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Studies on the Mechanism of Induction of Haem Oxygenase by Cobalt and Other Metal Ions

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Cobalt ions (Co^{2+}) are potent inducers of haem oxygenase in liver and inhibit microsomal drug oxidation probably by depleting microsomal haem and cytochrome *P*-450. Complexing of Co^{2+}ions with cysteine or glutathione (GSH) blocked ability of the former to induce haem oxygenase. When hepatic GSH content was depleted by treatment of animals with diethyl maleate, the inducing effect of Co^{2+} on haem oxygenase was significantly augmented. Other metal ions such as Cr^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, and Pb^{2+} were also capable of inducing haem oxygenase and depleting microsomal haem and cytochrome *P*-450. None of these metal ions had a stimulatory effect on hepatic haem oxidation activity *in vitro*. It is suggested that the inducing action of Co^{2+} and other metal ions on microsomal haem oxygenase involves either the covalent binding of the metal ions to some cellular component concerned directly with regulating haem oxygenase or non-specific complex-formation by the metal ions, which depletes some regulatory system in liver cells of an essential component involved in controlling synthesis or activity of the enzyme.

We have described the striking ability of Co^{2+} to stimulate the rate of microsomal haem oxidation in liver cells (Maines & Kappas, 1974). This action of Co^{2+} was observed in the course of studies in which it was possible to dissociate completely cytochrome *P*-450 from the oxidative degradation of haem by hepatic microsomal fractions (Maines & Kappas, 1974, 1975*b*). The lack of involvement of cytochrome *P*-450 in the haem oxygenase activity of spleen microsomal fractions was also reported by Yoshida *et al.* (1974), and suggestions that this cytochrome does not mediate haem oxidation have also been made by others (O'Carra & Colleran, 1969; Nichol, 1971). The differences in the developmental patterns for haem and drug oxidations also support the idea that these oxidations are mediated by separate enzyme systems (Maines & Kappas, 1975*c*).

The results of our previous experiments (Maines & Kappas, 1975*b*) were consistent with the view that Co^{2+} stimulation of haem oxidation in liver involved induction *de novo* of the enzyme which catalyses this reaction; however, the mechanism and site of action of Co^{2+} remained to be clarified. The present study was undertaken to investigate additional aspects of the inducing effect of Co^{2+} on hepatic haem oxygenase and to examine the ability of other metal ions to stimulate this oxidative system in liver cells.

Experimental

Materials

Male Sprague–Dawley rats (160–200g) were used throughout the study. Chemicals were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Solutions of haematin, bilirubin and biliverdin were prepared as described by Maines & Kappas (1975*a*), and were used immediately after their preparation. Rats were injected subcutaneously, 24h before killing, with a single dose of 25μmol/100g body wt. (unless otherwise indicated) of $CoCl_2$, or Co^{2+} in the same dose, mixed individually with 2% human serum albumin, glutathione (GSH), or the following amino acids: cysteine, cystine, methionine and taurine at a 3:1 molar ratio (complexing agent: $CoCl_2,6H_2O$). The other metal compounds studied were $NiCl_2$, $FeCl_2$, $FeCl_3$, $CdCl_2$ (2.5μmol/100g), $MnCl_2$, $CuCl_2$ (12.5μmol/100g), LiCl, the acetates of zinc, lead and mercury, (100μmol/100g), and potassium chromate (12.5μmol/100g). Where indicated, animals were treated with diethyl maleate (0.6ml/kg) 30min before injection of $CoCl_2/Co^{2+}$; bilirubin, biliverdin (4μmol/100g) or DL-α-tocopherol (0.1ml/100g) were administered similarly before the metal compound. The animals were starved for 24h after the injections and were then killed by decapitation. The control animals received an injection of 0.9% NaCl.

