Metal ion interactions in the control of haem oxygenase induction in liver and kidney

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(Received 22 April 1980/Accepted 31 July 1980)

Mn^{2+} and Zn^{2+} exhibit a striking ability to block the induction by Sn^{2+} and Ni^{2+} of haem oxygenase (EC 1.14.99.3) in kidney. The blocking effects of Mn^{2+} and Zn^{2+} were found to be greatest on simultaneous administration, time-dependent when administered up to 8 h before the inducing metal ions, and ineffective when administered as little as 10 min after the inducing metal ions. The decreases in cytochrome *P*-450 and haem contents and the sequential changes in δ -aminolaevulinate synthase (EC 2.3.1.37) activity that occur concomitant with haem oxygenase induction were largely eliminated with simultaneous or prior treatment with Mn^{2+} or Zn^{2+} , but not when Mn^{2+} or Zn^{2+} was administered after Sn^{2+} or Ni^{2+} . Mn^{2+} and Zn^{2+} did not increase the catabolism of the enzyme *in vivo*. Zn^{2+} on simultaneous administration was also able substantially to block the induction of haem oxygenase by Co^{2+} , Cd^{2+} and Ni^{2+} in liver. The Zn^{2+} blockade of Cd^{2+} induction was examined in detail, and prior or simultaneous administration of Zn^{2+} was found to be effective in blocking the induction of haem oxygenase and the concomitant decreases in cytochrome *P*-450 and haem contents, ethylmorphine demethylase activity and the sequential changes in δ -aminolaevulinate synthase activity. Zn^{2+} administration 10 min or more after Cd^{2+} was ineffective in preventing the occurrence of these perturbations in haem metabolism. These findings describe a new and striking biological property of Mn^{2+} and Zn^{2+} , and indicate the existence of significant metal ion interactions in the control of haem metabolism.

Previous studies from this laboratory have shown that the salts of a number of transition elements and heavy metals (Maines & Kappas, 1974, 1975, 1976a) can significantly enhance the synthesis *de novo* of microsomal haem oxygenase, the rate-limiting enzyme in the catabolism of haem to bile pigment (Tenhunen *et al.*, 1968, 1969). Metal ion induction of haem oxygenase is accompanied by various perturbations of haem metabolism, including alterations in δ -aminolaevulinate synthase activity, decreased microsomal contents of haem and haemoproteins and a diminished cellular capability to carry out biotransformations catalysed by cytochrome *P*-450 (Maines & Kappas, 1977c).

Metal ion induction of haem oxygenase in liver can be greatly altered by agents such as reduced glutathione, cysteine and diethylmaleate (Maines & Kappas, 1976a, 1977a), which presumably influence the availability of free metal ions to interact at regulatory sites for haem oxygenase (Maines & Kappas, 1976a). We have shown that hormones can also alter the ability of metal ions to induce this

enzyme in liver (Sardana *et al.*, 1980). The possibility that metal ions may themselves affect the induction of haem oxygenase by other metal ions has been actively explored (Drummond & Kappas, 1979), since metal ion interactions in various biological systems are well known. For example, Zn^{2+} and Se^{4+} diminish the toxic effects of cadmium salts in rats (Parizek, 1957, 1976), Mn^{2+} blocks the carcinogenic action of nickel salts (Sunderman *et al.*, 1974), and Zn^{2+} and Al^{3+} reverse the inhibition of δ -aminolaevulinate dehydratase produced by lead salts (Meredith *et al.*, 1977).

In the present paper we describe in detail the potent ability of Mn^{2+} and Zn^{2+} to block the induction of haem oxygenase elicited in kidney and liver by other metal ions. Mn^{2+} and Zn^{2+} are also shown to diminish or eliminate the perturbations of haem metabolism associated with the increases in haem oxygenase activity produced by the acute administration of other metal salts. The blockade of induction of haem oxygenase by Mn^{2+} and Zn^{2+} defines a new and striking biological property of

these elements, and suggests that the study of metal ion interactions in the regulation of haem oxygenase may provide valuable insights into the proximate mechanisms by which haem metabolism is regulated by metal ions.

Materials and methods

Materials

Male Sprague-Dawley rats (175–225 g) supplied by Holtzman (Madison, WI, U.S.A.) were used throughout this study. Rats were injected subcutaneously with the bivalent salts of manganese, zinc, tin, nickel, cobalt and cadmium in a single dose up to 250 $\mu\text{mol/kg}$ body wt. unless otherwise indicated. The animals were starved 16 h after injection and killed by decapitation. Cycloheximide was dissolved in a 25% (v/v) ethanol/0.9% NaCl solution and administered intraperitoneally at a dose of 18 mg/kg body wt. The control animals received an injection of an equivalent volume of 0.9% NaCl.

Tissue preparation

Livers were perfused *in situ* with ice-cold 0.9% NaCl and homogenized in 3 vol. of 0.1 M-potassium phosphate buffer, pH 7.4, containing 0.25 M-sucrose, and the microsomal fractions were prepared as described previously (Maines & Kappas, 1978) for determination of haem oxygenase and ethylmorphine demethylase activities. Microsomal fractions were prepared from whole kidneys in a manner identical with that used for those from liver. The microsomal supernatant from control animals served as the source of biliverdin reductase.

Enzyme assays

The activities of haem oxygenase, ethylmorphine demethylase and δ -aminolaevulinate synthase were assayed as previously described (Maines & Kappas, 1975, 1978; Sassa *et al.*, 1979a). Bilirubin formation was calculated by using an absorption coefficient of 40 $\text{mm}^{-1}\cdot\text{cm}^{-1}$ between 464 and 530 nm. The formaldehyde produced in the demethylase assay was measured by the method of Nash (1953).

Protein assay

Protein content was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard.

Spectral studies

Cytochrome *P*-450 was measured from the CO-induced difference spectrum with $\text{Na}_2\text{S}_2\text{O}_4$ as the reducing agent and an absorption coefficient of 91 $\text{mm}^{-1}\cdot\text{cm}^{-1}$ between 450 and 490 nm (Omura & Sato, 1964). 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 between 557 and 540 nm and an absorption coefficient of 20.7 $\text{mm}^{-1}\cdot\text{cm}^{-1}$. Spectral studies were performed with a Aminco-Chance DW2A spectrophotometer in the split-beam mode.

