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The Effect of Zinc on NADPH Oxidation and Monooxygenase Activity in Rat Hepatic Microsomes

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SUMMARY

This study confirms that zinc is able to inhibit hepatic microsomal drug metabolism and the related oxidation of NADPH. Zinc activates microsomal pyrophosphatase, a zinc-containing enzyme capable of metabolizing both NADPH and NADH. Although this reaction produces a potent inhibitor of drug metabolism, 2′,5′-ADP′, the activation of pyrophosphatase by zinc was found not to be solely responsible for the zinc-dependent inhibition of drug metabolism. Zinc was seen to inhibit drug metabolism in the presence of 5′-AMP, which inhibits pyrophosphatase. Incubation of zinc with microsomes prior to the addition of NADPH caused an interaction between zinc and the microsomal enzymes that was not reversed by NADPH. Zinc was found to exhibit noncompetitive inhibition of cytochrome *c* reduction and mixed inhibition of drug metabolism, with respect to NADPH. Zinc inhibition of drug metabolism was noncompetitive with drug substrate. Zinc was found to interact with cytochrome P-450, decreasing its ability to bind to both drugs and carbon monoxide. Zinc had a far greater effect on the reduction of cytochrome P-450 (90% inhibited) than on the reduction of exogenous cytochrome *c* (20% inhibited), although the same reductase is responsible for both reactions. It is concluded that zinc inhibits drug metabolism either by alteration of the oxidation-reduction potential of the flavoprotein or by interacting with a flavoprotein/cytochrome P-450 complex.

INTRODUCTION

The mixed-function oxidase system of rat hepatic microsomes is composed of a family of hemoproteins, cytochrome P-450 isozymes, and a single flavoprotein, NADPH-cytochrome P-450 reductase, embedded in the endoplasmic reticulum. During oxidative metabolism of drugs, oxygen and NADPH are consumed; water and $NADP^+$ are produced. In addition to undergoing oxidation, NADPH is hydrolyzed in microsomes by an NPPase[1] to 2′,5′-ADP and nicotinamide mononucleotide (1).

The mixed-function oxidase system is highly sensitive to zinc ions, exhibiting an inhibition of the rate of drug oxidation and a decrease in the rate of oxidation of NADPH in the presence of micromolar quantities of zinc (2). On the other hand, NPPase is stimulated by zinc, reaching a maximal velocity in the presence of 50 μM zinc (3, 4).

Chvapil and co-workers (5) have shown that zinc combines with NADPH to form both 1:1 and 2:1 zinc:NADPH complexes. They suggest that the inhibition of drug metabolism by zinc might be due to this complex formation. These authors carried out experiments consistently showing a 50% inhibition of drug metabolism in the presence of 70 μM zinc (2, 6). However, these incubations contained 330 μM NADPH, more than a 4-fold excess of NADPH over zinc. Thus, even if all of the zinc were bound to NADPH, there would still be uncomplexed NADPH far in excess of the 1 μM K_m estimated for the metabolism of drugs (7).

We therefore decided to repeat and extend the work of Chvapil and co-workers to determine whether zinc:NADPH complex formation, NPPase hydrolysis of NADPH, or an alternative mechanism was responsible for the zinc-dependent inhibition of NADPH oxidation and drug metabolism.

MATERIALS AND METHODS

Adult male Simonsen rats weighing between 190 and 230 g were given food and water ad libitum until the time of death. Animals were killed by cervical dislocation and the livers were perfused *in situ* with ice-cold isotonic KCl. The livers were homogenized in a dounce homogenizer and centrifuged at 9,000 × *g* for 20 min. The supernatant was recentrifuged at 104,000 × *g* for 60 min. The microsomal pellet was resuspended in cold isotonic KCl. This preparation was again centrifuged at 104,000 × *g* for 30 min, resuspended in fresh KCl, and used within 8 hr. Ethylmorphine *N*-demethylase and NADPH oxidation were measured as previously described (3) except

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[1] The abbreviation used is: NPPase, nucleotide pyrophosphatase.