Tissue preparations

Livers were perfused *in situ* with 0.9% NaCl, homogenized (1:4, w/v) in 0.1M-potassium phosphate buffer, pH7.4, and the hepatic microsomal fractions were prepared as described previously (Maines *et al.*, 1974). For determination of 5-aminolaevulinate synthetase activity, the livers were homogenized (1:2, w/v) in 0.05M-Tris/HCl buffer, pH7.4, containing 0.25M-sucrose. The microsomal pellets were resuspended in buffer and the protein concentration

was adjusted to 6–9mg/ml for the haem oxidation assay or 4–4.5mg/ml for the ethylmorphine *N*-demethylation assay. The liver microsomal supernatant fractions from the control animals served as the source of biliverdin reductase and the protein concentration of this fraction was adjusted to 10mg/ml.

Enzyme assays

The activity of hepatic 5-aminolaevulinate synthetase was assayed by the method of Marver *et al.* (1966) by using dichloromethane for extraction and the modified Ehrlich's reagent of Mauzerall & Granick (1956). Total microsomal thiol groups were measured by the micro-analytical method of Sakai (1968), Mercury Orange [1-(4-chloromercuriphenylazo)naphth-2-ol] being used as the thiol agent. Haem oxidation activity was measured as described in detail previously (Maines & Kappas, 1975*b*) in an incubation mixture (3ml) containing 6–9mg of microsomal protein, 5mg of microsomal supernatant protein, 17μM-haematin, an NADPH-generating system [$NADP^+$, 0.8mM; glucose 6-phosphate, 0.85mM; glucose 6-phosphate dehydrogenase (Sigma), 1.5 units, 0.2mM-$MgCl_2$ and 0.09M-potassium phosphate buffer, pH7.4]. The duration of incubation was 10min at 37°C. Bilirubin formation was calculated by using an extinction coefficient of $40mM^{-1}\cdot cm^{-1}$, $E_{464}-E_{530}$ (Maines & Kappas, 1974) and haem oxidative activity is presented as nmol of bilirubin formed/h per mg of protein. The oxidative activity of hepatic microsomal enzymes for drugs was monitored by measuring the oxidative demethylation of the prototype drug, ethylmorphine, as previously described (Maines & Kappas, 1975*b*). This assay was conducted in an incubation mixture (5ml) containing 4.5mg of microsomal protein, 2.0mM-ethylmorphine, an NADPH-generating system ($NADP^+$, 0.4mM; glucose 6-phosphate, 0.4mM; glucose 6-phosphate dehydrogenase, 2 units), 7.5mM-semicarbazide hydrochloride, 0.1mM-$MgCl_2$, 0.15M-KCl and 40mM-potassium phosphate buffer (pH7.4). Formaldehyde formed was measured by the method of Nash (1953).

Spectral studies

Cytochrome *P*-450 content was measured from the reduced-minus-CO difference spectrum by using sodium dithionite as the reducing agent and an extinction coefficient of $91mM^{-1}\cdot cm^{-1}$, $E_{450}-E_{490}$ (Omura & Sato, 1964). NADPH–cytochrome *c* reductase activity was measured as described by Williams & Kamin (1962) by using an extinction coefficient of $21mM^{-1}\cdot cm^{-1}$ for the difference between reduced and oxidized cytochrome *c* at 550nm. The concentration of microsomal haem was determined by the pyridine haemochromogen method of Paul *et al.* (1953) by using the reduced-minus-oxidized difference spectrum, $E_{557}-E_{575}$, and an extinction coefficient of $32.4mM^{-1}\cdot cm^{-1}$. All spectral studies were carried out with an Aminco–Chance DW-2 spectrophotometer.

Protein was determined by the method of Lowry *et al.* (1951) by using bovine serum albumin as standard. All experiments were performed at least three times and the results are reported as the mean of all experiments ± S.D. The data were analysed by the standard '*t*' test and the value of $P<0.05$ was regarded as denoting significance.

