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LOSS OF 3-METHYLCHOLANTHRENE-INDUCIBLE FORM OF CYTOCHROME P-450 IN LIVER MICROSOMES FOLLOWING ADMINISTRATION OF CARBON DISULFIDE IN C57BL/6 Cr MICE

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Abstract—Early after administration of CS_2 to untreated, phenobarbital (PB)- and 3-methylcholanthrene (3-MC)-pretreated C57BL/6 Cr mice: (1) the loss of cytochrome P-450 was enhanced by pretreatment with both inducers, but to a greater extent with 3-MC; (2) the decrease in 7-ethoxyresorufin (ER) O-deethylation activity was much greater than that of cytochrome P-450 in untreated and PB-pretreated mice, but both paralleled values in 3-MC-pretreated mice, in which ER O-deethylation activity was induced markedly, (3) the peak of the carbon monoxide-difference spectrum of microsomal reduced cytochrome P-450 (about 448 nm) in 3-MC-pretreated mice shifted toward 450 nm after administration of increasing doses of CS_2; (4) similar tendencies were observed *in vitro* in items (1) to (3); (5) electrophoresis of microsomal proteins revealed a loss of each protein band induced by PB and 3-MC following CS_2 administration; (6) in the reconstituted monooxygenase system using partially purified cytochrome P-450 and P-448 forms from PB- and 3-MC-treated rats, CS_2 suppressed the drug-metabolizing activities exhibited by the P-448 form but had little or no effect on those by the P-450 form; and (7) in *n*-octylamine difference spectra of microsomes, loss of the 3-MC-induced high spin form of cytochrome P-450 was evident. These results indicate that the 3-MC-inducible form of cytochrome P-450 was more susceptible to CS_2 than the PB-inducible form. The hepato-necrogenic action of CS_2 was not enhanced by PB or 3-MC pretreatment in mice.

Several lines of evidence indicate that administration of CS_2 decreases liver microsomal drug-metabolizing enzyme activities [1–5]. Phenobarbital (PB) pretreatment is reported to increase the loss of liver cytochrome P-450 content as well as hepatotoxicity following CS_2 administration in rats [1, 6–8]. The *in vitro* studies on the covalent binding of radiolabeled CS_2 suggest that CS_2 is metabolized by the microsomal cytochrome P-450-containing monooxygenase system and the reactive sulfur atoms released covalently bind to microsomal membranes, causing the destruction of the hemoprotein and hepatotoxicity [9–12].

On the other hand, Hunter and Neal [3] examined the ability of various thiono-sulfur-containing compounds including CS_2 to reduce liver microsomal cytochrome P-450 content and benzphetamine metabolism in PB- and 3-methylcholanthrene (3-MC)-pretreated rats, but no clear difference was found between such treatments. Obrebska *et al.* [13] reported that CS_2 produces a slightly smaller decrease of cytochrome P-450 in 3-MC-pretreated rats than in PB-pretreated rats, although hydroxylation activities of biphenyl and aniline were affected more in 3-MC-pretreated rats. Torres *et al.* [14] suggested an alteration of a CS_2-sensitive population of liver cytochrome P-450 hemoproteins, although this has not been demonstrated. Thus, it is still uncertain whether there is any inducer dependency in the action of CS_2 or what forms of cytochrome P-450 are involved.

In a recent study using normal ddY mice [15], we found that CS_2 produces a decrease in microsomal cytochrome P-450-dependent monooxygenase activities, NADPH–cytochrome P-450 reductase (but not NADPH–cytochrome *c* reductase) activity, and the magnitude of spectral changes induced by some substrates to a greater extent compared with the loss of cytochrome P-450 content. An explanation for this may be that cytochrome P-450 molecules are functionally more impaired than expected from the chemically determined loss of the hemoprotein content, but another possibility, that destruction of specific forms of cytochrome P-450 could also be involved, has been suggested.

We examined the profile of the cytochrome P-450 impairment following CS_2 administration in PB-inducible ddY strain mice and in 3-MC- and PB-inducible C57BL/6 Cr (BL) mice. The action of CS_2 in the reconstituted monooxygenase system was also tested using partially purified cytochrome P-450 and P-448 forms from the rat liver.

