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EARLY, SELECTIVE AND REVERSIBLE SUPPRESSION OF CYTOCHROME P-450-DEPENDENT MONOOXYGENASE OF LIVER MICROSOMES FOLLOWING THE ADMINISTRATION OF LOW DOSES OF CARBON DISULFIDE IN MICE

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Abstract—The effects of carbon disulfide (CS_2) on the liver microsomal drug-metabolizing enzyme system and other enzyme activities were studied 1 hr after the oral administration of 3–300 mg/kg of CS_2 in mice. Considerable decreases in drug-metabolizing enzyme activities (such as hydroxylation of aniline, O-dealkylation of *p*-nitroanisole, 7-ethoxycoumarin and 7-ethoxyresorufin, and N-demethylation of *N*,*N*-dimethylaniline), NADPH–cytochrome P-450 reductase (but not NADPH–cytochrome *c* reductase), and P-450-associated peroxidase activities were already observed at 3 and 30 mg/kg of CS_2, dose dependently. At the same dosage levels, the magnitudes of microsomal spectral changes induced by aniline and nicotinamide (type 2 substrates), but not those induced by hexobarbital and SKF-525A (type 1 substrates), were also reduced to a considerable extent. The degrees of these alterations were all greater than that of the measurable loss of P-450 content, i.e. the loss of functional activity of P-450 was much greater than simply expected from the apparent decrease in the hemoprotein content. Cytochrome b_5 content and NADH–ferricyanide reductase activity were unchanged at 30 and 300 mg/kg of CS_2, although NADH–cytochrome *c* reductase activity was increased at the latter dose. The following enzyme activities did not change significantly at up to 300 mg/kg of CS_2: flavin-containing monooxygenase, UDP-glucuronyl transferase, glucose-6-phosphatase and heme oxygenase in microsomes, and glutathione *S*-transferases in the soluble fraction. Microsomal conjugated diene levels and liver glutathione content were also unchanged. These observations support the theory that P-450 is a sensitive and selective site for CS_2 action, where CS_2 itself is bioactivated. It was also shown that the loss of P-450 was reversible after a single, or repeated, administration of CS_2.

As reported previously [1], the oral administration of diethyldithiocarbamate (DTC) protected mice from hepatic injury induced by a variety of hepatotoxicants that require metabolic activation and decreased various microsomal drug-metabolizing enzyme activities in the liver. Since these actions of DTC were reproduced by equimolar doses of carbon disulfide (CS_2), the action of DTC may be mediated by CS_2 produced under acidic conditions in the stomach. We have therefore studied the action of CS_2 in drug metabolism.

CS_2 is generally thought to be a hepatotoxicant as well as a neurotoxic agent. However, the hepatotoxic potential of CS_2 is reportedly weak in normal animals, although it is augmented by phenobarbital pretreatment [2–5]. Impairment of liver drug metabolism by CS_2, which is accompanied by a loss of cytochrome P-450, has been reported by many investigators [1, 6–14]. This action of CS_2 is likewise enhanced in phenobarbital-pretreated animals [4, 6, 11, 13].

From several lines of studies under different experimental conditions [6, 8, 10, 12, 14], impairment of the microsomal cytochrome P-450-containing monooxygenase system seems to be a specific event following the administration of CS_2. It is also proposed that CS_2 is metabolized by the hepatic monooxygenase system, liberating the reactive sulfur atom, which covalently binds to microsomal proteins to cause microsomal dysfunctions such as loss of P-450 [15–18]. However, *in vivo* evidence showing specific impairment of this hemoprotein may not be sufficient. For example, (1) the dose of CS_2 is sometimes so high that non-specific effects could ensue [6, 12], (2) exposure experiments may present difficulty in obtaining a dose–response relationship [8, 10], (3) phenobarbital pretreatment may also cause non-specific effects resulting from enhanced microsomal lipid peroxidation after high doses of CS_2 [5, 13, 19], and (4) not all of these indices were determined in parallel in most studies. In addition, it is still unclear why various microsomal drug-metabolizing enzyme activities are affected to a much greater extent as compared with the measurable loss of P-450 [1, 12].