Results

The state in which Co^{2+} induces microsomal haem oxygenase activity in liver was initially examined. The data in Table 1 suggest that the metal ions are

Table 1. *Effects in vivo of Co^{2+} complexes on microsomal enzyme activities and haem and cytochrome P-450 contents*

Rats were injected with $CoCl_2,6H_2O$ (25μmol/100g, subcutaneously) or Co^{2+} complexed as described in the text, and were starved for 24h. Haem oxidation was measured as described by Maines & Kappas (1975*b*). Ethylmorphine demethylation was assayed as described in the Experimental section. The rates of ethylmorphine demethylation are expressed as nmol of product (formaldehyde) formed/h per mg of protein. $Na_2S_2O_4$-reduced-minus-CO difference spectrum of the microsomal fraction was measured as described by Omura & Sato (1964). NADPH–cytochrome *c* reductase activity was measured by the method of Williams & Kamin (1962) and the results are expressed as nmol of cytochrome *c* reduced/min per mg of protein. Total microsomal haem content was determined by the pyridine haemochromogen method of Paul *et al.* (1953). * $P<0.05$.

Treatment	Bilirubin formed (nmol/h per mg of protein)	Ethylmorphine *N*-demethylase (nmol/h per mg of protein)	Cytochrome *P*-450 (nmol/mg of protein)	Microsomal haem (nmol/mg of protein)	NADPH–cytochrome *c* reductase (nmol/min per mg of protein)
Control	1.85 ±0.14	3.133 ±30.5	0.72 ±0.06	1.40 ±0.16	100.2
$CoCl_2$	12.59* ±1.25	130.3* ±12.7	0.43* ±0.07	0.98* ±0.22	97.5
$CoCl_2$+cysteine	1.52 ±0.47	242.2* ±37.9	0.61* ±0.04	1.23 ±0.09	97.6
$CoCl_2$+glutathione	1.49 ±0.10	292.0 ±29.5	0.65 ±0.05	1.25 ±0.11	103.5
$CoCl_2$+albumin	14.90* ±2.30	71.5* ± 7.1	0.37* ±0.04	0.78* ±0.02	97.6

Table 2. *Effects in vitro of Co^{2+} complexes on microsomal oxidations*

Rats were starved for 24h before killing. Hepatic microsomal fractions were prepared and the protein content was adjusted as described in the Experimental section. Co^{2+}, cobalt–cysteine complex, or cobalt–human serum albumin complex were added to the incubation mixture, and the assays were conducted as described in the Experimental section. Cysteine and albumin alone, at the same concentrations as used with the metal ion, did not significantly alter haem oxidation or drug-metabolizing activity. * $P<0.05$.

Treatment	Concn. (μM)	Bilirubin formed (nmol/h per mg of protein)	Ethylmorphine *N*-demethylase (nmol/h per mg of protein)
Control	0	1.76 ±0.19	254.0 ±28.9
$CoCl_2$	100	1.24*±0.11	298.1*±25.6
	250	0.84*±0.10	337.8*±30.0
$CoCl_2$+cysteine	100	1.70 ±0.15	253.0 ±20.4
	250	1.36 ±0.20	247.0 ±29.4
$CoCl_2$+albumin	100	1.53 ±0.19	304.8*±27.3
	250	0.86*±0.17	317.5*±18.0

effective in inducing this activity only in the free form, or bound to a reactive group from which they can be easily dissociated. As these data show, when Co^{2+} was administered in a tightly bound form such as, for example, covalently complexed (Weissbecker, 1956) with the thiol groups of cysteine or GSH, its inducing effect on haem oxygenase was totally nullified. The GSH–Co^{2+} complex was also unable to produce a significant decrease in the oxidative activity of the microsomal fraction for ethylmorphine or in the microsomal content of cytochrome *P*-450 and total haem ($P<0.05$). Complexing of Co^{2+} with cysteine also significantly inhibited the ability of the metal ions to decrease microsomal cytochrome *P*-450 and drug oxidative activity ($P<0.05$). However, this inhibition was not as complete as that with the GSH complex.