MATERIALS AND METHODS

Animals and treatments. Male SPF-grade mice of the ddY and BL strain (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Japan), 6 weeks old, were used, with food and water given

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ad lib. throughout the experimental period. Pretreatments with inducers were as follows: PB was dissolved in saline and given i.p. at a dose of 80 mg/kg once daily for 3 days and CS_2 was given 24 hr after the last administration; 3-MC (100 mg/kg, dissolved in olive oil) was given i.p. as a single dose 3 days before CS_2 administration. CS_2 (>99%, GC, Wako Pure Chemicals, Japan) was dissolved in olive oil and given p.o. at doses of 3, 30, 300 and 2000 mg/kg. All solutions were freshly prepared and given at a volume of 0.05 ml/10 g body weight. Control mice received the vehicle alone.

Preparation of microsomes and enzyme assays. Usually 1 hr after administration of CS_2, mice were killed by cervical dislocation and exsanguinated. The livers were well perfused *in situ* with cold 0.15 M KCl solution, and 20% liver homogenates in 0.15 M KCl–20 mM potassium phosphate (pH 7.4)–1 mM EDTA were prepared. The homogenate was centrifuged at 9,000 *g* for 20 min, and the supernatant fraction without the fluffy layer was further centrifuged at 105,000 *g* for 1 hr. The microsomal precipitate was suspended in 0.1 M potassium phosphate (pH 7.4)–0.1 mM EDTA at a concentration of 20 mg protein/ml and used for various assays while fresh. Protein was determined by the method of Lowry *et al.* [16].

Microsomal cytochrome P-450 and b_5 contents were determined by the method of Omura and Sato [17]. The molar extinction coefficient of 91 cm^{-1} mM^{-1} was used for calculation of the cytochrome P-450 content in any microsomal sample. 7-Ethoxyresorufin (ER) and 7-ethoxycoumarin (EC) O-deethylation, aniline (AN) hydroxylation and aminopyrine (AP) N-demethylation activities were assayed as described previously [5, 15].

Substrate-induced difference spectra of microsomes, which were mainly examined 2 hr after p.o. administration of 2 g/kg of CS_2, were recorded with 1 mM *n*-octylamine in 0.1 M potassium phosphate (pH 7.4)–50% glycerol, 1 mM metyrapone and 0.1 mM SKF-525A in 0.1 M potassium phosphate (pH 7.4) at a protein concentration of 2 mg/ml.

Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis (SDS–PAGE) of microsomes was conducted according to the method of Laemmli [18]. Microsomes (1 mg protein/ml) were dissolved in 50 mM Tris–HCl buffer (pH 6.8) containing 20% glycerol, 1% SDS, 1% β-mercaptoethanol, 1 mM EDTA, and 0.001% bromophenol blue and heated for 2 min at 100°. The separating gel (14 × 10 × 0.1 cm) contained 7.5% acrylamide. Electrophoresis was carried out at 10 and 30 mA/gel during stacking and separation respectively. The gels were fixed in 25% isopropylalcohol–10% acetic acid, stained in the fixative containing 0.05% Coomassie blue R-250, destained in 10% isopropylalcohol–10% acetic acid and immersed in 3% glycerol. The wet gel was placed on a glass plate, covered with a cellophane sheet, and dried in air. Densitometric scanning was done with a Shimadzu dual-wavelength TLC scanner CS-910.

Experiments with isolated microsomes. The complete reaction mixture contained 2 mg protein/ml of microsomes, 200 μM NADPH with its generating system (10 mM sodium isocitrate, 5 mM $MgCl_2$ and 0.25 units/ml of isocitrate dehydrogenase), 10^{-5} to 10^{-3} M CS_2 (added as methanol solution in a volume of 10 μl) and 2 μM diphenyl-*p*-phenylene diamine (DPPD) in a final volume of 10 ml of 0.1 M potassium phosphate (pH 7.4)–0.1 mM EDTA. DPPD was added to minimize the degradation of cytochrome P-450 due to endogenous lipid peroxidation. Control mixtures contained no NADPH and, at a zero concentration of CS_2, 10 μl of methanol was added. All reaction mixtures were prepared in duplicate in 50-ml capped flasks. Incubation was carried out at 37° for 30 min with shaking, and the reaction was stopped in ice. The microsomes were precipitated by centrifugation, suspended in 2.0 ml of the phosphate buffer, and assayed for protein, cytochrome P-450 content, and drug-metabolizing activities.