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that 0.06 M Tris-HCl buffer (pH 7.4 at 37°) replaced phosphate buffer because, in the presence of phosphate buffer, insoluble zinc phosphate formed and precipitated from solution. Aniline hydroxylase activity was measured by the method of Imai *et al.* (8). Total NADPH was measured by the method of Estabrook and Maitre (9) except that a spectrophotometer, rather than a fluorimeter, was used. NADPH- and NADH-cytochrome *c* reductase activity were measured by the method of Masters *et al.* (10) as modified by Jeffery *et al.* (11). NADPH-cytochrome P-450 reductase and cytochrome content were measured by the methods of Gigon *et al.* (12) and Omura and Sato (13), respectively. Linoleic acid hydroperoxide was prepared and used as previously described (11). Samples for zinc determination were lyophilized to dryness, ground to a fine powder, and digested in 1% nitric acid (14). The digest was centrifuged at 1,000 × *g* for 10 min, and the clear supernatant was used to determine zinc content by atomic absorption, using $ZnCl_2$ in nitric acid as standard. Protein was estimated by the method of Lowry *et al.* (15). Although zinc chloride was used in the studies presented, similar results were obtained using zinc acetate (data not shown).

RESULTS

Inhibitory effect of zinc on drug metabolism. When zinc chloride was incubated with microsomes for 2 min prior to the addition of an NADPH-generating system, ethylmorphine *N*-demethylase activity was found to be inhibited by 80% (Table 1). Similarly, the oxidation of NADPH was inhibited (Fig. 1B). Aniline hydroxylation and endogenous cytochrome P-450-dependent hydrogen peroxide production were also inhibited (data not shown).

Zinc complex formation. Chvapil and co-workers (5) have shown that, when zinc is mixed with NADPH, a complex forms. They have suggested that the depressant effect of zinc on rates of microsomal NADPH oxidation and drug metabolism is due to a sequestering of NADPH by zinc:NADPH complex formation. To test their theory, we incubated zinc alone or complexed to NADPH (200 μM zinc, 100 μM NADPH) with microsomes (1 mg/ml) for 2 min at 4° or 37°. The microsomes were then separated from the incubation medium by centrifugation (30 min at 104,000 × *g*) at 4°, and both fractions were assayed for NADPH and zinc. NADPH was found only in the supernatant fraction in all samples. The distribution of zinc varied with the treatment (Table 2). At 4°, whether added alone or as a complex, 50 nmoles of zinc were found associated with each milligram of microsomes [and thus not associated with NADPH (Table 2)]. Upon warming to 37°, the microsomal zinc content increased to 70 nmoles when added as the complex and 110 nmoles when added alone. NADPH added 2 min prior to zinc addition protected against zinc associated with the microsomes (70 nmoles/mg bound), whereas the addition of NADPH 2 min after the zinc was unable to reverse the zinc association with the microsomes (110 nmoles/mg). Doubling the NADPH concentration, and thus the proportion of NADPH to zinc, had no further effect on zinc distribution. These results suggest that zinc interacts with the microsome in a fashion that can be partially

TABLE 1

Effect of zinc on ethylmorphine N-demethylase

The incubation medium contained microsomes (1 mg/ml), Tris-HCl buffer (pH 7.4, 0.06 M), 5'-AMP (1 mM), NADPH (400 μM), glucose-6-phosphate dehydrogenase (2 units), glucose-6-phosphate (4 mM), and ethylmorphine (1 mM). The results are the mean ± standard error of four separate experiments, and were linear over 15 min. The sample containing zinc alone was found to be significantly different from all other treatments ($p < 0.01$).

Addition	HCHO formed
	nmoles/min/mg
None	6.0 ± 0.6
EDTA (0.2 mM)	5.4 ± 0.2
Zinc (17 μM)	1.2 ± 0.2
Zinc (17 μM) EDTA (0.2 mM)	5.6 ± 0.1