The present study was carried out to ascertain if the microsomal P-450-containing monooxygenase system is an early and selective target of CS_2 action and, at the same time, to gain some insight into the profile of the impairment of this enzyme system. From the toxicologic standpoint, the reversibility of the action of CS_2 was also examined.

MATERIALS AND METHODS

Animals and treatments. SPF-grade male mice of the ddY-strain (Shizuoka Agricultural Cooperative

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Association for Laboratory Animals, Japan), (5 weeks old) were used. Food and water were given *ad lib.* throughout the experimental period. CS_2 (>99%, GC, Wako Pure Chemicals, Japan) was freshly dissolved in olive oil and given at a volume of 0.05 ml/10 g of body weight. Routinely, animals were given 3–300 mg/kg of CS_2 p.o. and killed 1 hr later. For the acute hepatotoxicity test, up to 2 g/kg of CS_2 was given p.o. or i.p. 24 hr before sacrifice. In the subacute experiment, mice were treated with 30 and 300 mg/kg of CS_2 p.o. for 2 weeks and used for further experiments 24 hr after the last administration. Control mice received the vehicle alone.

Evaluation of hepatotoxicity. Animals were killed by exsanguination, the blood was collected into a heparinized syringe, and, after centrifugation, plasma glutamic pyruvic transaminase (GPT) activity was measured by the method of Reitman and Frankel [20]. For determination of liver calcium content, a portion of the liver was completely sonicated in 8% trichloroacetic acid (TCA), and the calcium content of the TCA extract was determined by the calcein-fluorometric method of Von Hattingberg *et al.* [21].

Preparation of microsomes. Mice were killed by decapitation and 20% liver homogenate was prepared in 0.15 M KCl–20 mM potassium phosphate (pH 7.4)–0.1 mM EDTA. The homogenate was centrifuged at 9000 *g* for 20 min, and the supernatant fraction, without containing the fluffy layer, was further centrifuged at 105,000 *g* for 1 hr. The precipitated microsomal fraction was suspended in the same medium at a protein concentration of 20 mg/ml and used for various assays while fresh. For glucose-6-phosphatase (G-6-Pase) assay, microsomes were isolated in medium buffered with 20 mM Tris–HCl (pH 7.4) instead of the phosphate buffer. For determination of the total heme content and heme oxygenase activity, the livers were perfused *in situ* with cold saline and the microsomal fraction was washed once.

Methods for various assays. The following microsomal enzyme assays were conducted essentially according to the methods given in the references: cytochrome P-450 [22], cytochrome b_5 [23] and total heme content [22]; NADPH–P-450 reductase [24]; NADPH–cytochrome *c* reductase [25]; NADH–ferricyanide reductase and NADH–cytochrome *c* reductase [23]; P-450-dependent peroxidase activity (aniline hydroxylation and *N,N*-dimethyl *p*-phenylene diamine oxidation) [26]; 7-ethoxycoumarin- and 7-ethoxyresorufin-*O*-deethylase [27]; substrate-induced spectral changes [28]; phenylthiourea *S*-oxidase [29]; *N,N*-dimethylaniline *N*-oxidase and *N*-demethylase [30]; UDP-glucuronyltransferase [31]; and G-6-Pase [32]. Glutathione (GSH) *S*-transferase activities in the soluble fraction were measured according to the method of Habig *et al.* [33]. For microsomal heme oxygenase assay, the reaction mixture as described by Maines and Kappas [34] was used, and the bilirubin formed was extracted with chloroform–methanol (1:1) at pH 6.5, and the absorbance at 453 nm was read. Microsomal lipid peroxidation was assessed by measuring conjugated diene levels as described by Recknagel and Ghoshal [35]. The liver GSH content was assessed by determining the non-protein sulfhydryl content [36]. Protein was determined by the method of Lowry *et al.* [37].

Statistical analysis was done by the method of Bonferroni after the one-way analysis of variance as described by Wallenstein *et al.* [38], and $P < 0.05$ was considered statistically significant.

