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SHORT COMMUNICATIONS

Covalent Binding of Sulfur to Microsomes and Loss of Cytochrome P-450 during the Oxidative Desulfuration of Several Chemicals

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SUMMARY

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When liver microsomes from phenobarbitone-treated rats are incubated with carbon disulfide, a marked loss of cytochrome P-450 is observed only when NADPH is present. By employing ^{14}C- and ^{35}S-labeled CS_2, it has been found that in the presence of NADPH labeled sulfur becomes covalently bound to microsomes: The binding of ^{35}S exceeds that of ^{14}C, indicating that sulfur itself becomes bound. A loss of cytochrome P-450 is also seen when parathion, phenylthiourea, or 1-naphthylisothiocyanate is incubated with liver microsomes in the presence of NADPH, whereas the oxygen-containing analogues of these compounds are all inactive. It is concluded that reactive sulfur liberated during the oxidative desulfuration of several chemicals may become bound to cellular components and initiate toxic changes in the liver. This may account not only for the loss of cytochrome P-450 but also for the centrilobular hydropic degeneration seen *in vivo*.

Previous work has shown that the toxicity of carbon disulfide to the liver is greatly enhanced when rats have been treated with phenobarbitone prior to CS_2 administration (1, 2). Prior treatment with phenobarbitone also results in a greater conversion of $^{14}CS_2$ to $^{14}CO_2$ by the whole animal (3). These findings and the observation that CS_2 causes a marked loss of cytochrome P-450 *in vitro* only in the presence of NADPH suggest that CS_2 may require metabolism before it damages the liver.

The conversion of CS_2 to CO_2 can be visualized as an oxidative desulfuration, possibly giving rise to elemental sulfur in two successive stages, through the monooxygenated intermediate COS. The possibility has therefore been considered (3) that reactive sulfur liberated from metabolism of CS_2 may become bound to components of the microsomes and the cell, thus initiating toxic liver changes.

The results reported here strongly support this interpretation. When liver microsomes are incubated with CS_2 in the presence of NADPH, there is a marked loss of cytochrome P-450 and sulfur becomes covalently bound to the microsomes. In addition, loss of cytochrome P-450 is observed during the oxidative desulfuration of parathion and of several other chemically unrelated compounds which contain either a P=S or a C=S grouping.

Washed liver microsomes were obtained

from male rats (230–250 g) which had been treated with phenobarbitone and fasted for 24 hr (3). A suspension of microsomes from the pooled livers of three rats was incubated for 15 or 30 min at 38° in air (100 cycles/min) in 25-ml conical flasks or in sealed Warburg flasks. Thunberg tubes were used when the incubations were carried out under N_2, and 1-oz screw-capped bottles (universal containers) for the incubations with labeled CS_2. Each flask, tube, or bottle contained the following components in a total volume of 3 ml (final concentrations in parentheses): the microsomes from 220 mg of wet liver (which gave an average protein and cytochrome P-450 concentration of 2 mg/ml and 4.5 nmoles/ml, respectively), sodium phosphate buffer, pH 7.4 (100.0 mM), KCl (66.6 mM), $MgCl_2$ (4.9 mM), EDTA (1 mM), and, where appropriate, NADPH (0.74 mM) and/or one of the drugs to be tested. The drugs were added either in phosphate buffer or in acetone (25 μl); CS_2 was delivered undiluted into small vials placed inside the screw-capped bottles, so that contact between microsomes and CS_2 could only take place through the air phase. In some experiments NADPH was generated in the incubation mixture from added NADP (0.19 mM) and an NADPH-generating system (4), with identical results. At the end of the incubation the samples were quickly transferred to an ice bath, diluted with ice-cold 0.1 M phosphate buffer, pH 7.4, and gassed for 30 sec with N_2. Their cytochrome P-450 content was then determined (5), using as the reference a similarly diluted suspension of the same microsomes which had been kept in an ice bath under N_2 and without addition of either NADPH or drugs.