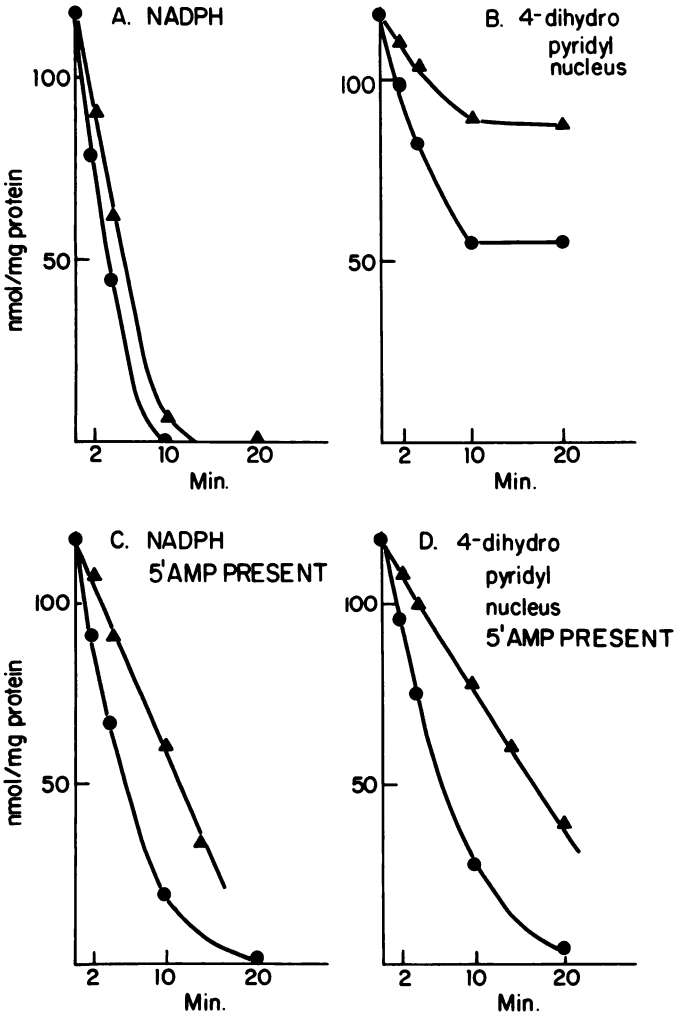

FIG. 1. *Effect of zinc on NADPH metabolism*

Microsomes (1 mg/ml in 0.06 M Tris-HCl buffer, pH 7.4 at 37°) were incubated with 119 μM NADPH. The disappearance of total NADPH was monitored by time point enzymatic estimation of NADPH (*9*, A). The oxidation of NADPH was monitored by the continual disappearance of absorbance at 340 nm (*1*, B). Incubations were carried out in the absence (●) and presence (▲) of 17 μM zinc sulfate. These measurements were repeated in the presence of 1 mM 5'-AMP (C and D) to inhibit NPPase activity. Results are the mean of three separate experiments; standard deviations were less than 10% of zero time.

TABLE 2

Zinc-NADPH complex formation

Zinc (20 mM) was mixed with NADPH (10 mM) and allowed to stand. Either this complex or zinc and NADPH separately were added to microsomal suspensions (with a 2-min incubation separating their addition) to a concentration of 200 μM zinc and 100 μM NADPH. The suspensions were then centrifuged at 104,000 × *g* for 30 min. The supernatant was poured off and assayed for NADPH (9) and zinc (10). Microsomes were resuspended in fresh medium, assayed for NADPH and zinc, and used to estimate cytochrome P-450 levels. NADPH was found only in the medium. Cytochrome levels were 60% of untreated levels, irrespective of treatment.

Addition	Temperature	Zinc concentration	
		Supernatant	Microsomes
		nmoles/ml	
Zinc, 200 μM	4°	150	50
Zinc, 200 μM	37	90	110
Complex (zinc, 200 μM; NADPH, 100 μM)	4	150	50
Complex (zinc, 200 μM; NADPH, 100 μM)	37	130	70
Zinc, 200 μM, 2 min; NADPH, 100 μM	37	90	110
Zinc, 200 μM, 2 min; NADPH, 200 μM	37	90	110
NADPH, 100 μM, 2 min; zinc, 200 μM	37	130	70

prevented by first allowing the zinc to complex with NADPH, limiting its availability to the microsome, but which cannot be reversed by NADPH. In all further studies, zinc was added to the microsomes 2 min prior to NADPH addition.

Effect of zinc on NADPH metabolism. Zinc has been reported to stimulate the hydrolysis of NADPH by microsomal NPPase (3). The presence of NPPase activity can be readily observed by comparing enzymatic estimations of NADPH concentration with non-enzymatic estimations of 4-dihydropyridyl nucleus concentration. Both estimations are based on the absorbance of the 4-dihydropyridyl nucleus at 340 nm. The non-enzymatic estimation uses the full absorbance of a sample of 340 nm, whereas the NADPH estimation uses only that absorbance that disappears after incubation with excess glutamic dehydrogenase, α-ketoglutarate, and ammonium chloride (9); enzymatic estimation avoids the interference of non-NADPH compounds containing the 4-dihydropyridyl nucleus. Since a product of NPPase hydrolysis of NADPH, reduced nicotinamide mononucleotide, contains this nucleus, this compound would be erroneously recognized as NADPH if the non-enzymatic estimation were used to estimate NADPH. The discrepancy between the two methods thus becomes a measure of reduced nicotinamide mononucleotide and therefore of NPPase activity. Experiments were carried out to show the effect of zinc on NPPase activity. Microsomes (1 mg/ml) were incubated with 119 μM NADPH at 37°. The disappearance of NADPH and of the 4-dihydropyridyl nucleus was monitored. More than 50% of the original absorbance at 340 nm (Fig. 1B) still remained when enzymatic estimations of NADPH had reached zero (Fig. 1A). This residual absorbance was increased in the presence of zinc, showing that zinc stimulates NPPase activity (Fig. 1B).