Experiments with reconstituted monooxygenase system. Cytochrome P-450 and P-448 forms were purified from livers of PB- and 3-MC-treated male Sprague–Dawley rats [hereafter referred to as P-450 (PB) and P-448 (3-MC)] according to the method of Guengerich and Martin [19]. Each major B_2 fraction was used. Our final preparations of P-450 (PB) and P-448 (3-MC), containing 9 and 13 nmol of cytochrome P-450/mg protein, respectively, had the absorption maxima of the reduced cytochrome P-450–carbon monoxide complex at approximately 450.0 and 447.5 nm respectively. By SDS–PAGE, P-450 (PB) exhibited virtually a single band at about 51,000 daltons, while P-448 (3-MC) showed a major band at about 55,000 daltons and a minor 54,000 dalton band (Fig. 1). These values of optical characteristics and subunit molecular weights are within the range of several reported values [20]. Judging from the specific hemoprotein content and electrophoresis pattern, both preparations were not completely pure; however, no mutual contamination was observed electrophoretically. NADPH–cytochrome P-450 reductase was purified from PB-treated rats by the method of Guengerich and Martin [19] and was electrophoretically pure (Fig. 1). Reconstitution and incubation with CS_2 were done as follows. P-450 (PB) or P-448 (3-MC) (each 2.5 nmol), NADPH-cytochrome P-450 reductase (0.5 units) and sonicated lauryl lecithin (50 μg) were mixed in a volume of 0.1 ml and, after a few minutes, were suspended in 0.1 M potassium phosphate buffer (pH 7.4) containing deoxycholate (50 μg), EDTA (1 mM), $MgCl_2$ (15 mM), NADPH (200 μM) with the generating system and CS_2 (5 mM) in a final volume of 0.5 ml. This mixture was prepared in a 10-ml capped test tube and incubated at 37° for 30 min with shaking. The reaction was stopped in an ice bath, and aliquots of the reaction mixture were directly assayed for drug-metabolizing activities.

Hepatotoxicity test. Untreated, PB- and 3-MC-pretreated mice received 0.5 to 1.5 g/kg of CS_2 i.p. and were killed by exsanguination 24 hr later. Blood was collected in a heparinized syringe, and plasma glutamic oxaloacetic transaminase (GOT) [21] and lactic dehydrogenase (LDH) [22] activities were measured. A portion of the liver was sonicated in 8% trichloroacetic acid (TCA), and the TCA supernatant fraction was determined for calcium by the calcein-fluorometric method of Von Hattingberg *et al.* [23].

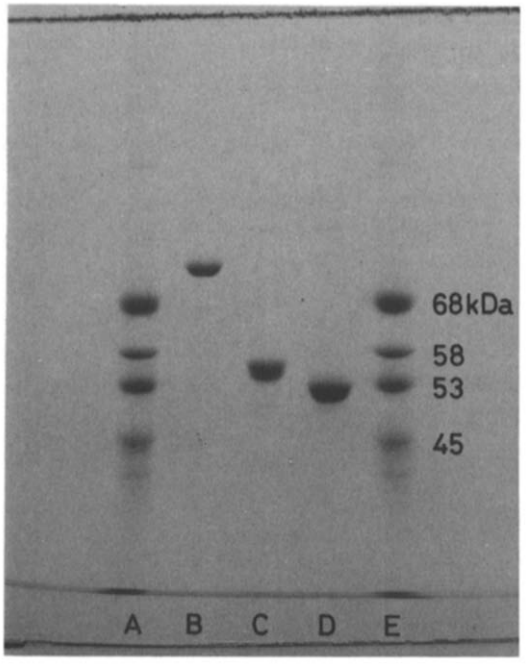

Fig. 1. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis of purified NADPH–cytochrome P-450 reductase, cytochrome P-450(PB) and P-448(3-MC) preparations from rat liver microsomes. For details, see text. The reductase (0.5 μg protein, well B), cytochrome P-448(3-MC) (0.8 μg, well C) and cytochrome P-450(PB) (1.0 μg, well D) were applied on 5-mm wide wells, Protein standards (wells A and E) used were bovine serum albumin (68 kD), catalase (58 kD), glutamate dehydrogenase (53 kD) and ovalbumin (45 kD).

Statistics. Statistical analysis was done by the method of Bonferoni as described by Wallenstein *et al.* [24] after the one-way analysis of variance, and a $P < 0.05$ was considered significant.