Three to six animals were used for each experimental point. The individual assays were performed in duplicate and the results are calculated as the means of all animals $\pm$ s.d. The data were analysed by the standard *t* test and a value of $P < 0.05$ was regarded as significant. All chemicals were of reagent grade.

Results

Time course of Sn^{2+} -induction of haem oxygenase in kidney

Sn^{2+} has previously been shown to be a potent inducer of haem oxygenase in the kidney 16 h after a single subcutaneous dose of the metal ion (Kappas & Maines, 1976). However, the time course of this enzyme induction, together with the associated perturbations of haem metabolism produced by Sn^{2+} in the kidney, have not been previously described. For this reason a time course of the responses to a single dose of Sn^{2+} on haem oxygenase and on other parameters of haem metabolism was studied, and the results are shown in Fig. 1. At 1 h after Sn^{2+} administration there was a small (approx. 30%) but significant increase in haem oxygenase activity, at 3 h haem oxygenase activity had increased by approx. 4-fold, and enzyme activity thereafter increased dramatically to a maximum at 16 h, at which time haem oxygenase activity had reached a value more than 20-fold that of the control. A decline in the enzyme activity then ensued, although at 24 h haem oxygenase activity was still some 7-fold above normal. The time course of haem oxygenase induction by Sn^{2+} was similar to that which we have previously described for Ni^{2+} and $\text{Pt}^{2+}, 4+$ in the kidney (Maines & Kappas, 1977b) and for Co^{2+} in the liver (Maines & Kappas, 1975).

δ -Aminolaevulinate synthase activity did not decline during the first 3 h, but remained constant before increasing at 16 h to a maximum 3–4-fold the initial value, after which this enzyme activity also declined. However, at 24 h δ -aminolaevulinate synthase activity was still greater than twice the initial value.

Cytochrome *P*-450 content decreased within the first 3 h by 20%, reaching by 12 h a low and relatively constant value that was approx. 40–50% of the starting value. Microsomal haem content declined gradually, reaching its lowest value (approx. 25% below normal) at 12 h, but, as with cytochrome *P*-450, did not decline thereafter.

A time-course study of the effects of Ni^{2+} on these enzymic activities and other parameters of haem

Fig. 1. Time course of the effect of Sn^{2+} on haem oxygenase and δ -aminolaevulinate synthase activities and on microsomal haem and cytochrome P-450 contents in kidney

Rats were injected subcutaneously with a single dose of Sn^{2+} ($250 \mu\text{mol/kg}$) and killed at the times indicated. Three rats were killed at each time point and the assays were performed in duplicate as described in the Materials and methods section. $\square$, Haem oxygenase activity (nmol of bilirubin formed/h per mg of protein); $\blacksquare$, δ -aminolaevulinate synthase activity (nmol/h per mg of protein); $\blacktriangle$, cytochrome P-450 content (nmol/mg of protein); $\bullet$, microsomal haem content (nmol/mg of protein).

Fig. 2. Dose-response of Mn^{2+} against Sn^{2+} and Ni^{2+} induction of haem oxygenase in kidney

Rats were injected subcutaneously with either Sn^{2+} or Ni^{2+} ($250 \mu\text{mol/kg}$) and Mn^{2+} (50, 100, 175 or $250 \mu\text{mol/kg}$), starved for 16 h and killed. Three rats were used at each concentration of Mn^{2+} . Haem oxygenase activity is expressed as nmol of bilirubin/h per mg of protein. For details see the Materials and methods section. C, Control.

metabolism in kidney was not performed, since we have reported such results previously (Maines & Kappas, 1977b).

Effects of various concentrations of Mn^{2+} on Sn^{2+} and Ni^{2+} induction of haem oxygenase in kidney

Exploratory studies indicated that Mn^{2+} could substantially inhibit the induction of haem oxygenase produced by Sn^{2+} and Ni^{2+} . Fig. 2 shows dose-response data of Mn^{2+} inhibition of Sn^{2+} and Ni^{2+} induction of haem oxygenase when the enzyme was measured at its peak value of induced activity (16 h) after the administration of the inducer. With Sn^{2+} ($250 \mu\text{mol/kg}$) induction of haem oxygenase, there was a slight inhibition of the induction response (Fig. 2) when the $\text{Mn}^{2+}/\text{Sn}^{2+}$ molar ratio was 1:5, but as the molar ratio of the metal ions approached 1:1 the blockade of enzyme induction by Mn^{2+} increased in a dose-dependent fashion until essentially total blockade of the induction response was achieved. With Ni^{2+} there was a somewhat greater initial blockade of enzyme induction compared with Sn^{2+} when the $\text{Mn}^{2+}/\text{Ni}^{2+}$ molar ratio was 1:5, this blockade of induction increased further as the $\text{Mn}^{2+}/\text{Ni}^{2+}$ molar ratio approached 1:1, but was not complete (Fig. 2). Total blockade of Ni^{2+}

induction of haem oxygenase by Mn^{2+} , however, was not achieved, despite the use of significantly higher concentrations of Mn^{2+} than of Ni^{2+} . The blocking effect of Mn^{2+} on haem oxygenase induction by Sn^{2+} or Ni^{2+} was independent of the manganese salt, i.e. chloride, sulphate or acetate, used.

