13.4	a,b)
+DAP	216 ±	112	a,b)
+putrescine	1574 ±	212	a,c)

Rats were injected twice with CoCl_2 (250 $\mu\text{mol/kg}$, s.c.) at 18 and 12 h before being killed. Some rats pretreated with CoCl_2 were injected intraperitoneally with DFMO (500 mg/kg) 4 h before, or with DAP (2 mmol/kg) and putrescine (2 mmol/kg) 4 and 1 h before being killed for the determination of hepatic ODC activity. Values are the mean $\pm$ S.E. for three rats. a) $p < 0.01$, different from control; b) $p < 0.01$ or c) $p < 0.05$, different from CoCl_2 .

Co^{2+} and diethyl maleate could act on ODC synthesis in an as yet unknown, specific manner, because the combined administration of the metal ion and the agent in either order produced synergistic enzyme induction, as shown in the previous⁵⁾ and present study.

Effects of DFMO, DAP and Putrescine on Co^{2+} -Induced ODC

To characterize the ODC induced by the repetitive administrations of Co^{2+} , the *in vivo* inhibitory effects of DFMO, a suicide inhibitor of the enzyme,²³⁾ and DAP and putrescine, inducers of an antizyme which binds to the enzyme and accelerates the enzyme breakdown,^{24,25)} were examined (Table VII). The administration of DFMO and DAP to Co^{2+} -pretreated rats resulted in almost complete inhibition of ODC activity. However, the inhibitory effect of putrescine on Co^{2+} -induced ODC was less extensive. The reason for such differences in the inhibitory potency of ODC induction between DAP and putrescine remains to be determined; however, the less potent effect of putrescine on the Co^{2+} -induced enzyme could be compatible with the results in Table IV, because there were highly increased levels of ODC activity and the amine content.

Discussion

This investigation has revealed that in addi-

tion to Co^{2+} as reported previously,⁵⁾ the administration of Co-protoporphyrin to rats resulted in a significant and persistent increase in ODC activity, as well as its known inducing effect on heme oxygenase and the decreasing effects on drug-metabolizing enzymes. This persistent increase of ODC activity elicited by Co-protoporphyrin is noteworthy, since the half life of the enzyme has been shown to be very short.⁶⁻⁹⁾ However, the reason for this long-lasting effect of Co-protoporphyrin on ODC activity remains to be determined. In accordance with the published reports on the effects of Co-protoporphyrin on ALA synthetase⁹⁾ and heme oxygenase,¹⁰⁾ the metalloporphyrin was also a more potent inducer of ODC than Co^{2+} in terms of the effective molar dose levels.⁵⁾ There is evidence indicating that Co-protoporphyrin is detectable *in vivo* in the liver after the administration of Co^{2+} to rats.^{9,11)} Taking these facts into consideration, it is likely that various biochemical changes that occur in the liver after the administration of Co^{2+} are mediated through the formation of Co-protoporphyrin. With respect to the induction of ODC by Co-protoporphyrin and Co^{2+} , however, there were differences between the two agents. Namely, Co-protoporphyrin was able to produce the persistent induction of ODC when compared with Co^{2+} , as shown previously.⁵⁾ In addition, the depletion of hepatic GSH by diethyl maleate did not augment Co-protoporphyrin-mediated induction of ODC, but did augment Co^{2+} -mediated induction of the enzyme.⁵⁾ A more striking difference between Co^{2+} and Co-protoporphyrin in the induction of ODC was that multiple administrations of the metal ion, but not metalloporphyrin, caused an extremely large increase in the enzyme activity. Such a super-additive induction of ODC by multiple administrations of Co^{2+} occurred in a dose- and time-dependent manner. Since such a super-additive induction of ODC did not occur on the administration of Co-protoporphyrin, both Co^{2+} and the metalloporphyrin could independently induce ODC. However, the possibility cannot be ruled out that such differences in the inducing effects of Co^{2+} and Co-protoporphyrin on ODC could be due to the differences in their pharmacokinetics

within the body and in access to the enzyme induction site when administered repeatedly. On the other hand, it is likely that the depression of ALA synthetase⁹⁾ and the induction of heme oxygenase¹⁰⁾ could take place in a similar manner.

The super-additive induction of ODC by multiple administrations of Co^{2+} was accompanied with a large increase in hepatic putrescine content, while spermine, but not spermidine, content was reduced significantly. The increased putrescine levels seen with various time intervals between the two dosages of Co^{2+} approached or exceeded the levels of spermidine and spermine. The facts suggest that the highly increased ODC could be functioning effectively for the synthesis of putrescine. In contrast, the reason for the decrease of spermine, but not spermidine, content is not clear at this time. However, such changed patterns of hepatic polyamine content have been observed after the administration of diethyl maleate²⁰⁾ and hepatotoxins,^{26,27)} associated with the induction of spermidine/spermine N^1 -acetyltransferase, which plays a role in the conversion of polyamines into putrescine^{26,27)} in the latter case. It is thus likely that Co^{2+} -induced changed patterns of polyamine content could be related to its possible ability to induce N^1 -acetyltransferase. In this respect, however, further detailed studies will be required.

Evidence has appeared that putrescine and other related amines are able to induce an antizyme, which binds to ODC and accelerates the enzyme breakdown, so negatively regulating the enzyme levels.^{24,25)} It is not clear at this time how Co^{2+} could produce the super-additive induction of ODC under conditions where putrescine content in the liver was increased remarkably by the administrations of Co^{2+} . The findings that ODC induced by multiple doses of Co^{2+} was rather insensitive to exogenous putrescine, but sensitive to DFMO and DAP, could be one possible explanation for the above question. Namely, the ability of putrescine to induce the antizyme, but not DAP, could be decreased in Co^{2+} -treated rat liver. In this respect, however, additional detailed studies will be required.

The super-additive induction of ODC in

Co^{2+} -pretreated rats occurred on the subsequent administration of the metal ion itself or diethyl maleate, but not Co-protoporphyrin, thioacetamide or partial hepatectomy. Thus, both Co^{2+} and diethyl maleate may induce ODC through a similar specific mechanism in the metal-pretreated rat liver. Co^{2+} , like diethyl maleate, has been shown to produce the depletion and the rebound increase of hepatic GSH content.^{28,29)} We have recently found that GSH depletors, diethyl maleate and phorone, are potent inducers of hepatic ODC and heme oxygenase, in addition to their biphasic effects on GSH content.^{20,30,31)} Further, Co^{2+} complexed with cysteine or GSH failed to induce both ODC and heme oxygenase.⁵⁾ All of these findings suggest that perturbation of hepatic GSH content may be related directly or indirectly to the induction of ODC and heme oxygenase. Thus, it may be useful to examine further the induction of ODC and heme oxygenase in relation to hepatic GSH metabolism.

It has been shown that there is a temporary refractoriness and thereafter a greater augmentation of ODC induction by phorbol esters in mouse skin³²⁾ or the maintenance of the initially increased enzyme activity by anthralin in rat liver³³⁾ following their multiple administrations. These facts are in marked contrast to the case of Co^{2+} , as shown in this study. It is thus likely that Co^{2+} is a very unique inducer of hepatic ODC and it may be a useful agent for investigating regulatory mechanism(s) for the enzyme synthesis.

In conclusion, this study has shown that both Co^{2+} and Co-protoporphyrin are able to induce ODC as well as heme oxygenase; specifically, multiple administrations of the metal ion elicit the super-additive induction of ODC.

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