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Accelerated Conversion of Heme to Bile Pigments Caused in the Liver by Carbon Disulfide and Other Sulfur-containing Chemicals

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SUMMARY

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After CS_2 administration a slight loss of heme was demonstrated from the microsomal fraction of rat liver, and when the microsomal heme was prelabeled with 5-amino[4-^{14}C]levulinate, a loss of heme radioactivity from the microsomes was observed after CS_2 treatment with accumulation of heme radioactivity in the cell sap. The conversion *in vivo* of 5-amino[4-^{14}C]levulinate into bile bilirubin and the activity of liver heme oxygenase were both stimulated by CS_2 treatment. Phenobarbital pretreatment of the rats, prior to CS_2 administration, potentiated the stimulation of heme oxygenase caused by CS_2, whereas cycloheximide pretreatment completely prevented it. A stimulation of liver heme oxygenase was also found after administration of diethylphenylphosphorothionate, pentothal, and phenylthiourea, whereas the oxygen-containing analogues of the last two drugs were inactive. It is concluded that the accelerated liver heme conversion to bile pigments caused by CS_2, pentothal, and other sulfur-containing drugs is related to their metabolism by oxidative desulfuration and ensuing microsomal toxicity: the damage of the apoprotein of cytochrome P-450 may result in a reduced affinity for heme, with more liver heme being available for degradation.

INTRODUCTION

An important feature of the acute liver toxicity of CS_2 is the loss of the hemoprotein cytochrome P-450 and the accompanying depression of microsomal drug-metabolizing enzymes (1-3). A current hypothesis is that CS_2 is oxidatively desulfurated to produce a reactive species of sulfur (4-6) which then becomes bound to the microsomal proteins, mostly to the apoprotein of cytochrome P-450 (7, 8); this may ultimately be responsible for the observed loss of the characteristic spectrum of the cytochrome and also for the inhibition of drug-metabolizing activity.

The present work was undertaken to study in detail the changes in liver heme metabolism caused by CS_2 exposure. The results suggest that after CS_2 the affinity of heme for binding sites within the microsomes is reduced: some of the pre-existing heme is rapidly lost from the microsomes and less of the newly formed heme is retained by the membranes in a stable form. A stimulation of heme oxygenase is also described after administration of either CS_2

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or of other sulfurcontaining compounds which are also metabolized by oxidative desulfuration and which damage cytochrome P-450 by a similar mechanism. Finally it is now found that CS_2 stimulates heme oxygenase much more effectively in rats pre-treated with phenobarbitone, where the damaging action of CS_2 on the cytochrome is also greater.

MATERIALS AND METHODS

Male Porton (Wistar-derived) rats of 160–180 g body weight were used throughout. They were all fasted overnight before killing or administration of radioisotopes.

CS_2 (20 or 50 μl per rat) was administered intraperitoneally in 2 ml arachis oil; control rats were injected with oil alone. Some rats were given two intraperitoneal injections of phenobarbital (80 mg/kg of the sodium salt in saline) the first 48 hr and the second 24 hr before CS_2.

The effect of CS_2 on the fate of prelabeled liver heme was studied in fasted rats (some of them pretreated with phenobarbital) which had received 5-amino[4-^{14}C]levulinate (2 μCi, 37.7 nmol/rat) intraperitoneally 2 hr before treatment with CS_2 or oil. The rats were killed 1 hr after CS_2, their livers homogenized in 0.25 M sucrose and the homogenate centrifuged at 9,000 *g* for 20 min. The 9,000 *g* supernatant was then centrifuged at 105,000 *g* for 1 hr to obtain a microsomal pellet and the cell sap. Samples of total liver homogenate, washed microsomal fraction, and cell sap were taken for determination of total radioactivity (in Insta-gel) and also for extraction and crystallization of hemin (9) after addition of horse erythrocytes lysate to provide carrier heme. The radioactivity of the crystalline heme was determined after combustion in an Intertechnique Oxymat apparatus by liquid scintillation counting in toluene/methanol/2-phenylethylamine/water (40:22:33:5, by vol), containing 1% of 2,5-diphenyloxazole.