Because only one route of NADPH metabolism, NADPH oxidation, causes loss of the 4-dihydropyridyl nucleus and thus of extinction at 340 nm, the rate of disappearance of absorbance at 340 nm provides a true estimate of the rate of NADPH oxidation, even though it cannot be used to calculate NADPH concentration. The rate of loss of NADPH (Fig. 1A) provides a measure of total NADPH metabolism, the combination of NADPH oxidation and NPPase activity. We found very little effect of zinc on the rate of NADPH loss (Fig. 1A), although there was a significant zinc-dependent decrease in the rate of oxidation of NADPH (Fig. 1B). The lack of any great effect of zinc on the rate of total NADPH disappearance reflects the fact that the increased consumption of NADPH due to a zinc-dependent increase in NPPase activity is partly balanced by a decreased consumption of NADPH by oxidation. Decreased oxidase activity could be due to several mechanisms: (a) a direct effect of zinc on NADPH-cytochrome P-450 reductase, the enzyme responsible for NADPH oxidation; (b) limiting substrate concentration due to removal of NADPH by NPPase; and (c) inhibition of oxidation by a product of NPPase, 2′,5′-ADP, which exhibits a K_i of 10^{-5} M for NADPH oxidation (3). Both of the latter two possibilities are secondary to the zinc-dependent increase in NPPase activity. The experiment was therefore repeated in the presence of 5′-AMP, an inhibitor of NPPase, in an attempt to differentiate between these possibilities. NPPase activity was abolished, as shown by the correlation between NADPH and 4-dihydropyridyl nucleus estimations (Fig. 1C and D) and by the lack of residual absorbance when no NADPH remained (Fig. 1D). Zinc was found to inhibit the rate of NADPH oxidation (Fig. 1D). It was therefore concluded that zinc affects oxidation directly rather than via its effect on NPPase. Subsequent studies were carried out in the presence of 5′-AMP (1 mM), which abolished NPPase activity while having no effect on oxidation, to avoid any interference from NPPase.

Effect of zinc on the reduction of cytochrome c by NADPH cytochrome P-450 reductase. During the mixed-function oxidation of drugs, the activity of the flavoprotein NADPH-cytochrome P-450 reductase is coupled to that of the cytochrome. Thus inhibition of either component would inhibit NADPH oxidation and drug metabolism. In order to test the possibility that zinc has its inhibitory effect by acting on the flavoprotein, we studied the effect of zinc on the ability of the flavoprotein to reduce an exogenous electron acceptor, cytochrome *c*. Zinc (17 μM) caused a slight but significant noncompetitive inhibition of reduction (Table 3) that was decreased, but not reversed, by 5′-AMP. Because it was possible that zinc was interacting with cytochrome *c* to interfere with the assay, the reduction of cytochrome *c* by NADH was also studied. This reaction involves similar techniques and identical cytochrome *c* concentrations, but different electron transfer components: electrons pass

TABLE 3

Effect of zinc on cytochrome C reduction

Microsomes (0.1 mg/ml) and cytochrome *c* (50 μM in 0.1 M Tris-HCl buffer, pH 7.4) were warmed to 37° in an Aminco DW2 spectrophotometer. The increase in absorbance at 550 nm was recorded after the addition of NADPH (1 μM–100 μM final concentration) or NADH (100 nM–100 μM final concentration) to the sample cuvette. Michaelis constants were estimated by the method of Wilkinson (16), using a Fortran program written by Cleland (17). Zinc, when present, was added to both cuvettes 2 min prior to addition of reductant.

Addition	Michaelis constant	Maximal velocity
	μM	*μmoles/min/mg*
NADPH	70	0.30 ± 0.02
NADPH + Zinc (17 μM)	69	0.13 ± 0.02
NADPH + 5′-AMP (1 mM)	70	0.27 ± 0.02
NADPH + 5′-AMP (1 mM) + zinc (17 μM)	70	0.19 ± 0.01
NADH	6.9	1.45 ± 0.04
NADH + zinc (17 μM)	7.4	1.43 ± 0.07

from NADH, via a specific flavoprotein (NADH-cytochrome b_5 reductase), to cytochrome b_5 and then to cytochrome *c*. No effect of zinc was observed on this system, confirming that the effect of zinc on NADPH-cytochrome P-450 reductase was specific (Table 3).