RESULTS

Acute hepatotoxicity of CS_2. Table 1 shows the acute hepatotoxic potential of CS_2 in normal mice. Intraperitoneal administration of 1 to 1.5 g/kg of CS_2 caused moderate hepatic injury, as indicated by not so great increases in plasma GPT activity and liver calcium content at 24 hr. Marked loss of body weight was also observed. At 2 g/kg, all mice died within 24 hr. With the oral route, however, all mice survived up to 2 g/kg of CS_2 with no signs of hepatic damage.

In the following experiments, far lower doses of CS_2 than that expected to cause hepatic damage were given orally.

Time-course of changes of drug-metabolizing enzyme activities. Following the oral administration of fairly low doses (3 and 30 mg/kg) of CS_2, the liver microsomal cytochrome P-450 content and drug-

Table 1. Acute hepatotoxic potential of CS_2 in ddY mice

Route of administration	Dose of CS_2 (mg/kg)	(N)	Body weight gain (g)	Plasma GPT (KU)	Liver Ca^{2+} (μmoles/g liver)
Untreated		(5)	0.3 ± 0.3	29 ± 14	0.74 ± 0.10
p.o.	500	(5/5)*	0.8 ± 0.2	25 ± 11	0.75 ± 0.08
	1000	(5/5)	0.3 ± 0.7	22 ± 6	0.72 ± 0.13
	1500	(5/5)	0.5 ± 1.3	35 ± 7	0.78 ± 0.13
	2000	(5/5)	0.2 ± 0.7	36 ± 11	0.72 ± 0.07
i.p.	500	(5/5)	0.3 ± 0.7	26 ± 20	0.96 ± 0.13
	1000	(5/5)	−2.2 ± 0.6†	98 ± 78	1.50 ± 0.36
	1500	(4/5)	−2.5 ± 0.4†	253 ± 243†	2.65 ± 1.75†
	2000	(0/5)	—	—	—

Animals were killed 24 hr after administration. Values represent the means ± S.D.
* Number survived.
† Significantly different from the control at $P < 0.01$.

metabolizing enzyme activities decreased rapidly, reaching their lowest levels at 1 hr, maintaining them for several hours, and then gradually rising to the control levels by 24 hr. The suppressive action of CS_2 was dose-dependent. It is also clear that the degree of the suppression of drug-metabolizing enzyme activities, especially that of aniline hydroxylase and *p*-nitroanisole *O*-demethylase, was much greater as compared with that of the loss of P-450 (Fig. 1).

Early alterations of P-450-associated enzyme activites. The microsomal cytochrome P-450 content and some enzyme activities associated with this hemoprotein were measured 1 hr after the administration of 3 and 30 mg/kg of CS_2 (Table 2). Although loss of P-450 was only slight with 3 mg/kg of CS_2, 30 mg/kg caused a significant decrease (16%) of this hemoprotein (similar to the results shown in Fig. 1). With the same microsomal preparation, NADPH–P-450 reductase activity was measured by the direct reduction of membrane-bound P-450 with NADPH under anaerobic conditions [24]. This activity decreased to a greater extent than that of the loss of P-450, i.e. 31 and 56% of the activity being lost at 3 and 30 mg/kg respectively. However, NADPH–cytochrome *c* reductase activity, i.e. the flavoprotein enzyme activity using the exogenous electron acceptor [25], was not affected at all. Therefore, the decrease in NADPH–P-450 reductase activity may be due to an alteration of P-450 molecules. In accordance with this, the peroxidase activity of P-450, a partial reaction of the monooxygenase system which was measured by aniline hydroxylation or tetramethylphenylene diamine as a hydrogen donor [26], also decreased to a greater extent than that of the P-450 loss. 7-Ethoxycoumarin- and 7-ethoxyresorufin-*O*-deethylase activities, the total monooxygenase reactions, were most markedly suppressed, although the degree of the suppression was not much different between the two substrates.