Effects of simultaneous administration of Sn^{2+} and Mn^{2+} on haem oxygenase activity, δ -aminolaevulinate synthase activity and cytochrome P-450 content in kidney

Mn^{2+} , when administered simultaneously and in equimolar amounts with Sn^{2+} , prevented the profound increase in haem oxygenase activity seen with the latter metal ion (Fig. 1); this blocking effect is shown in detail in Fig. 3. At 12, 16 and 24 h there was a slight increase in haem oxygenase activity in the presence of Mn^{2+} , but the blockade of Sn^{2+} induction remained substantial, being 86, 95 and 85% respectively at these time points. Simultaneous Mn^{2+} treatment prevented the decline in the content of cytochrome P-450 produced by Sn^{2+} , and also blocked the late rebound increase in δ -amino-

Fig. 3. Time course of the effect of simultaneous administration of Mn^{2+} on Sn^{2+} induction of haem oxygenase and the concomitant changes in δ -amino-laevulinate synthase activity and cytochrome P-450 content in kidney

Groups of rats were injected subcutaneously with either Sn^{2+} (250 μ mol/kg) ($\square$, $\circ$ and $\triangle$) or Sn^{2+} plus Mn^{2+} (250 μ mol/kg) ($\boxtimes$, $\bullet$ and $\blacktriangle$) and killed at the times indicated. Each time point represents three rats. The assays were performed in duplicate as described in the Materials and methods section. $\square$ and $\boxtimes$, Haem oxygenase activity (nmol of bilirubin formed/h per mg of protein); $\circ$ and $\bullet$, δ -amino-laevulinate synthase activity (nmol/h per mg of protein); $\triangle$ and $\blacktriangle$, cytochrome P-450 content (nmol/mg of protein).

Fig. 4. Time course of the effect of Mn^{2+} on the induction of haem oxygenase by Sn^{2+} in kidney

Rats were injected subcutaneously with a single dose of Sn^{2+} (250 μ mol/kg), starved for 16 h and killed. Mn^{2+} (250 μ mol/kg) was injected subcutaneously at the designated times, which ranged from 8 h before to 3 h after Sn^{2+} . Each time point represents a minimum of five rats. Three groups of rats were administered 0.9% NaCl, Sn^{2+} or Mn^{2+} , starved for 16 h and killed. The values for haem oxygenase activity (nmol of bilirubin formed/h per mg of protein) ($\bullet$) were 1.91 ± 0.12 , 26.28 ± 1.46 and 2.02 ± 0.21 respectively. (a) Entire time course; (b) the period ± 60 min from the time of Sn^{2+} administration (0 min; $\circ$).

laevulinate synthase activity observed at 12, 16 and 24 h after Sn^{2+} administration (see Fig. 1).

Time-dependent responses of Mn^{2+} inhibition of Sn^{2+} and Ni^{2+} induction of haem oxygenase and of related perturbations in haem metabolism in kidney

The effect of Mn^{2+} on Sn^{2+} and Ni^{2+} induction of renal haem oxygenase and the concomitant alterations in haem metabolism were examined at various intervals concurrent with, before and after administration of the inducing metal ions (Figs. 4a, 4b, 5, 6a and 6b). The specific effect of manganese on Sn^{2+} induction of haem oxygenase with time is shown in Figs. 4(a) and 4(b); the associated changes (at zero time, -60 min and $+60$ min) of microsomal haem and cytochrome P-450 content and δ -amino-laevulinate synthase activity that accompanied the effects of these metal ions on haem oxygenase activity are shown in Fig. 5.

When injected simultaneously with the inducer metal ion, Sn^{2+} , at zero time ($\circ$ in Fig. 4a), Mn^{2+} blocked Sn^{2+} induction of renal haem oxygenase activity, as measured at 16 h, essentially completely (98%). The blocking effect of Mn^{2+} pretreatment (single injection at each time point) diminished the longer the interval between Mn^{2+} and Sn^{2+} administration, as is clear from the data at the time points -60 to -480 min (Fig. 4a). Nevertheless,

even when administered as long as 8 h (-480 min) before Sn^{2+} , an equimolar single dose of Mn^{2+} was able to block the induction of haem oxygenase elicited by Sn^{2+} by nearly 50%.

Fig. 4(b) shows the time period (-60 to $+60$ min) in detail, and reveals a further aspect of the striking time-dependency of Mn^{2+} blockade of Sn^{2+} induction of the enzyme when Mn^{2+} was administered after Sn^{2+} injection (zero time, $\circ$). If the administration of Mn^{2+} was delayed for as little as 5 min after the Sn^{2+} injection, haem oxygenase induction was able to proceed to a value approx. 40% of that produced by Sn^{2+} alone; by 10 min the extent of induction was 75% of that of Sn^{2+} alone. Fig. 5 depicts the alterations produced in haem oxygenase activity by Sn^{2+} alone and combined treatment with Sn^{2+} and Mn^{2+} , as well as the concomitant changes in δ -aminolaevulinate synthase and microsomal haem and cytochrome P-450 contents at zero time and in the time period -60 min to $+60$ min. In the period zero time to -60 min (and also to -480 min; Fig. 4a), when Mn^{2+} was exerting a blocking effect on the induction of haem oxygenase, no consistent or substantial changes were evident in δ -amino-laevulinate synthase activity or microsomal haem and cytochrome P-450 contents. However, when Mn^{2+} administration was delayed in the post- Sn^{2+} -

treatment period (zero to +60 min), both cytochrome *P*-450 and microsomal haem contents declined to values produced by Sn^{2+} treatment alone, and δ -aminolaevulinate synthase activity showed the expected rebound increase. It should be noted, however, that the changes in microsomal haem and

Fig. 5. Effects of Mn^{2+} on the Sn^{2+} induction of haem oxygenase and the concomitant changes in δ -aminolaevulinate synthase activity and cytochrome *P*-450 and microsomal haem contents in kidney

Rats were treated as described in the legend to Fig. 4. Sn^{2+} administration is regarded as zero time. Three groups of rats were administered 0.9% NaCl, Sn^{2+} or Mn^{2+} , starved for 16 h and killed. The values for haem oxygenase activity (nmol of bilirubin formed/h per mg of protein) (●) were 1.90 ± 0.12 , 26.28 ± 1.46 and 2.02 ± 0.21 respectively, for δ -aminolaevulinate synthase activity (nmol/h per mg of protein) (○) 0.155 ± 0.05 , 0.389 ± 0.03 and 0.148 ± 0.06 respectively, for cytochrome *P*-450 (nmol/mg of protein) (△) 0.106 ± 0.007 , 0.070 ± 0.008 and 0.091 ± 0.003 respectively, and for microsomal haem content (nmol/mg of protein) (□) 0.27 ± 0.03 , 0.19 ± 0.03 and 0.28 ± 0.03 respectively.

cytochrome *P*-450 contents when Mn^{2+} administration was delayed for 10 min after the Sn^{2+} injection were negligible compared with the very marked rise (13-fold) in haem oxygenase activity that was observed.