In the bile cannulation experiments, rats were first given CS_2 (50 μl/rat) or oil, then their bile duct was cannulated under pentobarbital anesthesia. 5-Amino[4-^{14}C]levulinate (2 μCi, 37.7 nmol/rat) was injected intravenously 2 hr after CS_2 and bile collected for 300 min. The rats were kept under pentobarbital anesthesia throughout the experiment by injecting small amounts of the barbiturate, when appropriate, through an intraperitoneal cannula: their rectal temperature was continuously monitored and kept between 37–38° with the help of an electric bulb. A 0.9% NaCl solution containing 0.09% KCl was infused through a tail vein at the rate of 1 ml/hr to compensate for the ion losses due to the collection of bile. Bile was analyzed for total bilirubin content (10) and for radioactivity. Bilirubin was also isolated from the bile and crystallized (11) to constant specific radioactivity. The radioactivities of bile and of crystallized bilirubin were estimated by liquid scintillation counting, using Insta-gel as a scintillation fluid.

In the heme oxygenase experiment diethylphenylphosphorothionate (200 mg/kg), phenylurea (40 mg/kg) or phenylthiourea (40 mg/kg) was given orally in 10 ml arachis oil/kg, 17 hr before killing: pentothal or pentobarbital was injected intraperitoneally as the sodium salt in two doses, 40 mg/kg 22 hr and again 20 mg/kg 17 hr before killing. Cycloheximide (40 mg/kg) was injected subcutaneously in saline (10 ml/kg) 15 min before treatment with CS_2 or oil. All rats were fasted overnight before killing, then their livers were perfused *in situ* with ice-cold 0.9% NaCl and homogenized (20% w/v) in 0.25 M sucrose. Heme oxygenase was assayed using post-mitochondrial supernatants by a modification (12) of the method of Schacter *et al.* (13): in two experiments the bilirubin formed during the assay was identified by its characteristic spectrum with maximum absorption at about 460 nm.

Cytochrome P-450 and cytochrome b_5 were estimated in washed microsomal fraction by the method of Omura and Sato (14, 15) and total heme according to Falk (16). Proteins were estimated by Aldridge's (17) modification of the method of Robinson and Hogden (18).

RESULTS

Effect of treatment with CS_2 on the concentration of various heme components in rat liver microsomal fraction. When fasted

rats that had been pretreated with phenobarbital were given CS_2 intraperitoneally and killed 1 hr later, they showed a marked decrease in cytochrome P-450 and an accumulation in the microsomes of heme in a "noncytochromal" form, i.e., in a form which did not behave as either cytochrome P-450 or as cytochrome b_5 (Table 1). This confirms previous findings (1, 19). In addition, in most experiments the total heme concentration of the microsomes was slightly decreased, suggesting that although most of the heme which could not be accounted for as cytochrome P-450 was still in the microsomes, some of it may have been lost from the membranes; the same conclusion could be drawn whether the various heme components were expressed per g original liver (Table 1) or per mg microsomal protein. Similar microsomal changes were produced by CS_2 in rats which had not been pretreated with phenobarbital, but they were less pronounced: in these rats a slight loss of total microsomal heme was also observed after CS_2.

Effect of CS_2 on the metabolic fate of prelabeled and newly formed heme. In order to confirm that the pre-existing heme could be lost from the microsomal membranes after CS_2 treatment and also to investigate its fate, microsomal heme was prelabeled with 5-amino[4-^{14}C]levulinate. Two hours after injecting the label (when its incorporation into heme was complete [20]) CS_2 was administered and the animals were killed 1 hr later (Table 2).

In phenobarbital pretreated rats given 20 μl CS_2 and killed 1 hr later there was an apparent migration of radioactivity from the microsomes into the cell sap (Table 2): more radioactivity could by crystallized as hemin from the cell sap and less from the microsomes (as compared with the corresponding values of the controls), indicating that a partial redistribution of microsomal heme into the cell sap had taken place after treatment with CS_2. In addition, there was a small but significant loss of the radioactivity recovered as crystalline hemin from the total liver homogenate of treated rats.