In the experiments with radioactive CS_2, trichloracetic acid was added at the end of the incubation to a final concentration of 7%, and air was gently blown on the surface of the incubation mixtures for 30 min to displace the unchanged CS_2. The samples were then cooled and subjected to two 10-sec bursts of ultrasound. An aliquot corresponding to approximately 800 μg of protein was transferred onto a Whatman GF/A glass-fiber paper filter (2.5 cm). The precipitate was extensively washed on the filter first with 7% trichloracetic acid, then with chloroform—methanol (2:1 by volume), methanol, and ether. Washing with either acid or organic solvents was continued until no more counts could be removed from the precipitate. The filters were then dried at 50° for 30 min and transferred to a counting vial; 10 ml of Insta-Gel (Packard Instruments) were added to the vials, and the precipitate was solubilized by vigorous shaking. The radioactivity of both ^{14}C and ^{35}S was measured in a Packard Tri-Carb liquid scintillation spectrometer and corrected for quenching by the use of internal standards. Insta-Gel (10 ml) was also added to vials containing an aliquot (1 ml) of the acid washings or the residue obtained by evaporating the organic solvent washings in a stream of air.

When microsomes were incubated with either ^{14}C- or ^{35}S-labeled CS_2 in the presence of NADPH, labeled sulfur became covalently bound to the microsomes. Although some increase of ^{14}C binding (over the values obtained in controls incubated without NADPH) was also observed, the binding of ^{35}S was greatly in excess of that of ^{14}C (Table 1), indicating that sulfur itself became bound. An even greater excess of ^{35}S over ^{14}C radioactivity could be demonstrated in the trichloracetic acid washings from microsomal samples incubated with NADPH, and a slight excess of ^{35}S radioactivity was also found in the organic solvent washings (results not shown). In contrast, when fraction V from bovine plasma (2 mg/ml of incubation mixture) was substituted for the microsomes in the incubation experiments, no ^{35}S radioactivity in excess of ^{14}C radioactivity could be demonstrated in either the protein precipitate (Table 1) or the acid washings thereof. In addition, no radioactivity could be demonstrated in either precipitate or washings when trichloracetic acid was added to the incubation mixture before CS_2 (results not shown). These observations clearly indicated that in the presence of liver microsomes and NADPH, CS_2 was actively desulfurated and that a portion of the sulfur liberated became covalently bound to the microsomes. A 43%

TABLE 1

Covalent binding of labeled sulfur to microsomes and loss of microsomal cytochrome P-450 after incubation with CS_2 in the presence of NADPH

Rat liver microsomes or fraction V from bovine plasma were incubated aerobically in stoppered, screw-capped bottles for 15 min in an incubation mixture, whose composition is given in the text, with 33.3 μmoles of either $^{14}CS_2$ (6.5 nCi/μmole) or $C^{35}S_2$ (19.8 nCi/μmole). At the end of the incubation the protein precipitates from the microsomes or fraction V were extensively washed and the covalently bound radioactivity was determined. Values given refer to averages ± standard errors of four observations in the case of microsomes, or to one observation with fraction V. Both the total radioactivity of ^{35}S and ^{14}C and the ^{35}S radioactivity in excess of ^{14}C radioactivity are expressed as nanomoles of CS_2 equivalents recovered bound to the total protein precipitate of each bottle. Rat liver microsomes were also incubated under identical conditions with NADPH and/or unlabeled CS_2 (33.3 μmoles), and at the end of the incubation the cytochrome P-450 content was determined directly in the incubation mixture, using as a reference a microsomal suspension kept in an ice bath under N_2 without either NADPH or CS_2. Values of cytochrome P-450 in samples incubated with NADPH alone did not differ significantly from those of unincubated samples. Values of P-450 observed after incubation with CS_2 are given below and are averages ± standard errors of five observations.

S ource of protein	Addition		Bound radioactivity recovered			Cytochrome P-450 after incubation
	CS_2	NADPH	Total radioactivity recovered		^{35}S in excess of ^{14}C	
			^{35}S	^{14}C		
			nmoles CS_2 Eq/bottle			*% unincubated sample*
Rat liver microsomes	+	−	9.44 ± 0.93	8.79 ± 0.81	0.65	96.8 ± 1.6
	+	+	32.36 ± 2.51[a,b]	14.96 ± 1.82[c]	17.40	57.4 ± 3.4[d]
Fraction V from bovine plasma	+	−	2.99	3.00	0	
	+	+	3.20	3.10	0.10	

[a] $p < 0.001$ compared with radioactivity of the same isotope in samples incubated without NADPH.
[b] $p < 0.01$ compared with corresponding ^{14}C radioactivity of samples incubated with NADPH.
[c] $p < 0.05$ compared with radioactivity of the same isotope in samples incubated without NADPH.
[d] $p < 0.01$ compared with unincubated samples.

loss of cytochrome P-450 was observed in these experiments (Table 1) when microsomes were incubated with CS_2 in the presence of NADPH—that is, under conditions in which both desulfuration of CS_2 and covalent binding of sulfur to the microsomes were observed.