Figs. 6(a) and 6(b) show data from experiments comparable with those described above, but with Ni^{2+} as the inducer of haem oxygenase in kidney rather than Sn^{2+} . The results of combined Mn^{2+} and Ni^{2+} treatment on the induction of haem oxygenase were comparable with those produced by combined Mn^{2+} and Sn^{2+} administration, except that the extent of blockade of Ni^{2+} induction of haem oxygenase by Mn^{2+} was less than that observed with Sn^{2+} ; however, the ability of Mn^{2+} pretreatment to block Ni^{2+} induction of haem oxygenase was similar to that observed with Sn^{2+} . Mn^{2+} administration after Ni^{2+} (+5 and +10 min) appeared to exert a greater inhibitory effect (63 and 59% respectively) on Ni^{2+} induction of haem oxygenase than was the case with Sn^{2+} (60 and 25% respectively). The changes in other parameters of haem metabolism associated with administration of Ni^{2+} or Ni^{2+} plus Mn^{2+} were similar to those described for Sn^{2+} . Sn^{2+} and Ni^{2+} when incubated with renal cytochrome *P*-450 *in vitro* at concentrations as high as 0.25 mM did not cause decreases in cytochrome *P*-450 content (results not shown).

Fig. 6. Time course of the effect of Mn^{2+} on the induction of haem oxygenase by Ni^{2+} in kidney

Rats were treated as described in the legend to Fig. 4, except that Ni^{2+} (250 $\mu\text{mol/kg}$; zero time, ○) was the inducing metal ion. Mn^{2+} (250 $\mu\text{mol/kg}$) was the blocking metal ion. Three groups of rats were administered 0.9% NaCl, Ni^{2+} or Mn^{2+} , starved for 16 h and killed. The values for haem oxygenase activity (nmol of bilirubin formed/h per mg of protein) (●) were 1.73 ± 0.32 , 11.19 ± 1.03 and 1.32 ± 0.21 respectively. (a) Entire time period; (b) the period ± 60 min from the time of Ni^{2+} administration.

Time-dependent responses of Zn^{2+} inhibition of Sn^{2+} and Ni^{2+} induction of haem oxygenase in kidney

A dose-response study of the effects of Zn^{2+} on the induction of renal haem oxygenase by Sn^{2+} and Ni^{2+} showed results comparable with those described for Mn^{2+} inhibition of Sn^{2+} induction of the enzyme (results not shown). The time-dependency of the response of Zn^{2+} inhibition of the enzyme induction was therefore studied, and the associated perturbations in haem metabolism were examined at various times concurrent with, before and after the administration of the inducing metal ions in studies comparable with those performed with Mn^{2+} . The results of these studies are shown in Figs. 7(a), 7(b), 8(a), 8(b) and 9.

When administered simultaneously with Sn^{2+} , Zn^{2+} blocked the induction produced by the former metal ion to the extent of 98% (O in Fig. 7a). Zn^{2+} administration before Sn^{2+} resulted in blockade in haem oxygenase induction in a time-dependent manner similar to that observed with Mn^{2+} ; the closer to the time of the Sn^{2+} injection (zero time, O, in Fig. 7a) that Zn^{2+} was administered the greater was the blockade of enzyme induction. As with Mn^{2+} , Zn^{2+} when administered only 10 min after Sn^{2+} was essentially ineffective in preventing the full induction response produced by Sn^{2+} (Fig. 7b); when Zn^{2+} was administered 5 min after Sn^{2+} , more

than 80% of the blockade effect had already been lost (Fig. 7b). The changes in δ -aminolaevulinate synthase activity and cytochrome *P*-450 and microsomal haem contents were essentially similar to those described above for Mn^{2+} blockade of Sn^{2+} induction of renal haem oxygenase (Fig. 5); if Zn^{2+} administration was delayed for 5–10 min after Sn^{2+} , the changes in these parameters of haem metabolism were essentially equal to those produced by Sn^{2+} alone.

Figs. 8(a), 8(b) and 9 show results from experiments similar to those described above, but with Ni^{2+} as the inducing ion. The results of combined Zn^{2+} and Ni^{2+} administration on haem oxygenase induction were comparable with those of Mn^{2+} and Ni^{2+} (Figs. 6a and 6b), in that the most profound blockade of enzyme induction occurred with simultaneous administration of both metal ions (zero time, O, in Fig. 8a), and the extent of blockade of Ni^{2+} induction of haem oxygenase by Zn^{2+} in the time period (+5 min and +10 min) when Zn^{2+} was administered after Ni^{2+} (Fig. 8b) was less than when Sn^{2+} was the inducing metal ion (respectively 42 and 37% for Ni^{2+} and 20 and 0% for Sn^{2+} as inducing metal ion). In addition, Zn^{2+} pretreatment substantially diminished the decrease in cytochrome *P*-450 and microsomal haem contents and the rebound increase in δ -aminolaevulinate synthase activity associated with Ni^{2+} induction of haem oxygenase in kidney (Maines & Kappas, 1977b).

Fig. 7. Time course of the effect of Zn^{2+} on the induction of haem oxygenase by Sn^{2+} in kidney

Rats were treated as described in the legend to Fig. 4, except that Zn^{2+} (250 μ mol/kg) was the blocking metal ion and was administered at the designated times, which ranged from 5 h before to 1 h after Sn^{2+} (zero time, O). The values for haem oxygenase activity (nmol of bilirubin formed/h per mg of protein) of 0.9%-NaCl-, Sn^{2+} - or Mn^{2+} -treated rats were 1.27 ± 0.22 , 22.22 ± 1.31 and 1.38 ± 0.15 respectively. (a) Entire time period; (b) the period ± 60 min from the time of Sn^{2+} administration.

Fig. 8. Time course of the effect of Zn^{2+} on the induction of haem oxygenase by Ni^{2+} in kidney

Rats were treated as described in the legend to Fig. 7, except that Ni^{2+} (250 μ mol/kg) was the inducing metal ion. The values of 0.9%-NaCl-, Ni^{2+} - or Zn^{2+} -treated animals for haem oxygenase activity (nmol of bilirubin formed/h per mg of protein) (●) were 1.54 ± 0.14 , 10.14 ± 1.70 and 1.62 ± 0.10 respectively. (a) Entire time period; (b) the time period ± 60 min from the time of Ni^{2+} administration (0 min, O).