RESULTS

Loss of microsomal cytochrome P-450 and drug-metabolizing activities in vivo. The suppressive action of CS_2 on the liver microsomal cytochrome P-450-containing monooxygenase system was examined in untreated or inducer-pretreated ddY and BL mice 1 hr after administration of CS_2 when the monooxygenase activity decreases rapidly [15].

As shown in Fig. 2, in BL mice, PB-treatment increased the cytochrome P-450 content, AN hydroxylation and AP N-demethylation activities in parallel, whereas ER and EC O-deethylation activities were increased to a greater extent. Administration of CS_2 to untreated and PB-pretreated animals produced the following changes: (1) loss of cytochrome P-450, which was slightly greater in PB-pretreated mice, was generally less as compared with the decreases in drug-metabolizing activities, (2) the decrease in ER O-deethylation activity was greatest in both untreated and PB-pretreated mice, especially in the latter, whereas EC O-deethylation was less affected in PB-pretreated mice, and (3) AN hydroxylation activity also decreased to a considerable degree as compared with the loss of cytochrome P-450 irrespective of the PB pretreatment, whereas the decrease in AP N-demethylation activity was similar with that of the hemoprotein content.

In 3-MC-pretreated BL mice (1) the degree of the loss of cytochrome P-450 was greater than that observed with untreated and PB-pretreated mice, (2) ER O-deethylation activity, which was increased approximately thirty times by 3-MC pretreatment, was much less affected as compared with the untreated and PB-pretreated mice, and (3) the degree of the suppression of ER and EC O-deethylation and AP N-demethylation activities was similar to the loss of cytochrome P-450, although the decrease in AN hydroxylation activity was still greater.

Originally these experiments were started with ddY mice. Untreated and PB-pretreated ddY mice responded to CS_2 almost as BL mice did (data not shown), but a marked variation in the degree of the induction by 3-MC was observed with ddY mice. In the following study, therefore, we used the BL-mouse strain, which has been shown to be genetically responsive to 3-MC invariably accompanying the induction of cytochrome P-448 [25, 26].

In vitro *action of CS_2 on isolated microsomes*. Microsomes isolated from PB- or 3-MC-pretreated mice (PB-microsomes or 3-MC-microsomes) were incubated with CS_2 in the presence or absence of NADPH. In the absence of NADPH, 10^{-5} to 10^{-3} M CS_2 had little effect on microsomal cytochrome P-450 content and drug-metabolizing activities. In the presence of NADPH, however, these variables were variably suppressed (Fig. 3). With PB-microsomes, the suppression of drug-metabolizing activities, especially ER O-deethylation and AN hydroxylation, was greater as compared with the loss of cytochrome P-450. In the case of 3-MC-microsomes, loss of cytochrome P-450 was greater than that in PB-microsomes and was almost paralleled by the decrease in drug-metabolizing activities except for AN hydroxylation which was suppressed to a greater extent. These *in vitro* observations were nearly in accordance with the *in vivo* results.

Microsomal spectral studies. During the preceding *in vivo* experiments, the peak of CO difference spectrum of the reduced 3-MC microsomes, about 448 nm, shifted to a higher wavelength after administration of CS_2, which is shown in Fig. 4 together with the data obtained under a higher dose (2 g/kg) of CS_2. *In vitro*, 10^{-3} M CS_2 also shifted the peak to 450 nm only in the presence of NADPH.

The following spectral studies were done with untreated, PB- and 3-MC-microsomes isolated 2 hr after administration of a high dose of CS_2 (2 g/kg, p.o.), to fully lower the cytochrome P-450 level. Under such conditions, loss of cytochrome P-450 was still greater in both PB- and 3-MC-microsomes, but the cytochrome b_5 content was unaffected (Fig. 5).