Covalent binding of sulfur to microsomes has already been reported during the oxidative desulfuration of parathion (6, 7). Experiments were therefore carried out to discover whether a loss of microsomal cytochrome P-450 would also occur during the desulfuration of parathion and other chemicals. Three pairs of drugs were studied, each pair consisting of a compound containing sulfur (as either P=S or C=S) and the corresponding oxygen analogue. The concentration of cytochrome P-450 was estimated in samples incubated with drug alone or with drug together with NADPH and compared with the appropriate controls incubated under identical conditions (with and without NADPH, respectively) but without drug (Table 2). All three sulfur-containing drugs caused loss of cytochrome P-450 in the presence of NADPH. With two of them a loss of the cytochrome was also observed in the absence of NADPH, but when the cofactor was present the loss was far greater. In clear contrast, the oxygen-containing analogues either did not cause any loss (not even in the presence of NADPH) or, when they caused a loss of the cytochrome (as with 1-naphthylisocyanate), it could not be increased significantly by

TABLE 2

Effects of several chemicals on levels of cytochrome P-450 of liver microsomes in vitro

Rat liver microsomes were incubated aerobically in 25-ml conical flasks (sealed Warburg flasks in the case of 1-naphthylisocyanate) for 30 min in an incubation mixture as described in the text. At the end of the incubation the cytochrome content was determined directly in the incubation mixture, using as a reference a microsomal suspension kept in an ice bath under N_2 and without either NADPH or drugs. Values of cytochrome P-450 in samples incubated without drugs (either with or without NADPH) did not differ significantly from those of unincubated samples. Results given are averages, with individual values in parentheses, or averages ± standard errors of the number of observations in parentheses.

Drug added	Final concentration	Cytochrome P-450 after incubation with drugs	
		−NADPH (A)	+NADPH (B)
	mM	*% control without drug*	
None (controls)		100	100
Sulfur-containing drugs			
Phenylthiourea	1.00	103.4 (102, 104.8)	88.3 (88.9, 87.8)
	0.10	103.2	95.9
	0.04	101.0 (101.6, 100.6)	94.5 (100.3, 88.8)
Parathion	1.00	78.1 ± 4.4 (4)	32.6 ± 7.1[a] (4)
	0.10	89.0 ± 4.0(3)	55.3 ± 14.1(3)
1-Naphthylisothiocyanate	0.10	89 (96, 82)	65 ± 6.2(3)
	0.01	95	76
Oxygen-containing analogues			
Phenylurea	1.15	101 (101.5, 100.6)	101.7 (101.3, 102.2)
Paraoxon	1.20	97 (94.3, 99.7)	100.9 (101.5, 100.3)
	0.12	108	109
1-Naphthylisocyanate	0.08	69.8 (70.2, 69.5)	69.4 (70.3, 68.6)
	0.04	88.9	88.4
	0.008	101.2	103.1
Sodium deoxycholate	1.00	67.2 (67.7, 66.8)	67.7 (67.6, 67.8)

[a] $p < 0.01$ compared with results obtained without NADPH.

addition of NADPH. In this respect the effect of 1-naphthylisocyanate was similar to that of a detergent, sodium deoxycholate (Table 2). The difference between the two groups of drugs is best illustrated by the differences between the values given in columns A and B of Table 2, which represent the fractional loss of P-450 dependent on NADPH and, therefore, probably due not to the drugs themselves but to any reactive derivative which might be produced through their oxidative metabolism.