Similar results were obtained after administration of CS_2 (50 μl/rat) to animals that had not been pretreated with phenobarbital: an apparent migration of radioactivity was seen from the microsomes into the cell sap and a loss of radioactivity was observed from the heme isolated in a crystalline form from the liver homogenate (data not shown), suggesting that as a result of treatment some of the prelabeled heme had been lost from the liver, presumably after conversion into bile pigments.

In order to obtain direct evidence for increased conversion of liver heme into bilirubin after CS_2 treatment *in vivo,* the bile ducts of CS_2-treated rats and of control rats were cannulated and the animals were then injected with 5-amino[4-^{14}C]levulinate, and the radioactivity appearing in their bile was determined. CS_2-treated rats excreted a greater proportion of the label into their bile than the corresponding control rats (Fig. 1). CS_2-treated rats also showed a greater conversion of the injected 5-amino[4-^{14}C]levulinate into bilirubin, isolated in a crystalline form from their bile (Table 3) but there was no change in either volume of bile or amount of bilirubin ex-

TABLE 1

Effect of treatment with CS_2 on the concentration of various heme components in rat liver microsomal fraction

Phenobarbital pretreated rats were fasted overnight, then given CS_2 (20 μl in 2 ml arachis oil) or oil alone, intraperitoneally and killed 1 hr later. Results presented are averages ± S.E.M. of 5 observations.

Treatment	Heme component(s) (nmol/g wet liver)				
	Cytochrome P-450	Cytochrome b_5	Sum of cytochromes (A)	Total Heme (B)	Non-cytochromal Heme (B − A)
Oil	71.7 ± 2.4	15.2 ± 0.54	86.9 ± 2.9	100.4 ± 4.1	13.4 ± 1.4
CS_2	49.4 ± 2.7**	17.6 ± 0.34*	67.0 ± 2.7**	88.1 ± 3.5	21.3 ± 0.9*

* $p < 0.01$

** $p < 0.001$, when compared with corresponding control values (Student's t test).

TABLE 2

Loss of radioactivity from prelabeled microsomal heme caused by CS_2 in phenobarbital pretreated rats

Rats were pretreated with phenobarbital for 2 days and fasted overnight before receiving 5-amino[4-^{14}C]levulinate intraperitoneally. Two hours after the isotope administration the rats were treated with CS_2 (20 μl in 2 ml arachis oil) or with oil alone and were killed 1 hr later. Their liver homogenates were fractionated and the homogenates and their microsomal or cell sap fractions were assayed for total radioactivity and for heme radioactivity (the latter after isolation of crystalline hemin). Results are given as means ± S.E.M. of 4 observations and are all expressed as percentage of the injected isotope recovered in the appropriate liver fraction of the whole liver.

Treatment	Radioactivity recovered in: (% of injected dose)				
	Total liver homogenate	Microsomal fraction of total liver		Cell sap fraction of total liver	
	(heme counts)	*Total counts*	*Heme counts*	*Total counts*	*Heme counts*
Oil	13.2 ± 0.4	9.9 ± 0.2	6.9 ± 0.2	2.3 ± 0.1	1.1 ± 0.03
CS_2	12.0 ± 0.3*	7.9 ± 0.2**	5.1 ± 0.2**	3.0 ± 0.2*	1.3 ± 0.07*