(Fig. 9). However, Zn^{2+} , like Mn^{2+} , when administered 60 min after Ni^{2+} (+60), was unable to prevent the decreases in cytochrome *P*-450 and haem contents and the rebound increase in δ -aminolaevulinate synthase activity produced by the latter metal ions (Fig. 9).

Effects of Mn^{2+} and cycloheximide on the decay of induced haem oxygenase in kidney

In order to determine whether Mn^{2+} affected the

Fig. 9. Effect of Zn^{2+} on the Ni^{2+} induction of haem oxygenase and the concomitant changes in δ -aminolaevulinate synthase activity, cytochrome *P*-450 content and microsomal haem content in kidney

Rats were treated as described in the legend to Fig. 8. Three groups of rats were administered 0.9% NaCl, Ni^{2+} or Zn^{2+} . The values for haem oxygenase activity (nmol of bilirubin formed/h per mg of protein) (●) were 1.54 ± 0.14 , 10.14 ± 1.71 and 1.62 ± 0.10 respectively, for δ -aminolaevulinate synthase activity (nmol/h per mg of protein) (□) 0.137 ± 0.014 , 0.240 ± 0.034 and 0.146 ± 0.03 respectively, for cytochrome *P*-450 content (nmol/mg of protein) (△) 0.072 ± 0.006 , 0.041 ± 0.002 and 0.059 ± 0.003 respectively, and for microsomal haem content (nmol/mg of protein) (○) 0.21 ± 0.05 , 0.17 ± 0.02 and 0.19 ± 0.03 respectively.

rate of decay of haem oxygenase activity after the enzyme had been maximally induced, an equimolar amount of Mn^{2+} ($250 \mu\text{mol/kg}$) was administered to animals 16 h after Sn^{2+} and the decay of activity of microsomal haem oxygenase was compared over the subsequent 8 h time period in Mn^{2+} -treated animals with that in untreated animals. There was no difference in the rate of decrease of haem oxygenase activity in animals treated with Mn^{2+} , as compared with those not treated, indicating that Mn^{2+} administration did not influence the catabolism of the induced enzyme (Fig. 10). Similar results were noted for Zn^{2+} (results not shown). In another group of animals, cycloheximide was administered 16 h after Sn^{2+} induction of haem oxygenase, and the decline in enzyme activity was found to be similar to that observed for the maximally induced enzyme in the presence and in the absence of Mn^{2+} (Fig. 10). From these findings the half-life of pretreated renal haem oxygenase appeared not to be affected by Mn^{2+} , and thus the blocking effect of Mn^{2+} , and presumably of Zn^{2+} as well, is thought to reflect an action at some cellular level involving the synthesis of the enzyme.

Fig. 10. Effects of Mn^{2+} and cycloheximide on the rate of decay of maximally induced haem oxygenase

Rats were injected subcutaneously with Sn^{2+} ($250 \mu\text{mol/kg}$) and starved. At 16 h after Sn^{2+} administration the rats were divided into three groups, three rats per time point, and one group was injected subcutaneously with Mn^{2+} ($250 \mu\text{mol/kg}$) (△), one group with 0.9% NaCl (○) and one group intraperitoneally with cycloheximide (18 mg/kg) (□) and then killed at 2 h intervals.

Effects of Zn^{2+} and Mn^{2+} on the induction of haem oxygenase by Cd^{2+} , Co^{2+} and Ni^{2+} in liver

In view of the demonstrated metal ion interactions found with respect to haem oxygenase induction in kidney, several studies involving an examination of the effects of Mn^{2+} or Zn^{2+} on the induction of hepatic haem oxygenase by Co^{2+} , Cd^{2+} and Ni^{2+} were also performed; the results of these studies are shown in Figs. 11–13 and Table 1. Co^{2+} , Cd^{2+} and Ni^{2+} , as we have previously demonstrated, are potent inducers of hepatic haem oxygenase (Maines & Kappas, 1975, 1976a, 1977a). Zn^{2+} at very high concentrations (1 mmol/kg) also induces haem oxygenase slightly (Maines & Kappas, 1976a); however, at 250 μ mol/kg Zn^{2+} does not significantly increase haem oxygenase activity in liver (Table 1).

The simultaneous administration of equimolar amounts of Mn^{2+} had no effect on the induction response of hepatic haem oxygenase to either Co^{2+} or Cd^{2+} (Table 1). However, Mn^{2+} did significantly diminish, by approx. 40%, the induction of hepatic haem oxygenase by Ni^{2+} (Table 1). Zn^{2+} , when administered simultaneously with Co^{2+} , Cd^{2+} or Ni^{2+} , was able to block the induction of haem oxygenase by these metal ions by 50, 80 and 60% respectively by 16 h (Table 1).

Time-dependent responses of Zn^{2+} inhibition of Cd^{2+} induction of haem oxygenase and perturbations of haem metabolism in liver

When Zn^{2+} was administered simultaneously with (zero time, O, in Fig. 11a) or before (–60 min) Cd^{2+} , the induction of hepatic haem oxygenase was blocked by approx. 75% (Fig. 11a); interestingly, the extent of blockade of enzyme induction became greater with increasing time of Zn^{2+} administration before Cd^{2+} (–180 min to –480 min; Fig. 11a). This pattern of blockade from –180 min to –480 min was different from that observed in kidney with Zn^{2+} blockade of Sn^{2+} and Ni^{2+} induction of haem

oxygenase (Figs. 7a and 8a). Zn^{2+} , when given 10 min after Cd^{2+} , was completely ineffectual in blocking the induction of haem oxygenase (Fig. 11b). δ -Aminolaevulinic synthase activity did not change significantly when measured 16 h after Cd^{2+} administration. Cd^{2+} treatment alone resulted in a modest (30%) decline in cytochrome P-450 content that was diminished by prior Zn^{2+} administration (Fig. 12). Zn^{2+} pretreatment protected against the decrease in microsomal haem content produced by Cd^{2+} , and also the decrease in ethylmorphine demethylase activity that occurred concomitant with

Fig. 11. Time course of the effect of Zn^{2+} on the induction of haem oxygenase by Cd^{2+} in liver

Rats were injected subcutaneously with Cd^{2+} (25 μ mol/kg), starved for 16 h and killed. Zn^{2+} (250 μ mol/kg) was injected subcutaneously at the designated times, a minimum of five rats per time point, ranging from 8 h before to 1 h after Cd^{2+} treatment (0 min, O). Three groups of rats were treated with 0.9% NaCl, Cd^{2+} or Zn^{2+} , and the values for haem oxygenase activity (nmol of bilirubin formed/h per mg of protein) (●) were 1.74 ± 0.10 , 10.59 ± 0.52 and 2.58 ± 0.33 respectively. (a) Entire time period; (b) the period ± 60 min from the time of Cd^{2+} administration.