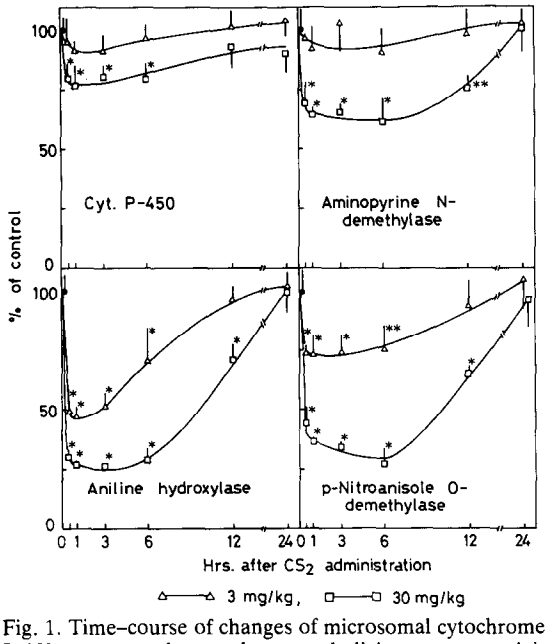

Fig. 1. Time-course of changes of microsomal cytochrome P-450 content and some drug-metabolizing enzyme activities after the oral administration of CS_2. Each point represents the mean ± S.D. of five mice. The control values at 0 hr were as follows: cytochrome P-450, 0.891 ± 0.056 nmoles/mg protein; aminopyrine *N*-demethylase, 0.337 ± 0.037 μmoles formaldehyde formed/mg protein/hr; aniline hydroxylase, 66.8 ± 5.3 nmoles *p*-aminophenol formed/mg protein/hr; and *p*-nitroanisole *O*-demethylase, 134.1 ± 13.0 nmoles *p*-nitrophenol formed/mg protein/hr. Key: Significantly different from the control at (*)$P < 0.01$ or (**)$P < 0.05$.

The microsomal substrate-binding profile was examined next. The magnitude of the microsomal spectral change (ΔO.D. values) induced by hexobarbital and SKF-525A (type 1 substrates) was not changed significantly by *in vivo* treatment with CS_2, although the ΔO.D. value with the former substrate tended to increase. On the contrary, the ΔO.D. values with aniline and nicotinamide (type 2) were decreased by CS_2 treatment and the degree of the decrease likewise exceeded that of the P-450 loss. Shapes of the spectra induced by these substrates were unchanged after CS_2 treatment.

The relationship between the loss of P-450 and total heme content was studied at 30 and 300 mg/kg of CS_2, as the P-450 content was only slightly decreased by 3 mg/kg of CS_2. As shown in Table 3, the total heme content decreased dose dependently, but to a lesser extent as compared with the loss of P-450. Conversion to P-420 was not observed after CS_2 treatment.

Effects on other microsomal enzymes and constituents. In the following experiments, the doses of CS_2 were raised to 30 and 300 mg/kg p.o., which were given 1 hr before the animals were killed.

In agreement with previous reports [6, 13, 14], cytochrome b_5 content and NADH–cytochrome b_5 reductase activity, which was measured by using ferricyanide as an electron acceptor, were not changed significantly following up to 300 mg/kg of CS_2. NADH–cytochrome *c* reductase activity, however, significantly increased at the higher dose (Table 4).

As shown in Table 5, microsomal flavin-containing monooxygenase activities, as determined by phenylthiourea S-oxidation and *N*,*N*-dimethylaniline N-oxidation, were not affected significantly by CS_2, whereas N-demethylation of the latter substrate, which is dependent on P-450, was suppressed markedly. This specific inhibition of P-450-dependent, but not flavin-dependent, monooxygenase was confirmed by assaying the activities in the presence of *n*-octylamine, which is known to suppress the former but enhance the latter enzyme activity [30].

Other microsomal enzyme activities such as UDP-glucuronyltransferase, G-6-Pase and heme oxygenase activities, conjugated diene levels of microsomal lipids, GSH *S*-transferase activities of the soluble fraction, and the liver GSH content were not affected significantly by CS_2 administration (Table 6).

Effects of repeated administration of CS_2. Hepatotoxicity and P-450-dependent monooxygenase activities were determined after repeated administration of 30 and 300 mg/kg (p.o.) of CS_2 for 2 weeks.