The oxidative desulfuration of parathion to paraoxon *in vitro* (and the covalent binding of sulfur to microsomes which accompanies it) is known to take place also in the presence of NADH (though to a lesser extent than with NADPH) and to be inhibited by the addition of piperonyl butoxide (*6*). Table 3 shows that the loss of cytochrome P-450 due to parathion was also inhibited by piperonyl butoxide and stimulated by either NADH or, to a larger extent, by NADPH. Furthermore, both desulfuration of parathion (8) and loss of cytochrome P-450 caused by parathion in the presence of NADPH (Table 4) required the presence of O_2 and were inhibited when N_2 replaced air. Piperonyl butoxide caused an apparent increase in the amount of cytochrome P-450 (Table 3); this has already been reported by Franklin (9) as a turbidity effect. The reason why NADPH led by itself to a loss of the cytochrome under N_2 but not in air (Table 4) is not known.

In further experiments microsomes were incubated aerobically with 1-naphthylisothiocyanate or 1-naphthylisocyanate, and both aerobically and under N_2 with parathion. At the end of the incubation the mi-

TABLE 3

Effects of NADH, NADPH, and piperonyl butoxide on loss of microsomal cytochrome P-450 caused by parathion

Rat liver microsomes were incubated aerobically in the presence and absence of parathion (0.1 mM) in 25-ml conical flasks for 15 min in an incubation mixture described in the text with the additions indicated below. At the end of the incubation the cytochrome content was determined in the incubation mixture as indicated in the legend to Table 2. Results are averages ± standard errors of three observations.

Additions (final concentration)			Cytochrome P-450 after incubation		p (B vs. A)
Piperonyl butoxide (0.4 mM)	NADPH (0.74 mM)	NADH (0.77 mM)	−Parathion (A)	+Parathion (B)	
			% unincubated sample		
−	−	−	99.0 ± 3.0	98.0 ± 3.2	>0.8
−	+	−	97.8 ± 2.7	53.0 ± 1.0	<0.001
−	−	+	102.0 ± 1.5	76.3 ± 2.0	<0.001
+	−	−	109.0 ± 1.1	107.0 ± 2.3	>0.4
+	+	−	107.0 ± 1.1	72.6 ± 0.9	<0.001

TABLE 4

Requirement of oxygen for loss of microsomal cytochrome P-450 caused by parathion in vitro

Rat liver microsomes were incubated in sealed Thunberg tubes under air or N_2 for 30 min in an incubation mixture described in the text with the additions indicated below. At the end of the incubation the cytochrome P-450 content was estimated directly in the incubation mixtures as indicated in the legend to Table 2. Results are averages ± standard errors of three observations or individual observations.

Atmosphere and addition (final concentration)	Cytochrome P-450 after incubation		p (B vs. A)
	−Parathion (A)	+Parathion (B)	
	% unincubated sample		
Air			
No addition	100.1 ± 0.47	93.1 ± 0.55	<0.01
NADPH, 0.74 mM	100 ± 0.33	55.7 ± 1.5	<0.001
N_2			
No addition	100.9 ± 0.5	93.1 ± 2.22	<0.05
NADPH, 0.74 mM	80 ± 2.6	72.2 ± 1.94	>0.05
N_2, 30 min; air, 30 min			
No addition	93	94.4	
NADPH, 1.48 mM[a]	80.7	57.5	

[a] In two equal additions at the beginning of each 30-min incubation.

crosomes were isolated by centrifugation and resuspended in phosphate buffer. The cytochrome was then estimated by the conventional method of assay, with both reference and sample cells containing the same suspension of incubated microsomes. The results were very similar to those obtained (Tables 2 and 4) when the cytochrome was estimated directly in the incubation mixture against a reference of unincubated microsomes. In most experiments the loss of cytochrome P-450 seen during the oxidative desulfuration was accompanied by the appearance of a peak at about 420 nm, so it is possible that, as suggested for CS_2 (3), the loss of the cytochrome caused by parathion and the other sulfur-containing compounds studied here may also occur through conversion of P-450 to the labile heme-containing species known as P-420, followed by degradation of some of the heme.

It is concluded from these findings that

reactive sulfur liberated during the oxidative desulfuration of CS_2 and of several other compounds may become bound to cellular components and initiate toxic changes in the liver. This may account not only for the loss of cytochrome P-450 but also for the centrilobular hydropic degeneration which is seen *in vivo* after CS_2 (2) or phosphorothionates.[1] This interpretation is in line with the findings from several laboratories [reviewed by Uehleke (10)] that covalent binding of reactive metabolites to macromolecules accompanies, and may be responsible for, the toxicity of several other chemicals, which require metabolic activation for their toxicity.

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[1] A. A. Seawright, unpublished observations.

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