Table 1. Effects of Mn^{2+} and Zn^{2+} on the induction of hepatic haem oxygenase by Co^{2+} , Cd^{2+} and Ni^{2+}
Rats (four to six for each metal ion) were injected subcutaneously with Co^{2+} , Ni^{2+} , Mn^{2+} , Zn^{2+} (250 μ mol/kg) or Cd^{2+} (2.5 μ mol/kg), starved for 16 h and killed. Hepatic microsomal fractions were prepared and haem oxygenase activity was assayed as described in the Materials and methods section.

Inducing metal ion	Blocking metal ion ...	Haem oxygenase activity (nmol of bilirubin formed/h per mg of protein)		
		Saline control	Zn^{2+}	Mn^{2+}
0.9% NaCl		1.77 ± 0.11	2.58 ± 0.33	5.87 ± 0.60
Co^{2+}		14.87 ± 0.96	$8.37^* \pm 0.82$	13.80 ± 2.10
Cd^{2+}		11.32 ± 0.61	$3.89^* \pm 0.42$	13.56 ± 1.15
Ni^{2+}		11.97 ± 1.54	$5.91^* \pm 0.68$	$7.94^* \pm 1.09$

* $P < 0.05$.

the induction of haem oxygenase by Cd^{2+} (Fig. 12). Cd^{2+} *in vitro* at concentrations as high as 0.25 mM did not decrease the cytochrome *P*-450 content or

Fig. 12. Effect of Zn^{2+} on the Cd^{2+} induction of haem oxygenase and the concomitant changes in δ -aminolaevulinate synthase activity, ethylmorphine *N*-demethylase activity, cytochrome *P*-450 content and microsomal haem content in liver

Rats were treated as described in the legend to Fig. 11; Cd^{2+} administration was regarded as zero time. The values for 0.9%-NaCl-, Cd^{2+} - or Zn^{2+} -treated animals for haem oxygenase activity (nmol of bilirubin formed/h per mg of protein) (●) were 1.74 ± 0.10 , 10.59 ± 0.52 and 2.58 ± 0.33 respectively, for δ -aminolaevulinate synthase activity (nmol/h per mg of protein) (□) 0.261 ± 0.01 , 0.274 ± 0.03 and 0.211 ± 0.04 respectively, for cytochrome *P*-450 content (nmol/mg of protein) (Δ) 0.75 ± 0.04 , 0.52 ± 0.03 and 0.66 ± 0.06 respectively, for microsomal haem content (nmol/mg of protein) (○) 1.25 ± 0.07 , 0.95 ± 0.06 and 1.29 ± 0.12 respectively, and for ethylmorphine *N*-demethylase activity (nmol/h per mg of protein) (▲) 0.582 ± 0.09 , 0.387 ± 0.02 and 0.567 ± 0.03 respectively.

inhibit ethylmorphine demethylase activity (results not shown).

*Effects of simultaneous administration of Co^{2+} and Zn^{2+} on haem oxygenase activity, δ -aminolaevulinate synthase activity and cytochrome *P*-450 content in liver*

Co^{2+} -mediated induction of haem oxygenase followed the previously described time course (Maines & Kappas, 1975), reaching a maximum at 16 h before beginning to decline (Fig. 13). Increased haem oxygenase activity was noted as early as 1 h after Co^{2+} administration. Zn^{2+} , when administered simultaneously with Co^{2+} , diminished the extent of induction of haem oxygenase throughout the time course. This diminution was most evident at 5, 8 and 16 h after the combined metal ion administration when haem oxygenase activity was increasing towards a maximum at 16 h. With Co^{2+} treatment alone, δ -aminolaevulinate synthase activity exhibited the initial decline followed by the rebound induction that we have previously described (Maines & Kappas, 1975). Simultaneous Zn^{2+} administration with Co^{2+} diminished the extents of both the initial decrease and the rebound increase in δ -aminolaevulinate synthase activity (Fig. 13). Cytochrome *P*-450 content exhibited near-normal values for the first 5 h after Co^{2+} treatment, at a time when haem oxygenase activity was rising rapidly. Simultaneous Zn^{2+} administration with Co^{2+} maintained

Fig. 13. Effect of simultaneous Zn^{2+} administration on the changes in hepatic haem oxygenase activity, δ -aminolaevulinate synthase activity and cytochrome *P*-450 content associated with Co^{2+} administration in liver. Rats were injected subcutaneously with either Co^{2+} (250 $\mu\text{mol/kg}$) or Co^{2+} plus Zn^{2+} (250 $\mu\text{mol/kg}$), starved and killed at the designated times. The changes in haem oxygenase activity (nmol of bilirubin formed/h per mg of protein) (□, Co^{2+} -treated; ▤, Co^{2+} -plus- Zn^{2+} -treated), δ -aminolaevulinate synthase activity (nmol/h per mg of protein) (○, Co^{2+} -treated; ●, Co^{2+} -plus- Zn^{2+} -treated) and cytochrome *P*-450 content (nmol/mg of protein) (Δ, Co^{2+} -treated; ▲, Co^{2+} -plus- Zn^{2+} -treated) were followed.

the cytochrome *P*-450 content at normal values up to 16 h before the microsomal content of this haemoprotein finally declined to the value observed with Co^{2+} treatment alone at 24 h. Mn^{2+} did not inhibit the induction of haem oxygenase by Co^{2+} , nor were the initial inhibition and rebound changes in δ -aminolaevulinate synthase activity or the decline in cytochrome *P*-450 content associated with Co^{2+} administration altered by Mn^{2+} in the liver (results not shown).