The gross appearance of the liver in the CS_2-treated groups was almost normal. Plasma GPT activity, liver calcium content and body weight gain

Table 2. Changes of the liver microsomal cytochrome P-450-containing monooxygenase system 1 hr after administration of CS_2

	Control	CS_2 (3 mg/kg)	CS_2 (30 mg/kg)
Cytochrome P-450 content	0.800 ± 0.018	0.764 ± 0.014	0.668 ± 0.047*
(nmoles/mg protein)	(100)	(96)	(84)
NADPH–cytochrome P-450 reductase activity	4.24 ± 0.18	2.94 ± 0.40*	1.88 ± 0.13*
(nmoles cyt. P-450 reduced/mg protein/min)	(100)	(69)	(44)
NADPH–cytochrome *c* reductase activity	79.6 ± 4.9	79.9 ± 1.4	77.6 ± 5.5
(nmoles cyt. *c* reduced/mg protein/min)	(100)	(100)	(98)
Peroxidase activity			
Aniline hydroxylation	5.20 ± 0.45	4.60 ± 0.58	3.70 ± 0.05*
(nmoles *p*-aminophenol formed/mg protein/min)	(100)	(88)	(71)
Tetramethylphenylenediamine (TMPD) oxidation	35.3 ± 3.1	27.6 ± 4.5†	16.2 ± 3.3*
(nmoles Wurster's blue free radical formed/mg protein/min)	(100)	(78)	(46)
Integral monooxygenase reaction			
7-Ethoxycoumarin *O*-dealkylase activity	0.776 ± 0.050	0.391 ± 0.046*	0.210 ± 0.012*
(nmoles 7-hydroxycoumarin formed/mg protein/min)	(100)	(50)	(27)
7-Ethoxyresorufin *O*-dealkylase activity	0.099 ± 0.009	0.073 ± 0.008*	0.031 ± 0.005*
(nmoles resorufin formed/mg protein/min)	(100)	(74)	(31)
Substrate-induced spectral changes			
Hexobarbital (5 mM)	6.5 ± 0.9	8.0 ± 1.2	7.5 ± 1.6
($\Delta O.D._{385-419}$: $\times 10^{-3}$/mg protein)	(100)	(123)	(115)
SKF-525A (0.1 mM)	16 ± 1	16 ± 1	16 ± 1
($\Delta O.D._{385-420}$: $\times 10^{-3}$/mg protein)	(100)	(100)	(100)
Aniline (5 mM)	26 ± 1	23 ± 2	20 ± 1*
($\Delta O.D._{430-392}$: $\times 10^{-3}$/mg protein)	(100)	(88)	(77)
Nicotinamide (40 mM)	25 ± 3	19 ± 2†	17 ± 1*
($\Delta O.D._{425-390}$: $\times 10^{-3}$/mg protein)	(100)	(76)	(68)

Microsomal preparation was carried out by using the pooled livers from three mice. Values represent the means ± S.D., N = 3 or 4. Values in parentheses are the percentages of the controls.
*,† Significantly different from the controls at * $P < 0.01$, and † $P < 0.05$.

were all within the normal range (data not shown).

Relatively small decreases in the P-450 content and drug-metabolizing enzyme activities were observed in a dose-dependent manner 24 hr after the last administration of CS_2 (Fig. 2, open columns). To examine the susceptibility of such mice to CS_2, half of the animals in each group were again challenged with 30 mg/kg of CS_2 and killed 1 hr later. The results shown in Fig. 2 (dotted columns) indicate that susceptibility to 30 mg/kg of CS_2 was almost unchanged even after the repeated administration of a ten times higher dose of CS_2. Thus, the suppressive action of CS_2 on microsomal drug metabolism *in vivo* seems to be rather reversible and not cumulative with low dosage levels.

DISCUSSION

Although it is well known that CS_2 impairs the liver drug-metabolizing enzyme system under various experimental conditions [1, 6–14], detailed knowledge on the profile of the enzyme inhibition *in vivo* still seems to be lacking.