Because we were able to show a specific, noncompetitive inhibition of NADPH-cytochrome P-450 reductase activity by zinc using cytochrome *c* as terminal electron acceptor, a kinetic study of the effect of zinc on drug metabolism was carried out to determine whether this would confirm that the flavoprotein was the site of inhibition of zinc. When NADPH was varied, zinc was found to inhibit drug metabolism in a mixed fashion with a strong competitive component (Table 4). When drug substrate (ethylmorphine) was varied rather than NADPH, the inhibition was found to be noncompetitive (Table 4). These results, rather than confirming the site of action as the flavoprotein, suggest that zinc may act at more than one site, affecting both the flavoprotein and the cytochrome, or that zinc may act at a site that is common to the flavoprotein and cytochrome P-450. We therefore turned to a study of the reduction of cytochrome P-450 to test the possibility that the interaction between the flavoprotein and cytochrome P-450 required for the reduction of cytochrome is altered by zinc.

TABLE 4

Effect of zinc on the kinetic parameters of ethylmorphine N-demethylase

Ethylmorphine *N*-demethylation was carried out as described previously (3), and zinc (when present) was added 2 min prior to reductant. Either ethylmorphine was held constant (2 mM) and NADPH was varied (10 nM–100 μM) or NADPH was held constant (100 μM) and ethylmorphine was varied (0.1 mM–2.0 mM). 5′-AMP and a glucose-6-phosphate/glucose-6-phosphate dehydrogenase NADPH-regenerating system were present in all incubates. Results were analyzed as described in Table 3.

Variable	Michaelis constant	Maximal velocity
	μM	*nmoles/min/mg*
NADPH	1	9.4 ± 0.4
NADPH, zinc (17 μM)	15	6.6 ± 1.5
Ethylmorphine	93	11.2 ± 0.6
Ethylmorphine, zinc (17 μM)	91	2.3 ± 0.2

Effect of zinc on the reduction of cytochrome P-450. The rate of reduction of cytochrome P-450 by NADPH, mediated by the flavoprotein, under totally anaerobic conditions is biphasic and made up of two reactions with widely differing rate constants; approximately equal amounts of cytochrome P-450 are involved in the two phases (12). Microsomal preparations treated with zinc revealed no change in rate constants, but there was a dramatic decrease in the amount of cytochrome P-450 involved in the fast reaction (Table 5). This inhibition, although evident at 17 μM zinc, was not evident at 10 μM zinc. The effect of zinc on the various spectral properties of cytochrome P-450 was studied, to ascertain whether zinc is able to bind directly to the cytochrome, to cause this inhibition. Zinc was found to have no effect on the spectral properties of cytochrome b_5 (Fig. 2). Zinc was found to inhibit the formation of the reduced cytochrome P-450-CO spectrum, with a maximal inhibition of 35% at a concentration of 17 μM zinc (Fig. 2). Zinc might act by binding to the oxidized cytochrome and preventing reduction, or by binding to reduced cytochrome to inhibit CO ligand formation. Since reduction of cytochrome P-450 is estimated by appearance of the reduced CO spectrum, the experiment described in Table 5 does not differentiate between these two possibilities. The effect of zinc on the oxidized and reduced spectra of cytochrome P-450 was therefore studied (Fig. 3). This was accomplished by difference spectrometry of microsomes, with the cytochrome P-450 in the reference cuvette partially destroyed by lipid peroxides (11). Oxidized cytochrome P-450 gave a spectrum with an absorbance maximum at 418 nm, and this spectrum was unaffected by the presence of zinc. Reduced cytochrome P-450 gives a slightly smaller absorbance maximum, at 414 nm. In the presence of zinc, an absorbance maximum appeared at 428 nm with a shoulder at 414 nm. As the concentra-

TABLE 5

Effect of zinc on NADPH-cytochrome P-450 reductase

The reduction of cytochrome P-450 was carried out and the methods analyzed essentially according to Gigon *et al.* (12). To ensure the absence of oxygen, the microsomes and reductant were evacuated and gassed with nitrogen three times prior to mixing; nitrogen and CO were passed over activated BASF copper catalysts at 200° before use. The NADPH concentration (87 μM) was maintained by a glucose-6-phosphate/glucose-6-phosphate dehydrogenase-regenerating system.