Discussion

The present study demonstrates that Mn^{2+} and Zn^{2+} have a potent ability to block the induction of haem oxygenase, which is mediated by other metal ions in kidney and liver. When administered in equimolar amounts, Mn^{2+} and Zn^{2+} entirely blocked the induction of the enzyme produced by Sn^{2+} and 80% of that produced by Ni^{2+} in kidney; Zn^{2+} , in addition, was able to block substantially the induction of the enzyme produced in liver by Co^{2+} , Cd^{2+} and Ni^{2+} . Metal ions that induce haem oxygenase exhibit a degree of tissue-specificity in their action, Co^{2+} being a potent inducer in liver, Sn^{2+} in kidney and Hg^{2+} in heart (Maines & Kappas, 1977c). Similarly Zn^{2+} and Mn^{2+} exhibit some tissue-specificity as well as metal-ion-inducer-specificities in blocking haem oxygenase synthesis, and these may reflect differential tissue-distribution patterns of inducer and blocking metal ions, differences in binding affinities or cellular concentrations of chemical substituents which complex these ions, etc.

The blocking effects of Mn^{2+} and Zn^{2+} on haem oxygenase induction were shown to be both dose-related (Fig. 2) and time-dependent (Figs. 4, 6, 7, 8 and 11); with respect to the latter, blockade was greatest when these blocking ions were administered simultaneously with inducer metal ions, but decreased with time when Mn^{2+} and Zn^{2+} were administered before the inducers. These findings make it clear that Mn^{2+} and Zn^{2+} have the capacity to remain localized *in situ*, or to initiate cellular responses, in such a way as to inhibit for long periods of time the ability of certain metal ions to induce haem oxygenase. Also, once the enzyme-induction response to specific metal ions is triggered, the ability of Mn^{2+} or Zn^{2+} to block the response is quickly lost (Figs. 4, 6, 7, 8 and 11). The rapid and substantial loss of this blocking effect (within 5–10 min in the case of the Mn^{2+} – Sn^{2+} interaction, for example) indicates that metal-ion inducers of haem oxygenase must very quickly (i.e. within minutes; Fig. 4b) reach the regulatory site(s) for enzyme induction and initiate the complex cellular responses that lead to the marked increases (e.g. 20-fold; Fig. 1) in enzyme synthesis that they evoke.

The time-dependent blockade of Cd^{2+} induction of haem oxygenase in liver by Zn^{2+} is of specific interest in that the time course of blockade of enzyme induction (Fig. 11a) was different from that observed in kidney with Mn^{2+} and Zn^{2+} (Figs. 4a, 6a, 7a and 8a); namely, with increasing time of administration of Zn^{2+} before Cd^{2+} , blockade of enzyme induction by the latter metal ion increased, and in fact became complete. Zn^{2+} in excess amounts is known to induce metallothionein formation in the liver of male rats (Webb, 1972; Cherian & Goyer, 1978), and the subsequent injection of Cd^{2+} results in the accumulation of the metal ion in this protein (Webb, 1972). Zn^{2+} when administered 180 min or more before Cd^{2+} (Fig. 11), at a time when metallothionein formation is known to be increasing (Squibb *et al.*, 1977), may therefore block the induction of haem oxygenase through metallothionein binding of Cd^{2+} . In the Zn^{2+} – Cd^{2+} interaction two adaptive responses may possibly occur: with simultaneous administration of Zn^{2+} and Cd^{2+} an ion–ion interaction, perhaps at the regulatory site(s) for synthesis of the enzyme, may underlie the mechanism of blockade; and with administration of Zn^{2+} 180–480 min before Cd^{2+} metallothionein formation may confer an additional blocking effect against the inducing action of Cd^{2+} .

Thus metallothionein formation, as well as metal ion interactions and metal-ion-complexing agents such as cysteine and reduced glutathione (Maines & Kappas, 1977a), may influence the extent of haem oxygenase induction and the associated perturbations of haem metabolism that can be elicited by certain metal ions. The demonstration by Kotsonis & Klaasen (1979) that alkylating agents such as bromobenzene and diethylmaleate, which alter cellular thiol status, can also increase the synthesis of metallothionein in liver is significant in this respect. Their suggestion that such agents could change the distribution of intracellular metal ions and thereby enhance their ability to increase protein (i.e. metallothionein) synthesis may have relevance to the mechanisms of other metal-ion-regulated (i.e. haem oxygenase) protein-synthetic processes.

We have previously shown that concomitant with the metal-ion-mediated induction of haem oxygenase there occurs a decrease in microsomal haem and cytochrome *P*-450 concentrations and a diminished catalytic activity of the cytochrome *P*-450-dependent mixed-function oxidase system (Maines & Kappas, 1975). Mn^{2+} and Zn^{2+} , while blocking the haem oxygenase induction produced by inducer metal ions, also blocked the decreases that occur in microsomal haem and cytochrome *P*-450 contents (Figs. 3, 5, 9, 12 and 13), thus permitting maintenance of normal activity of chemical oxidations mediated by the cytochrome *P*-450 system (Fig. 12). The sequential changes in δ -amino-

laevulinate synthase that may occur in association with metal-ion induction of haem oxygenase were also diminished or eliminated by the concurrent administration of Mn^{2+} and Zn^{2+} (Figs. 3, 5, 9, 12 and 13). By blocking the induction of haem oxygenase, therefore, Mn^{2+} and Zn^{2+} , which do not inhibit this enzyme activity *in vitro*, also substantially diminished or prevented the perturbations in haem metabolism that are usually associated with the acute increases of this enzyme produced by certain metal ions. Thus evidence derived from experiments in which metal ions induce, as well as block, the induction of haem oxygenase supports the view that the alterations in haem metabolism that occur concomitantly with haem oxygenase induction are closely inter-related with, if not dependent on, the enzyme induction process.