* $p < 0.05$

** $p < 0.01$, when compared with corresponding control values in rats given oil alone (Student's t test).

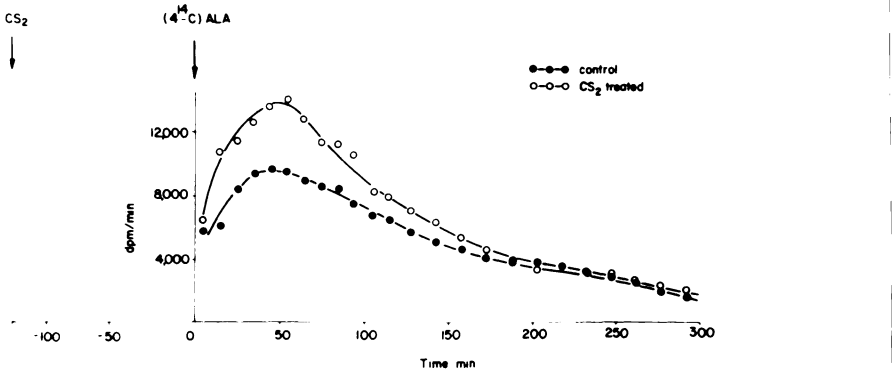

FIG. 1. *Effect of CS_2 pretreatment on the biliary excretion of radioactivity from administered 5-amino[4-^{14}C]levulinate.*

Fasted rats were given CS_2 in oil or oil alone (control) intraperitoneally, then their bile duct was cannulated and 5-amino[4-^{14}C]levulinate (2 μCi/rat) was injected intravenously. Their bile was collected for 300 min in successive fractions of 10–15 min, the total volume of each bile fraction was measured and an aliquot taken for determination of radioactivity. Results presented are those of a typical experiment and are expressed as total radioactivity excreted in bile per min.

creted (results not shown).

Stimulation of liver heme oxygenase caused by CS_2 and other sulfur containing chemicals. The results presented above indicated that CS_2 stimulated the conversion of liver heme to bile pigments, presumably by making more heme available for degradation by heme oxygenase. Since heme oxygenase is an inducible enzyme (21), further experiments were carried out to ascertain 1) whether a stimulation of heme oxygenase could be observed after administration of CS_2; 2) whether other sulfur-containing chemicals, which depress cytochrome P-450 by a similar mechanism, could also stimulate liver heme oxygenase; and 3) whether prior treatment with phenobarbital, which is known to potentiate the microsomal toxicity of CS_2, could also potentiate the stimulation of heme oxygenase caused by CS_2.

CS_2 administration was followed by a stimulation of liver heme oxygenase and this effect was found to be significantly potentiated by pretreatment of rats with phenobarbital (Table 4). In phenobarbital pretreated rats cycloheximide completely abolished the stimulation of heme oxygenase caused by CS_2 (Table 4), without preventing the loss of cytochrome P-450: values of cytochrome P-450 observed in these

animals, expressed as nmol of cytochrome per total liver of 100 g body wt (average ± S.E.M. of 3 observations), were as follows: treatment with cycloheximide and oil, 211 ± 14; treatment with cycloheximide and CS_2, 106 ± 16. The loss of cytochrome P-450 caused by CS_2 in these animals was of the same order of magnitude as that observed in the absence of cycloheximide treatment in another study (4).

Diethylphenylphosphorothionate, a chemical containing sulfur as P=S, has been reported to cause loss of cytochrome P-450, both *in vivo* and *in vitro,* and to lead, when given to rats pretreated with phenobarbital, to a liver lesion indistinguishable from that caused by CS_2 in similarly pretreated rats (22). Similar effects have been reported for other sulfur-containing chemicals (6, 23). We now find that diethylphenylphosphorothionate and two other chemicals containing sulfur, pentothal and phenylthiourea, all stimulate heme oxygenase, whereas the oxygen-containing

TABLE 3

The effect of treatment with CS_2 on the conversion of 5-amino[4-^{14}C]levulinate into bile bilirubin by the rat in vivo

Rats were fasted overnight, then they were injected intraperitoneally with CS_2 (50 μl in 2 ml oil) or with oil alone (controls). Their bile duct was cannulated and 2 hr after administration of CS_2, 5-amino[4-^{14}C]levulinate was injected intravenously and bile collected in 3 successive fractions over 300 min. Results given are averages ± S.E.M. of 3 observations and are calculated from the specific activity of the bilirubin isolated in a crystalline form from the bile and from the total amount of bilirubin excreted in each of the three fractions.