In the present study, administration of fairly low doses of CS_2 (3 and 30 mg/kg, p.o.) to mice caused rapid and dose-dependent reductions in the cyto-

Table 3. Loss of microsomal total heme and cytochrome P-450 content 1 hr after CS_2 administration

	Total heme content (nmoles/mg protein)	Cytochrome P-450 content (nmoles/mg protein)
Control	1.73 ± 0.11	1.072 ± 0.051
CS_2 (30 mg/kg)	1.62 ± 0.11 (−0.11)	0.865 ± 0.059* (−0.21)
CS_2 (300 mg/kg)	1.56 ± 0.03 (−0.17)	0.760 ± 0.026* (−0.31)

Microsomes were isolated from well-perfused livers of three mice for each sample and washed once. Data are given as means ± S.D. (N = 6 for control and N = 3 for CS_2-treated groups). Values in parentheses are the difference from the controls.
* Significantly different from control at $P < 0.01$.

Table 4. Effects of CS_2 on the components of microsomal cytochrome b_5-containing electron transport system

	Cytochrome b_5 content*	NADH–ferricyanide reductase activity†	NADH–cytochrome *c* reductase activity‡
Control	0.37 ± 0.02	5.82 ± 0.48	0.97 ± 0.10
CS_2 (30 mg/kg)	0.33 ± 0.03	5.23 ± 0.17	1.09 ± 0.03
CS_2 (300 mg/kg)	0.37 ± 0.06	5.37 ± 0.49	1.39 ± 0.23§

Mice were killed 1 hr after CS_2 administration. Microsomes were isolated from three pooled livers. Values are the means ± S.D., N = 3.

* Expressed in nmoles/mg protein.

† Expressed in μmoles ferricyanide reduced/mg protein/min.

‡ Expressed in μmoles cytochrome c reduced/mg protein/min.

§ Significantly different from the control at $P < 0.01$.

Table 5. Effects of CS_2 on phenylthiourea *S*-oxidase, *N*,*N*-dimethylaniline *N*-oxidase and *N*,*N*-dimethylaniline *N*-demethylase activities

	Octylamine* (3 mM)	Phenylthiourea *S*-oxidase activity†	*N*,*N*-Dimethylaniline *N*-oxidase activity‡	*N*,*N*-Dimethylaniline *N*-demethylase activity§
Control	−	188 ± 45	450 ± 89	384 ± 7
	+	354 ± 42	499 ± 84	21 ± 7
CS_2 (30 mg/kg)	−	177 ± 9	529 ± 60	197 ± 25‖
	+	342 ± 21	527 ± 59	22 ± 5
CS_2 (300 mg/kg)	−	169 ± 30	510 ± 61	118 ± 2‖
	+	325 ± 13	511 ± 60	7 ± 0¶

Mice were killed 1 hr after CS_2 administration. Microsomes were prepared from the pooled livers of two mice. Values represent the means ± S.D., N = 3.

* Enzyme activities were measured in the absence or presence of 3 mM octylamine.

† Expressed in nmoles substrate oxidized/mg protein/hr.

‡ Expressed in nmoles *N*-oxide formed/mg protein/hr.

§ Expressed in nmoles of HCHO formed/mg protein/hr.

‖,¶ Significantly different from control at $\|P < 0.01$, and $\P P < 0.05$.

Table 6. Other microsomal or non-microsomal enzyme activities and constituents that are not affected by CS_2

	Control	CS_2 (30 mg/kg)	CS_2 (300 mg/kg)
UDP-glucuronyltransferase activity (nmoles 3-methyl-2-nitrophenol glucuronide formed/mg protein/min)	4.00 ± 0.21	3.96 ± 1.42	3.53 ± 0.76
Heme oxygenase activity (nmoles bilirubin formed/mg protein/hr)	0.290 ± 0.017	0.293 ± 0.032	0.345 ± 0.059
Glucose-6-phosphatase activity (μmoles P_i released/mg protein/hr)	16.8 ± 1.0	17.9 ± 0.3	18.0 ± 0.5
Conjugated diene levels ($\Delta O.D._{243}$/mg phospholipids/ml)	0.140 ± 0.016	0.128 ± 0.003	0.142 ± 0.017
GSH *S*-transferase activity (μmoles substrate conjugated/mg protein soluble fraction/min)			
3,4-Dichloronitrobenzene	0.065 ± 0.007	0.066 ± 0.003	0.056 ± 0.006
2,4-Dinitrochlorobenzene	4.73 ± 0.67	4.89 ± 0.64	5.27 ± 0.29
p-Nitrophenoxypropane	0.116 ± 0.004	0.118 ± 0.010	0.116 ± 0.010
GSH content (mg/g liver)	2.84 ± 0.25	2.94 ± 0.18	3.05 ± 0.24

Mice were killed 1 hr after CS_2 administration. Microsomes were isolated from two or three pooled livers. Values are the means ± S.D. (N = 3–4, except for GSH content in which 6 mice per group were used).