	K	Cytochrome P-450 involved	Rate
	sec^{-1}	*nmoles/ml*	*nmoles/min*
Untreated			
Fast	0.49 ± 0.01	0.4 ± 0.04	11.7 ± 2.0
Slow	0.083 ± 0.006	0.35 ± 0.03	1.5 ± 0.4
Zinc, 10 μM			
Fast	0.37 ± 0.02	0.51 ± 0.07	11.3 ± 1.5
Slow	0.071 ± 0.020	0.23 ± 0.05	1.0 ± 0.3
Zinc, 17 μM			
Fast	ND[a]	ND	ND
Slow	0.087 ± 0.013	0.21 ± 0.01	1.0 ± 0.3

[a] ND, Not detectable.

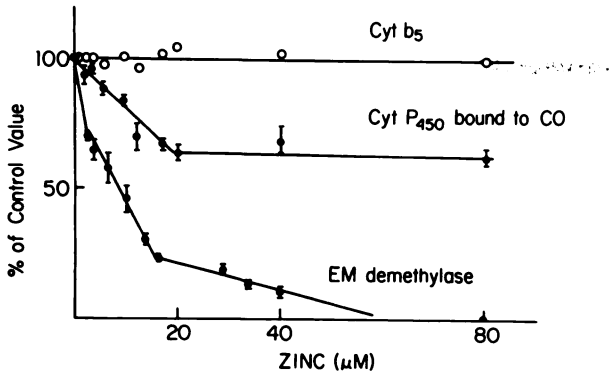

FIG. 2. *Effect of increasing zinc concentrations on the N-demethylation of ethylmorphine and the measurement of microsomal cytochromes*

Ethylmorphine (*EM*) *N*-demethylase was carried out as described in the legend to Table 1. Cytochromes b_5 and P-450 were measured by difference spectrometry of their reduced versus oxidized and reduced plus CO versus reduced spectra, respectively (13), with zinc sulfate added to both reference and sample cuvettes. The results are the mean ± standard error of three separate experiments.

tion of zinc was increased, absorbance at 428 nm increased, and absorbance at 414 nm decreased. Upon addition of CO, a peak appeared at 450 nm at the expense of absorbance at 414 nm; the absorbance at 428 nm was unaffected by the presence of CO.

Although the spectral properties of the oxidized cytochrome were unchanged by zinc, we studied the effect of zinc on interactions of drugs with the oxidized cytochrome P-450 and found that zinc also inhibited this interaction (Table 6). Surprisingly, shifts from low to high spin (Type 1 spectra) were as equally affected as shifts from high to low spin (Type II spectra). The percentage of cytochrome in each spin form prior to drug addition was not affected by zinc. Oxidized cytochrome P-450 in the absence of drugs consists of substrate-free cytochrome (low spin) and endogenous substrate-bound cytochrome (high spin) in equilibrium, portrayed by a

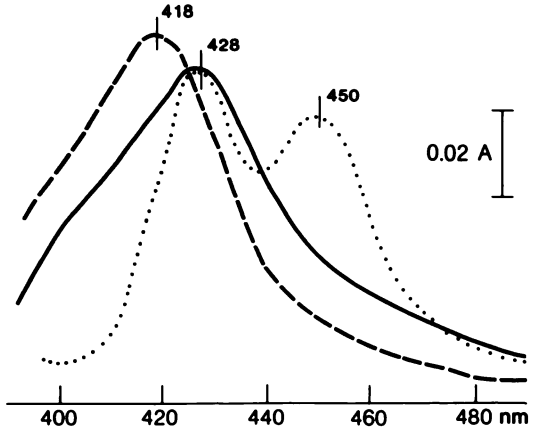

FIG. 3. *Effect of zinc on the absorption spectrum of cytochrome P-450*

Rat hepatic microsomes (1 mg/ml in Tris-HCl, buffer pH 7.4 at 37°) containing 17 μM zinc sulfate were divided between two cuvettes, and linoleic acid hydroperoxide (100 μM) was added to the reference cuvette only (– – –). Dithionite was added to both cuvettes (——); CO was then added to the sample cuvette only (·····).

spectrum with a large (418-nm) component (low-spin) and a small (393-nm) component (high-spin). The oxidized cytochrome exhibited no change in spectrum in the presence of zinc (Fig. 3), showing that zinc does not alter the balance between high- and low-spin forms of the cytochrome. Similarly, when the equilibrium between high- and low-spin forms of cytochrome P-450 was altered by the addition of drugs prior to the introduction of zinc, no effect of zinc on the new high- to low-spin equilibrium was found (data not shown). However, the addition of drug prior to zinc was not able to protect the cytochrome P-450 from zinc-dependent changes in the reduced and reduced/CO spectra. This suggests either that zinc acts at two separate sites or that the interaction of zinc with the cytochrome masks the drug binding site, although drugs are unable to mask the zinc-binding site. Since the same percentage of cytochrome is involved in both the effect of zinc on drug binding and the effect of zinc on CO binding, the latter suggestion seems the most plausible.