Bile Fraction	Time after injection of 5-amino[4-^{14}C]levulinate	Total radioactivity excreted as bilirubin (% of injected isotope recovered as crystalline bilirubin in total fraction)	
		Controls	CS_2-treated
	min		
1	0–70	4.8 ± 0.4	5.3 ± 1.4
2	71–165	5.1 ± 0.3	10.0 ± 1.1*
3	166–300	4.5 ± 0.7	4.2 ± 0.3

* $p < 0.02$, when compared with corresponding control value (Student's *t*-test).

TABLE 4

Stimulation of liver heme oxygenase activity caused by CS_2. Effect of pretreatment with phenobarbital and with cycloheximide

Rats (some pretreated with phenobarbital) were fasted overnight, then injected with CS_2 (50 μl/rat) in 2 ml arachis oil or with oil alone and killed 2 hr or 4 hr later. Results given are averages ± S.E.M. of the number of observations in parentheses or averages of 2 observations, with individual results in parentheses. The enzyme activity was related to the protein content of the post-mitochondrial supernatant used as the source of the enzyme.

Pretreatment	Treatment	Time of killing	Heme oxygenase activity	
		hr after CS_2 or oil	*pmol bilirubin/min/mg protein*	*Stimulation caused by CS_2 %*
None	Oil	2	17.8 (15.0, 20.7)	43.8
	CS_2	2	25.6 (22.4, 28.8)	
	Oil	4	24.0 ± 2.3 (4)	114.6
	CS_2	4	51.5 ± 9.9 (4)*	
Phenobarbital	Oil	4	24.4 ± 1.6 (5)	383.6
	CS_2	4	118.0 ± 11 (5)**	
Phenobarbital and cycloheximide	Oil	4	19.4 ± 2.4 (3)	None
	CS_2	4	14.7 ± 2.2 (3)	

* $p < 0.05$

** $p < 0.001$, when compared with corresponding values in animals treated with oil.

TABLE 5

Effect of treatment of rats with diethylphenylphosphorothionate, pentothal, phenylthiourea, or the oxygen-containing analogues of the last two drugs on the activity of liver heme oxygenase

Rats were treated as indicated in MATERIALS AND METHODS, with controls receiving the appropriate solvent alone. All rats were fasted overnight before being killed. The activity of heme oxygenase was related to the protein content of the post-mitochondrial supernatant used as the source of the enzyme. Results given are averages ± S.E.M. of the number of observations in parentheses, or averages of two observations with individual observations in parentheses.

Treatment	Heme oxygenase activity
	pmol bilirubin/min/mg protein
Saline (controls)	18.0 ± 2.2 (4)
Pentothal	40.5 ± 8.4 (4)*
Pentobarbital	18.1 ± 2.2 (4)
Oil (Controls)	19.5 ± 2.2 (4)
Phenylthiourea	57.1 ± 9.4 (3)**
Phenylurea	19.2 ± 1.1 (3)
Diethylphenylphosphorothionate	75.9 (76.5, 75.3)

* $p < 0.05$

** $p < 0.02$, when compared with corresponding control values (Student's *t*-test).

analogues of the last two chemicals are inactive (Table 5).

DISCUSSION

The present work has shown that CS_2, a chemical previously known to depress cytochrome P-450 through damage to its apoprotein, causes a loss of liver heme by accelerating its breakdown to bile pigments. This has been indicated by three different lines of evidence. 1) CS_2 has been shown to decrease the total heme content of the microsomes, with an apparent migration of radioactivity from prelabeled microsomal heme into the cell sap and with loss of radioactivity from the liver. 2) The biliary excretion of radioactivity from 5-amino[4-^{14}C]levulinate was found to be increased by CS_2 treatment and more radioactivity could be isolated as crystalline bilirubin from the bile of treated rats. 3) CS_2 was also found to stimulate the activity of the enzyme heme oxygenase, an effect which may itself contribute to the loss of liver heme and to the accelerated conversion of 5-amino[4-^{14}C]levulinate into bilirubin, by accelerating liver heme breakdown.