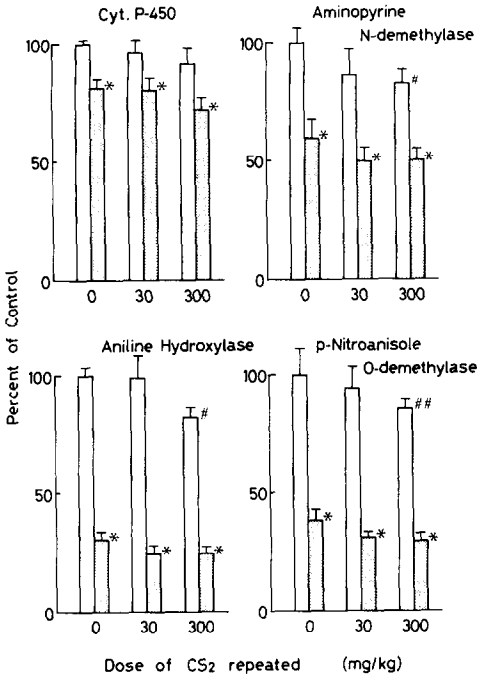

Fig. 2. Effects of repeated administration of CS_2 on liver microsomal cytochrome P-450 content and some drug-metabolizing enzyme activities. CS_2 was given orally at 0, 30 and 300 mg/kg, daily for 2 weeks. Each group of mice was subdivided into two groups 24 hr after the last administration. Each one of the subdivided groups received an additive challenge of 30 mg/kg of CS_2 and was killed 1 hr later (dotted columns), with the other half as a control (open columns). Values are the means ± S.D. of five mice. Absolute values for the control group receiving neither repeated nor single administration of CS_2 were as follows: cytochrome P-450, 0.858 ± 0.015 nmoles/mg protein; aminopyrine *N*-demethylase, 0.425 ± 0.026 μmoles formaldehyde formed/mg protein/hr; aniline hydroxylase, 74.3 ± 2.8 nmoles *p*-aminophenol formed/mg protein/hr; and *p*-nitroanisole *O*-demethylase, 128.7 ± 14.6 nmoles *p*-nitrophenol formed/mg protein/hr. Key: (*)Significantly different from the corresponding control group receiving no additive challenge of CS_2 at $P < 0.01$. (#, ##)Significantly different from the control group receiving no repeated CS_2 at (#)$P < 0.01$ or (##)$P < 0.05$.

chrome P-450 content and P-450-associated activities (NADPH–P-450 reductase, peroxidase, drug-metabolizing enzyme activities and spectral changes due to substrate binding), whereas contents or activities of other microsomal enzymes such as cytochrome b_5, NADH–ferricyanide reductase, NADPH–cytochrome *c* reductase, flavin-containing monooxygenase, UDP-glucuronyltransferase, G-6-Pase and heme oxygenase, and supernatant GSH *S*-transferase were not affected significantly even at a dose of 300 mg/kg of CS_2. Microsomal carboxyesterase [39] and epoxide hydrolase [11] activities are also reported to be unaffected. Thus, impairment of P-450 may be an early and rather selective event in the action of CS_2.

Since the doses used in these experiments were much lower than the hepatotoxic dose (>2 g/kg, p.o.), the losses of P-450 and the associated enzyme activities may not result from a pathological effect on the liver. Supporting this are the fact that (1) microsomal G-6-Pase activity, which is known to be very sensitive to various hepatotoxicants [40, 41], was unaffected at a dose up to 300 mg/kg of CS_2, (2) conjugated diene levels of microsomal lipids were not increased by CS_2 in the present experimental conditions, although lipid peroxidation is involved in the development of CCl_4 hepatotoxicity [42] and CS_2 is also reported to enhance microsomal lipid peroxidation in phenobarbital-pretreated rats [5, 19], and, in addition, (3) liver GSH content, which is known to be depleted by some hepatotoxic agents [43], remained unchanged. Microsomal heme oxygenase activity, a heme-degradating enzyme, was also unchanged.