DISCUSSION

We have confirmed the findings of Chvapil and coworkers (2) that low levels of zinc inhibit microsomal NADPH oxidation and cytochrome P-450-dependent drug metabolism (Table 1; Fig. 2). These workers have shown that zinc forms a 2:1 complex with NADPH (5). They suggest that complexed NADPH may be unavailable for NADPH oxidation and that when zinc is present NADPH oxidation and drug metabolism are inhibited because of a lack of uncomplexed NADPH. We found that zinc binds to microsomes (Table 2) and that, whereas prior complexing of NADPH with zinc did partially prevent zinc binding to microsomes, NADPH was unable to reverse binding of zinc to microsomes (Table 2) or to prevent the detrimental effect of zinc on cytochrome P-450 estimation. When added to microsomes as the complex, a significant portion of the zinc was released from NADPH and bound to the microsome, leaving free NADPH well in excess of 5 μM, sufficient for maximal rates of drug metabolism. We conclude that zinc does not act by sequestering NADPH into an unavailable complex. These results do not preclude the possibility that

TABLE 6

Binding of drugs to cytochrome P-450

Microsomes (1 mg/ml in 0.1 M Tris-HCl buffer, pH 7.4) were placed in each of two cuvettes and warmed to 37°. Drug was added to the sample cuvette, and a spectrum was run between 350 nm and 450 nm. The experiment was repeated with zinc (17 μM) in both cuvettes. Carbon monoxide binding was measured by the method of Omura and Sato (13).

Drug	Δ Absorbance/mg of microsomal protein[a]		
	Control	+Zinc, 17 μM	%[b]
Aniline, 10 mM	0.029 ± 0.001	0.023 ± 0.001	79
Hexobarbital, 2.5 mM	0.021 ± 0.001	0.015 ± 0.003	71
Ethylmorphine, 2.5 mM	0.011 ± 0.001	0.008 ± 0.001	73
CO (reduced microsomes)	0.43 ± 0.03	0.31 ± 0.02	72

[a] Absorbance was measured from peak to trough.

[b] Binding in the presence of zinc as a percentage of binding in the absence of zinc.

zinc is bound to an enzyme complex containing NADPH at the site of inhibition.

The stimulation of NPPase activity by zinc was found not to be responsible for the inhibition of NADPH oxidation or drug metabolism, since addition of 5′-AMP, which effectively abolished NPPase activity, did not abolish the effect of zinc on drug metabolism or NADPH oxidation (Fig. 1).

Zinc was found to cause a small, specific, noncompetitive inhibition of the reduction of cytochrome *c* by the NADPH-cytochrome P-450 reductase. This alone could not explain the effect of zinc on drug metabolism, since kinetic studies revealed that the inhibition of drug metabolism was mixed with a strong competitive component with respect to reduction and noncompetitive with drug substrate (Table 4).

A study of the reduction of cytochrome P-450 in the presence of zinc revealed two findings. First, a significant portion of cytochrome P-450 no longer exhibited a 450-nm peak when treated with CO in a reducing environment (Figs. 2 and 3). Second, although there was no change in either rate constant for the biphasic reduction of cytochrome P-450, loss of cytochrome was involved almost exclusively with the fast reaction, leading to dramatic decreases in the over-all visible rate of reduction. The lack of a simple linear relationship between the concentration of zinc employed and the extent of inhibition (Table 5) is consistent with the type of inhibition of reduction being mixed with a strong competitive component, as seen for drug metabolism (Table 4). Because reduction is measured by the rate of formation of the reduced CO complex, these results could indicate inhibition either at the site of CO addition to cytochrome P-450 or at the site of reduction.