The most likely interpretation for these findings is that CS_2, by damaging the apoprotein of cytochrome P-450, decreases its affinity for heme within the microsomal membranes. This will entail two main consequences: first, some of the heme preexisting as the complete hemoprotein at the time of poisoning will subsequently dissociate from the damaged apoprotein and become available for degradation; and secondly, less of the heme that is formed after the apoprotein has been damaged will be stabilized in the membranes. The net result will be an accelerated conversion of liver heme to bile pigments and a substrate-mediated induction of heme oxygenase. This interpretation is supported by the following findings: a) pretreatment with phenobarbital, which increases the microsomal damage caused by CS_2 (1), also potentiated the stimulating effect of CS_2 on heme oxygenase (Table 4); b) the stimulation of heme oxygenase caused by CS_2 could be completely abolished by cycloheximide (Table 4), even though this inhibitor did not prevent the loss of cytochrome P-450 due to CS_2 treatment; c) diethylphenylphosphorothionate, phenylthiourea and pentothal, other sulphur-containing chemicals which, like CS_2, are metabolized by oxidative desulfuration and are known to damage cytochrome P-450 by a similar mechanism (6, 22, 23), also induce heme oxygenase (Table 5). This interpretation may also account for the hitherto unexplained finding that α-naphthylisothiocyanate stimulates the incorporation of 5-amino[^{14}C]levulinate into bile bilirubin *in vivo* (24), as this drug has been found to cause loss of cytochrome P-450 (6) and might be expected, like CS_2, to reduce the affinity of hemoprotein binding sites for heme and lead therefore to accelerated liver heme breakdown.

The present findings are compatible with the hypothesis that induction of heme oxygenase may result, at least in some cases, from a labilization of cytochrome P-450 and dissociation of its heme complement in a form that can be accepted as a substrate by heme oxygenase and can also act as an

inducer for this enzyme (25, 26). Bissell and Hammaker (27, 28) have also obtained evidence that with endotoxin, another stimulator of heme oxygenase, the heme that dissociates from cytochrome P-450 can also be utilized to regulate tryptophan pyrrolase and 5-aminolevulinate-synthetase, by increasing the activity of the former and depressing that of the latter: the same effects might be expected for CS_2 and related chemicals, and in this respect might be relevant to note that a slight inhibition of 5-aminolevulinate-synthetase after CS_2 treatment has been reported (29).

It can therefore be concluded that a compound like CS_2, which alters the apoprotein of cytochrome P-450, generates a relative redundancy of heme in the liver, of which increased formation of bile pigments, stimulation of heme oxygenase, and possibly depression of 5-aminolevulinate-synthetase are all consequences. This contrasts with the liver effects of 2-allyl-2-isopropylacetamide, where cytochrome P-450 is also depressed but through an effect that is specific to the heme moiety (29, 30). With 2-allyl-2-isopropylacetamide, the liver heme is converted to abnormal pigments that are irreversibly lost from the heme oxygenase pathway of heme degradation and also from the regulatory functions that heme exercises on its own metabolism: the net result is one of relative heme deficiency affecting the same parameters discussed above for CS_2, but in the opposite direction. Thus, the activity of liver 5-aminolevulinate-synthetase is stimulated (31), that of heme oxygenase is depressed (32), and the rate of liver bilirubin synthesis is decreased (33).

In the mechanism discussed above for the stimulation of heme oxygenase caused by CS_2 and related chemicals, liver heme has been envisaged not only as a substrate for heme oxygenase but also as the inducer for this enzyme. The possibility should also be considered, however, that the induction of heme oxygenase caused by CS_2 and other sulfur-containing chemicals may be mediated by a mechanism unrelated to heme, a mechanism perhaps reflecting the liver toxicity of these chemicals. This possibility appears less likely at present but cannot be completely discounted.

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