The effects of other sulphur-containing amino acids on the changes produced by Co^{2+} in hepatic haem oxidation activity were investigated. Taurine was unable to inhibit the effect of Co^{2+} on microsomal haem oxidation (results not shown) indicating that the presence of free thiol groups is necessary for binding of Co^{2+} and its subsequent inactivity. Cysteine and methionine had partial inhibitory effects (30–50%) on the enhancement of haem oxidation and on decreased ethylmorphine *N*-demethylation and microsomal contents of cytochrome *P*-450 and total haem (results not shown). In contrast, when Co^{2+} was administered in complexed form with albumin there was a small augmentation of the effect of the metal ions on the microsomal parameters measured (Table 1).

The effects of Co^{2+} and its complexes *in vitro* were quite different from the effects *in vivo*. At high concentrations *in vitro* (Table 2) metallic cobalt or Co^{2+} complexed with albumin had similar inhibiting effects on hepatic haem oxidation. Co^{2+} complexed with cysteine was ineffective in altering haem oxidation activity *in vitro*. Table 2 shows that the cysteine–Co^{2+} complex was also ineffective *in vitro* in altering ethylmorphine *N*-demethylase activity at the concentrations used; however, when cobalt was added in the ionic form, or complexed with albumin, there was an enhancement of demethylase activity. The mechanism of this enhancement is unknown.

The direct participation of free Co^{2+} in the alterations described above is strongly suggested from the experiments in which the livers of rats were depleted of GSH by treatment *in vivo* with diethyl maleate before injection of the Co^{2+} (Fig. 1). At the dose used in this experiment (0.6ml/kg) diethyl maleate has been shown by Boyland & Chausseaud (1970) to deplete total hepatic cell content of GSH by 94%. As Fig. 1 shows, the depletion of GSH by diethyl maleate greatly enhanced haem oxidation by the hepatic microsomal fraction. Concomitant with the observed increase in haem oxygenase activity there were exaggerated decreases in microsomal cytochrome *P*-450 and haem contents.

The microsomal contents of total thiol groups at 4, 8, and 16h following Co^{2+} injection, as well as the effect of pretreatment with diethyl maleate on this parameter, were measured. There were no significant differences between the values obtained for animals treated with Co^{2+} alone and those obtained with Co^{2+} plus diethyl maleate. However, a slight but significant decrease of 16% (0.247±0.023μg/mg as compared with 0.200±0.021μg/mg, $P<0.05$) in the total number of thiol groups at 4h following either treatment was observed, which may reflect the binding of small amounts of Co^{2+} to the thiol groups of the microsomal fraction. At 8 and 16h following Co^{2+} injection there were no significant changes in the total number of thiol groups, although at 16h there was a slight increase (13%) in this value; however, this value was not statistically significant.

Apparently the substrate binding site for the haem oxygenase system does not involve a thiol moiety; this conclusion follows from experiments in which both the un-induced as well as the Co^{2+}-induced enzymes retained essentially all their activity when incubated *in vitro* with *N*-ethylmaleimide, or with *p*-chloromercuribenzoate (10–100μM final concn.) before (2min) or after (2min) incubation of the microsomal fraction with the substrate, haematin.

To explore the possibility that cobalt degradation of cytochrome *P*-450 and of drug-metabolizing activity is mediated *in vivo* through free-radical formation, experiments with DL-α-tocopherol were conducted. This vitamin inhibits free-radical formation and the damaging effects on membranes and membrane components which they mediate. Table 3 shows that in animals treated with the vitamin, the inhibiting effect of Co^{2+} on drug oxidation or on microsomal haemoprotein content was not changed, indicating that free-radical formation was not involved in the mechanism of these metal-ion effects. Further, the animals receiving the vitamin alone displayed a small but significant ($P<0.05$) increase in their haem oxidation activity as compared with control animals.

To determine whether the end product of microsomal haem oxidation, i.e. biliverdin or its own reduction product, bilirubin, could regulate hepatic haem oxygenase activity, the experiments described in Fig. 2 were carried out. In these experiments rats were treated with Co^{2+} to increase the rate of haem oxidation, and were simultaneously treated with biliverdin or bilirubin. Neither substance altered the inducing effect of Co^{2+} on haem oxygenase activity. However, biliverdin provided essentially complete protection against the ability of Co^{2+} to degrade cytochrome *P*-450 and to diminish the content of total microsomal haem. The mechanism of this protective effect is not known.