The possible mechanisms by which foreign chemicals as well as natural substances, such as haem compounds and certain trace metal ions, induce haem oxygenase have been summarized by De Matteis (1978). We have postulated that metal ions may act directly in the enzyme-induction process and that the associated decreases in cellular contents of haem and cytochrome *P*-450 largely reflect the increase in this enzyme activity (see review by Maines & Kappas, 1977c). Evidence supporting this hypothesis derives in part from the fact that, in the whole animal, metal-ion-induced loss of radioactivity from prelabelled haem is observed after, not before, metal-ion induction of haem oxygenase activity (De Matteis & Unseld, 1976; Maines & Kappas, 1976b).

Certain metal-ion inducers of haem oxygenase, such as Fe^{2+} and Co^{2+} , can enzymically form metalloporphyrin complexes that might then be considered to initiate the induction process. Iron-porphyrin compounds (Tenhunen *et al.*, 1970) as well as cobalt-porphyrin (Maines & Kappas, 1975) have, for example, been shown to induce the enzyme.

Co^{2+} , which has been particularly studied in this respect, is known to be a substrate for haem chelatase (Labbe & Hubbard, 1961), is able to form cobalt-porphyrin in isolated mitochondrial preparations (Jones & Jones, 1969) and cobalt-porphyrin in rat liver *in vivo* (Sinclair *et al.*, 1979). The demonstrated formation of trace amounts of cobalt-porphyrin *in vivo* after $CoCl_2$ treatment, and the reported direct relationship of this process to the inhibition of iron-porphyrin (haem) synthesis in liver, prompted the suggestion that Co^{2+} incorporation into cobalt-porphyrin was responsible for the inhibitory action of the metal ion on δ -aminolaevulinate synthase (Sinclair *et al.*, 1979). However, the Co^{2+} -associated impairment in haem synthesis reported in that paper does not conform with the results of earlier work (Guzelian & Bissell,

1976), in which no direct effect of Co^{2+} on haem synthesis in cultured rat hepatocytes could be detected. Moreover, if $CoCl_2$ administration to animals leads to the hepatic formation of biologically significant amounts of cobalt-porphyrin *in vivo*, then a profound and sustained repression of δ -aminolaevulinate synthase would be expected to occur, since cobalt-porphyrin is not utilizable for haemoprotein synthesis, nor is it apparently metabolically degraded (Yoshida & Kikuchi, 1978; Sassa *et al.*, 1979b). $CoCl_2$ administration, however, is well known (Maines & Kappas, 1975; De Matteis & Gibbs, 1976) to produce only a transient (approx. 1–3 h) decrease of hepatic δ -aminolaevulinate synthase activity, this then being followed by a substantial rebound induction of the enzyme (Maines & Kappas, 1975; De Matteis & Gibbs, 1976). The latter rebound induction of δ -aminolaevulinate synthase does not occur when Co^{2+} is administered as cobalt-porphyrin even at a dose less than 1% that of $CoCl_2$ (Drummond & Kappas, 1980), despite the fact that cobalt-porphyrin produces a marked initial repression of the enzyme activity. Thus, although the formation of cobalt-porphyrin *in vivo* can occur, the functional significance of this process, in terms of regulatory effects on haem oxygenase induction and the associated alterations in δ -aminolaevulinate synthase activity and cytochrome *P*-450 content, cannot be assessed until its quantitative aspects have been defined.

Moreover, in considering a putative regulatory role for haem oxygenase induction of metalloporphyrin complexes which may be derived from administered inorganic metal ions, it should be noted that induction of this enzyme, with concomitant perturbations of δ -aminolaevulinate synthase activity and cytochrome *P*-450 content, can also be mediated by a variety of metal ions that, unlike Fe^{2+} or Co^{2+} , are known not to be enzymically incorporated *in vivo* into haem compounds (Maines & Kappas, 1977a,b,c). This consideration supports the idea that metal ions can regulate haem metabolism directly, rather than acting only through the intermediate formation of metalloporphyrin complexes *in vivo*.

It is possible that metal ions might induce haem oxygenase by labilizing the haem moiety from cytochrome *P*-450, with the released haem then initiating the enzyme-induction process (Bissell & Hammaker, 1976). However, with respect to this mechanism it should be emphasized that high concentrations (approx. 0.25 mM) of potent inducers of haem oxygenase, such as Cd^{2+} , Ni^{2+} , Sn^{2+} and Co^{2+} , do not degrade cytochrome *P*-450 in microsomal preparations or impair cytochrome *P*-450-dependent chemical oxidations *in vitro*; the latter may even be enhanced somewhat (Maines &

Kappas, 1975). It is also implicit from radiotracer studies in bile-fistula animals that cytochrome *P*-450 haem *in vivo* is resistant to direct degradation by metal ions, since accelerated metabolism of this haem moiety only becomes evident after and not before metal ions have initiated the induction of haem oxygenase (De Matteis & Unseld, 1976; Maines & Kappas, 1976b).

Finally, the simultaneous administration of Mn^{2+} or Zn^{2+} with inducing metal ions completely blocks haem oxygenase induction, as we have shown in these studies, whereas this blocking effect is largely lost if Mn^{2+} or Zn^{2+} treatment is delayed by more than 10 min after administration of the inducer ion. No experimental evidence exists to date that indicates that subcutaneously injected inducer metal ions, such as Sn^{2+} , Co^{2+} , Ni^{2+} or Cd^{2+} , could within the time described in these observations (i.e. several minutes) release the haem moiety from cytochrome *P*-450 *in vivo* rapidly enough and in amounts sufficient to induce hepatic and renal haem oxygenase to the very high activities characteristic of treatment with these ions. It is evident that it is important to clarify the metabolic events that occur within the first few minutes after administration of metal ion *in vivo*, since these very early events, as we show here, are crucial to the induction process for haem oxygenase that is initiated by inducer metal ions, and to the blockade of this process that can be produced by Mn^{2+} and Zn^{2+} , and perhaps by other metal ions as well.

This study was supported by U.S. Public Health Service grant ES-01055 and by a grant from the Scaife Foundation. We are indebted to Miss Susan Spinner for technical assistance and Mrs. Heidemarie Robinson for preparation of the manuscript.

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