The decrease that we report in the reduced cytochrome P-450-CO complex levels after zinc treatment was not expected. Chvapil *et al.* (6) and Colby *et al.* (18) report no significant changes in cytochrome content on addition of zinc to microsomes. The former studies were carried out in phosphate buffer, and therefore the zinc might have precipitated out of solution. The latter studies employed guinea pigs rather than rats. Also, it was possible to carry out a cytochrome P-450 estimation very rapidly, without warming, and see no decrease in cytochrome P-450-CO complex formation in the presence of zinc. When zinc was added to the sample cuvette only, oxidized microsomes showed no absorbance change due to the zinc. Upon reduction in both cuvettes a peak was evident that did not shift when CO was introduced into the sample. We interpret these results to mean that zinc complexes with cytochrome P-450, inhibiting its interaction with CO. Since CO binds to cytochrome P-450 at the same site as oxygen, it is likely that the oxygenation of cytochrome P-450 is also altered in the presence of zinc. Possible mechanisms for the interaction of zinc with cytochrome P-450 might include liganding across carboxyl or sulfhydryl groups or displacement of the iron from the porphyrin center. However, protoporphyrin-Zn(II) has a soret maximum at 412 nm (19), thus replacement of iron seems an unlikely possibility.

The mechanisms leading to the observance of two rate constants for reduction of cytochrome P-450 have not yet been elucidated. Logic favors their dependence on the flavoprotein rather than on the cytochrome, since purified systems, which contain identical flavoprotein but an altered lipid matrix and an altered cytochrome population in comparison to microsomal preparations, exhibit the same pattern of reduction (20). However, the present results appear to support a dependence of these phases on the cytochrome population: some particular fraction of the cytochrome interacts with zinc (Fig. 2). This same particular fraction is normally rapidly reduced (Table 5). One possible explanation is that by binding to the reductase, zinc has changed the oxidation-reduction potential of the reductase, changing the percentage of cytochrome P-450 reduced at the steady state achieved with the NADPH concentrations used. This possibility is consistent with the finding that ethylmorphine *N*-demethylase, aniline hydroxylase, and cytochrome P-450-dependent H_2O_2 production were equally affected by zinc, a fact that points to inhibition at a common site in drug metabolism rather than to the interaction of a specific isozyme with zinc. An alternative explanation for these results is that this fraction of the cytochrome population may differ from the remainder because the flavoprotein has complexed to it in a certain manner, which bestows upon it the ability to be reduced more rapidly than cytochromes not complexed or differently complexed by different forms of the flavoprotein, and that zinc binds only to this "rapid reduction complex." Both the noncompetitive inhibition of cytochrome *c* reduction and competitive inhibition of metabolism would fit such a model. However, the idea that this fraction of the cytochrome is bound only to zinc because of its interaction with flavoprotein is difficult to reconcile with the fact that zinc binds to the same amount of cytochrome P-450 when either NADPH or dithionite is used as the reducing agent. Dithionite is thought to reduce cytochrome P-450 directly, without the aid of the flavoprotein.

The significance of these findings to whole animal studies has not yet been addressed. Saldeen (21) and Aronson *et al.* (22) have reported protection against CCl_4 toxicity by zinc, possibly indicative of a depressed rate of metabolism. Several laboratories (23–25) have reported deposition of zinc in the liver as zinc-metallothionein after injection of zinc. This zinc might be available to the cytochrome either in the bound form or prior to binding. Metallothionein formation requires 6 hr (26) whereas zinc uptake by the liver is complete within 2 hr (25). We are presently involved in studying the effect of zinc administration on hepatic cytochrome P-450-dependent activity.

In conclusion, zinc is able to interact with NADPH, nucleotide pyrophosphatase, NADPH-cytochrome P-450 reductase, and cytochrome P-450. The interaction of zinc with either NADPH or nucleotide pyrophosphatase does not explain the inhibitory effect of zinc on microsomal NADPH oxidation and drug metabolism. The interaction of zinc with cytochrome P-450 probably blocks entrance to the substrate site, since substrate binding is decreased but not reversed by zinc treatment. However, zinc probably does not bind directly to the binding site, since inhibition was not competitive with respect to ethylmor-

phine. The interaction of zinc with cytochrome P-450 gives rise to a novel reduced spectrum, which is unchanged by CO. Zinc causes a small noncompetitive inhibition of cytochrome *c* reduction and a specific inhibition of CO binding to the fraction of cytochrome P-450 that is rapidly reduced. These results are compatible with the possibility either that zinc inhibits drug metabolism by binding to a flavoprotein-cytochrome P-450 complex or that zinc alters the oxidation-reduction potential of the flavoprotein so that a greater NADPH:NADP ratio is required before the flavoprotein is sufficiently reduced to initiate the rapid phase of cytochrome P-450 reduction.

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