The data obtained in the preceding experiments suggested that complexing of Co^{2+} with thiol-containing cellular components was involved directly, or indirectly, in the ability of the metal ion to induce hepatic haem oxygenase. To explore this question further, studies were undertaken to determine whether administration *in vivo* of other metal ions having a strong ability to complex with thiol groups

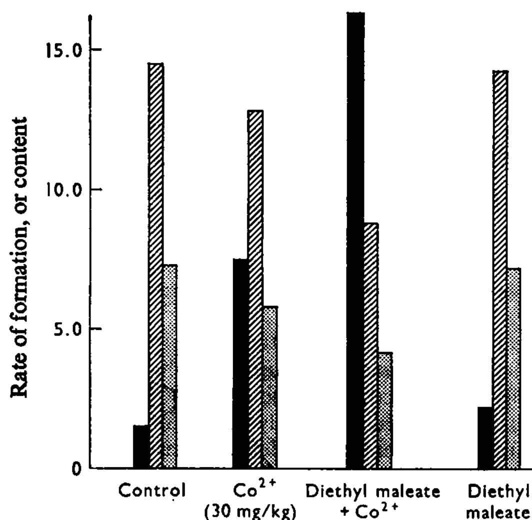

Fig. 1. *Influence of pretreatment with diethyl maleate on the effect of Co^{2+} on haem oxidation activity and on microsomal haem and cytochrome P-450 contents*

One group of rats was injected intraperitoneally with diethyl maleate (0.6ml/kg) 30min before the injection of Co^{2+} (12.5μmol/100g). Other groups were injected with diethyl maleate or Co^{2+} at the above dosages. The dosage of Co^{2+} was decreased to half of that usually used, as diethyl maleate treatment decreased the LD_{50} (median lethal dose) of Co^{2+} greatly. The animals were starved for 24h and the formation of bilirubin as well as the hepatic microsomal haem and cytochrome *P*-450 contents were measured. ■, Bilirubin formed (nmol/h per mg of protein); ▨, 10^{-1} × microsomal haem content (nmol/mg of protein); ▩, 10^{-1} × cytochrome *P*-450 content (nmol/mg of protein).

Table 3. *Effects in vivo of tocopherol on Co^{2+}-induced changes in microsomal oxidation and haem and cytochrome P-450 contents*

Rats were given DL-α-tocopherol orally (0.1 ml/100g) 5h before the injection of $CoCl_2$ (25μmol/100g, subcutaneously), and 16h later the animals were killed, hepatic microsomal fractions were prepared and various assays were conducted as described in the Experimental section. * $P<0.05$.

Treatment	Bilirubin formed (nmol/h per mg of protein)	Ethylmorphine *N*-demethylase (nmol/h per mg of protein)	Cytochrome *P*-450 (nmol/mg of protein)	Microsomal haem (nmol/mg of protein)
Control	1.30 ±0.22	228.2 ±29.4	0.76 ±0.08	1.53 ±0.14
$CoCl_2$	9.70* ±1.40	96.6* ±17.4	0.50* ±0.04	1.13* ±0.09
$CoCl_2$+tocopherol	11.00* ±1.10	73.6* ± 7.8	0.51* ±0.05	1.16* ±0.10
Tocopherol	2.14* ±0.03	284.2 ±34.2	0.75 ±0.02	1.53 ±0.16