Functional damage of P-450 was greater than expected simply from the measurable loss of the hemoprotein as determined by the usual chemical method [22], i.e. spectral change due to carbon monoxide binding to dithionite reduced microsomes. This was shown by the fact that, following CS_2 administration, enzyme activities associated with P-450, such as NADPH–P-450 reductase, peroxidase and various drug-metabolizing enzyme activities, and in addition, spectral changes (ΔO.D. values) induced by some type 2 substrates, were all suppressed to a greater extent than the apparent loss of P-450 content (Table 2). These findings indicate that, after the administration of CS_2, even the measurable P-450 molecules may be somewhat injured, possibly at the vicinity of the heme moiety, which may lead to a decrease in the functional abilities of P-450 to bind substrates, to accept electrons from NADPH through the flavoprotein, and to oxidize substrates in the final oxidation step. Consequently, the total monooxygenase reaction may be most affected, as represented by the marked decrease in aniline hydroxylation and other drug-metabolizing enzyme activities. Increased NADH–cytochrome *c* reductase activity, which is evident at a high dose of CS_2, may be partly explained by a reduced electron flow from cytochrome b_5 to impaired P-450.

Since the loss of total heme content accounted for only a part of the P-450 loss (Table 3), the impairment of P-450 molecules would proceed as follows: first, loss of functional activity by slight modification of P-450 molecules, but without loss of measurable P-450 content; second, destruction of P-450, but with heme structure being preserved; and finally, heme degradation.

On the other hand, Torres *et al.* [14] suggested the presence of a CS_2-sensitive population of P-450 isozymes. We also attempted to check this point by measuring deethylation of 7-ethoxycoumarin and 7-ethoxyresorufin, the former being preferentially dealkylated by phenobarbital-inducible P-450, but the latter by 3-methylcholanthrene-inducible P-448 in rats [27]. However, no clear difference between the degrees of the suppression of both dealkylating activities was observed after CS_2 administration. Thus, further experiments may be necessary to prove if this mechanism is involved or not.

Reversibility of the inhibition may be an important factor in a toxicological sense. In agreement with the results of exposure experiments by Fruendt *et al.* [10], the inhibition of drug-metabolizing enzyme

activities following a single administration of fairly low doses of CS_2 continued for several hours, but was completely reversed by between 12 and 24 hr. Järvisalo *et al.* [44], on the other hand, reported that mice subacutely exposed to CS_2 exhibit a decreased responsiveness of microsomal enzymes to CS_2. In our experiments, however, the P-450 content and drug-metabolizing enzyme activities dependent on this hemoprotein were still sensitive and restorable even after the repeated administration of CS_2. Because of the difference in the route of administration, comparison of both results is difficult. The mechanism of the loss of P-450 is proposed to involve irreversible covalent binding of sulfur atoms liberated during oxidative desulfuration of CS_2 [15–18]. Therefore, the *in vivo* reversibility observed in the present study may be noteworthy. Whether mechanisms of the restoration involve new synthesis of the hemoprotein or repair of injured P-450 or both are of interest to us.

Finally, CS_2 at low oral doses may serve experimentally as a rather selective inhibitor of P-450 *in vivo* and seems to be superior to the well-known P-450 inhibitors, SKF-525A, piperonyl butoxide and metyrapone, which are reported to have a variety of non-specific actions *in vivo* [45, 46]. It has already been reported that CS_2 and CS_2-producing agents like DTC suppress P-450-dependent monooxygenase reactions not only in the liver [1, 39], but also in the kidney [47, 48] and lung [49], and thereby may modify toxicities or pharmacologic actions of chemical agents in organs that contain this hemoprotein. In addition, CS_2 may also be useful in distinguishing flavin-containing monooxygenase reactions from P-450-dependent reactions, since CS_2 does not inhibit the former monooxygenase.

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