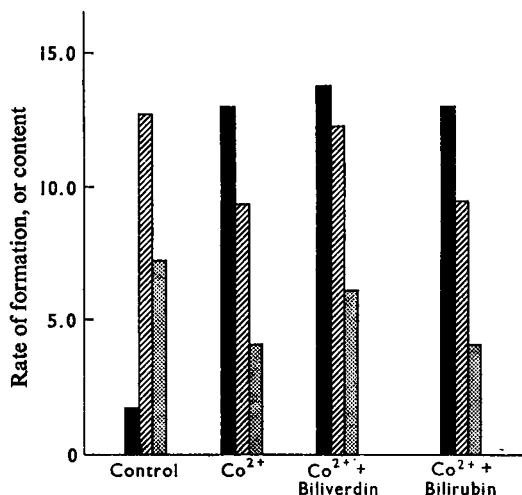

Fig. 2. *Effect of biliverdin and bilirubin on the alterations produced by Co^{2+} in oxidative activities of the microsomal fraction and of microsomal haemoprotein content*

Alkaline solutions (pH 7.4) of bilirubin and biliverdin (Maines & Kappas, 1975*a*) were injected intraperitoneally in rats (4 μmol/100 g body wt.) 1 h before and 1 h after the injection of Co^{2+} (25 μmol/100 g); 24 h later the starved animals were killed and haem oxidation activities as well as haemoprotein contents were measured in the hepatic microsomal fraction. ■, Bilirubin formed (nmol/h per mg of protein); ■, 10^{-1} × microsomal haem content (nmol/mg of protein); ▨, 10^{-1} × cytochrome *P*-450 content (nmol/mg of protein).

could influence the rate of microsomal haem oxidation in liver. The ions selected for study were those of certain metals of the first transition series, namely Cr^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Cu^{2+} and Zn^{2+}, and those of certain heavy metals, namely Cd^{2+}, Pb^{2+} and Hg^{2+}. The univalent metal ion Li^{+} was also investigated.

These studies revealed that a number of such metal ions, including those of iron, as De Matteis (1975) has shown, had the capacity to induce haem oxygenase in liver and that several had a potent capacity in this respect. On the other hand, the univalent metal ion, Li^{+}, which does not have a high affinity for thiol groups, did not exhibit an inducing effect on haem oxidation (results not shown). Cytochrome *P*-450 and haem contents of microsomal fractions were greatly decreased in animals treated with the metal ions even in the presence of normal or higher-than-normal 5-aminolaevulinate synthetase activity, with the exceptions of Zn^{2+} and Hg^{2+}, which are known to inhibit directly the activity of this enzyme. Concomitant with significant decreases in cytochrome *P*-450 contents (Table 4), microsomal ethylmorphine demethylase activities (results not shown) of animals treated with metal ions were diminished by 40–75%, depending on the metal ion. NADPH–cytochrome *c* reductase activities were also significantly decreased (20–40%) by Mn^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+} and Cd^{2+} treatment (results not shown). The effects of other metal ions on this enzyme activity were smaller (5–10%); Co^{2+} had no effect on this activity.

The effects *in vitro* on haem oxygenase activity of certain metal ions examined in this study (Mn^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Zn^{2+}, Pb^{2+}, Hg^{2+}) were examined. None had stimulatory effects on the enzyme at the concentrations used (12.5–250 μM) indicating that the responses to the metal ions found *in vivo* did not result from direct activation of preformed enzyme. Fe^{2+} displayed a marked degradative effect on haem oxygenase, such that with concentrations as low as 12.5 μM the enzyme activity was almost totally eliminated; a comparable effect of this metal ion on microsomal drug metabolism was shown by De Matteis & Sparks (1973). Cu^{2+}, Ni^{2+} and Hg^{2+} at concentrations of 100 μM or higher inhibited haem oxygenase significantly.

Discussion

The present study provides information relating to the mechanism of induction by Co^{2+} of microsomal haem oxygenase activity in liver and demonstrates that this action of the metal ions extends to a number of other trace- or heavy-metal ions as well. It appeared that, among the metal ions studied, those generally recognized to complex readily with thiol groups of amino acids and proteins also had the ability to induce hepatic haem oxygenase. This idea was studied in detail with respect to Co^{2+}, which was first shown by us to induce this enzyme activity (Maines & Kappas, 1974, 1975*b*), and the results of the studies with this inducer of haem oxygenase provide strong evidence that complexing of ionic cobalt with thiol groups is directly or indirectly involved in the mechanism and extent of its enzyme-inducing action. This conclusion is based on studies which correlated the relative effectiveness of amino acids to block Co^{2+} induction of haem oxygenase, and their ability to complex the metal ion. Bivalent reactive Co^{2+} covalently binds with thiol moieties to form a trithiol–cobalt complex, and the metal ion is converted to the trivalent, unreactive state. Such a complex is formed upon the reaction of Co^{2+} with GSH and cysteine, and as shown in these studies, the complex is unable to bring about alterations in haem oxygenase activity.

On the other hand Co^{2+} does not covalently bind to the disulphide groups of cysteine or the thioether moiety of methionine, and these amino acids did not completely block the ability of the metal ions to

Table 4. *Effect of bivalent cations of some first-transition-series metals and of some heavy metals on hepatic 5-aminolaevulinate synthetase, microsomal haem oxidation and cytochrome P-450 content*

Rats were treated subcutaneously with 25 μmol/100 g of the metal ion (with the exceptions designated) 24 h before killing, and were starved during this period. Thereafter they were decapitated, livers were perfused and homogenized, and portions were taken for the measurement of 5-aminolaevulinate synthetase (Marver *et al.*, 1966; Mauzerall & Granick, 1956). Microsomal fractions were prepared and assayed for haem oxidation activity, and the contents of haem and cytochrome *P*-450. The effects of Fe^{2+} and Fe^{3+} on the measured parameters were not significantly different; therefore only those of Fe^{2+} are presented.

Treatment	Bilirubin formed (nmol/h per mg of protein)	Cytochrome *P*-450 (nmol/mg of protein)	Microsomal haem (nmol/mg of protein)	5-Aminolaevulinate synthetase (nmol/g wet wt. of liver)
Control	1.52 ±0.29	0.88 ±0.09	1.54 ±0.17	44.92 ± 5.95
Cr^{2+}	3.81* ±0.50	0.66* ±0.08	1.31 ±0.09	53.04 ± 6.02
Mn^{2+}	4.64* ±0.50	0.55* ±0.08	1.02* ±0.08	58.38* ± 4.01
Fe^{2+}	5.53* ±1.04	0.59* ±0.10	1.24* ±0.11	49.49 ± 8.78
Co^{2+}	12.09* ±1.91	0.42* ±0.07	0.94* ±0.11	109.76* ±15.97
Ni^{2+}	4.27* ±0.87	0.53* ±0.06	1.52 ±0.06	65.23* ± 9.69
Cu^{2+}†	7.73* ±2.10	0.58* ±0.11	1.02* ±0.13	41.84 ± 5.23
Zn^{2+}‡	6.46 ±0.99	0.47* ±0.09	0.92* ±0.08	30.09* ± 5.09
Cd^{2+}§	10.18* ±1.11	0.41* ±0.09	0.88* ±0.09	40.37 ± 6.63
Hg^{2+}	6.79* ±1.04	0.39* ±0.10	0.99* ±0.09	17.31* ± 4.98
Pb^{2+}‖	4.41* ±1.98	0.52* ±0.13	1.11* ±0.14	74.79* ±13.21

* $P<0.05$.
† 12.5 μmol/kg.
‡ 100 μmol/kg.
§ 2.5 μmol/kg.
‖ intraperitoneally.

induce haem oxygenase, although they did have a partial effect in this regard. The ability of these amino acids to partially inhibit metal-ion induction of the enzyme may possibly result from some reduction of the disulphide bond or demethylation reaction which may occur at the site of injection. Co^{2+} forms much less stable complexes with reactive groups of albumin such as carbonyl, free amino or carboxyl groups (Weissbecker, 1956). This, plus the slowing of Co^{2+} excretion into urine when in the protein-bound form, and the presumably slower rate of Co^{2+} absorption from the injection site, could together result in the observed enhancement of its effect on haem oxygenase when complexed with albumin.

The involvement of thiol groups in the mechanism of action of Co^{2+} is further suggested by the experiments showing that, when the animals were pretreated with the GSH-depleting agent diethyl maleate, the ability of the metal ion to induce hepatic haem oxygenase activity was greatly enhanced. These findings indicate that GSH and possibly other cellular thiol-containing compounds such as cysteine, serve as a protective mechanism against this action of Co^{2+} by binding the free metal ion; these data also suggest that the metal ion, to initiate the induction effect on haem oxygenase, must react directly with some cellular component which has a metal-ion binding or complexing site and which is involved in the regulation of haem oxygenase synthesis or activity.

The absence of differences in the residual thiol groups in microsomal fractions from animals treated with Co^{2+} or diethyl maleate plus Co^{2+} metal, in spite of the great differences which these two treatments produced in haem oxygenase activity, may indicate that the greatly enhanced induction of haem oxygenase in the latter animals reflects Co^{2+} interaction with thiol groups elsewhere than in endoplasmic-reticulum membranes. The depleting effect of diethyl maleate on total cellular GSH must therefore make it possible for Co^{2+}, at a higher concentration, to reach active, presumably thiol-dependent, binding sites in the cell.

The cellular components which bind Co^{2+} must either complex the metal ion in the cytosol for transport to its active site, or the metal-ion binding may take place directly at the regulatory site itself. The latter is apparently neither in the cytosol nor in the membranes of the endoplasmic reticulum, as noted above (Maines & Kappas, 1974); thus if Co^{2+} acts directly to evoke the synthesis of haem oxygenase *de novo*, the binding of the metal ion at some regulatory site in the liver-cell nucleus may reasonably be postulated.

Alternatively the possibility must be considered that Co^{2+}, and the other haem oxygenase-inducing metal ions described here, may in the course of forming an intracellular complex of non-specific type, indirectly induce haem oxygenase by depleting some regulatory system of an essential component involved in repressing synthesis of the enzyme, or synthesis of some substance controlling its activity. It is not possible with the evidence at hand to define the proximate mechanism through which the metal ion can induce this liver enzyme, but it is clear that this enzyme-inducing action is a prevalent and potent one among the series of transition-metal ions studied.

As was observed throughout this study, a common pattern was always followed, in which increases in haem oxidation activities were accompanied by decreases in microsomal cytochrome *P*-450 and haem contents, indicating that these phenomena are closely and probably causally related. It can be postulated from these findings that the mechanism by which Co^{2+} and other metal ions decrease microsomal haem concentrations probably involves at least two independent processes; one is the known ability of certain metal ions to inhibit haem synthesis (Ericksen *et al.*, 1961; Tephly & Hibbein, 1971), and the second and perhaps more significant is the induction of haem oxygenase activity, i.e., as the enzyme activity become elevated, endogenous haem is degraded at an accelerated rate.

A causal relationship between the increases of hepatic haem oxygenase activity and the decreases in microsomal contents of haem and cytochrome *P*-450 is further suggested by the fact that the latter decreases could be observed in the presence of normal or even elevated hepatic 5-aminolaevulinate activity (Table 4). Since 5-aminolaevulinate synthetase is rate-limiting for haem synthesis, these observations indicate that enhanced degradation of endogenous haem oxygenase, in addition to any interference with the synthesis of haem at a post-5-aminolaevulinate synthetase step, must account for the observed decreases in microsomal haem and cytochrome *P*-450 contents. The metal ions in general caused a greater decrease in the microsomal content of cytochrome *P*-450 than of total haem; this probably reflects the shorter half-life of cytochrome *P*-450 as compared with the other known microsomal haemoprotein cytochrome b_5. In addition, it is possible that the metal ions also inhibit the formation of cytochrome *P*-450 through inhibition of synthesis of the apoprotein moiety of the cytochrome, and/or interference with complex-formation between the apoprotein and the haem prosthetic group.

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