The magnitude of spectral changes induced by metyrapone and SKF-525A was increased by PB pretreatment and reduced by additional CS_2 administration nearly in parallel with cytochrome P-450 content. However, 3-MC pretreatment increased only the metyrapone-induced spectral change, and rather

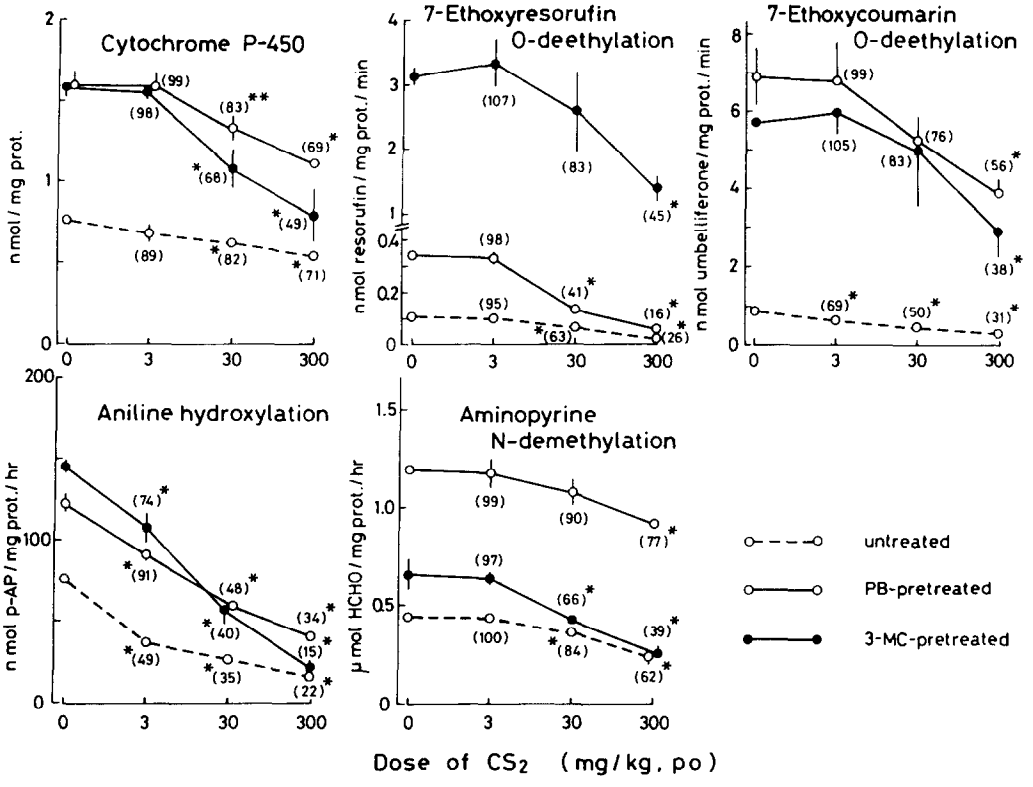

Fig. 2. Effects of CS_2 administration on microsomal cytochrome P-450 content and drug-metabolizing activities in untreated, PB- and 3-MC-pretreated BL mice. Mice were killed 1 hr after administration of CS_2. Each microsomal sample was prepared from pooled livers of two mice. For details, see text. Each point represents mean ± SD (N = 3). Key: Significantly different from the controls at $^*P < 0.01$ and $^{**}P < 0.05$.

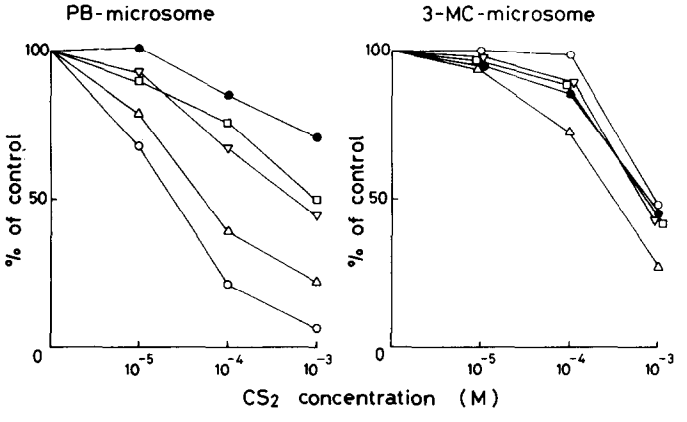

Fig. 3. *In vitro* effects of CS_2 on microsomal cytochrome P-450 content and drug-metabolizing activities in isolated microsomes. Microsomes were prepared from pooled livers of fifteen PB- and fifteen 3-MC-treated BL mice and incubated in the presence of NADPH and CS_2. For details, see text. With CS_2 alone, i.e. in the absence of NADPH, no suppression was observed. The values are expressed as percent of the controls. Each point represents the mean of duplicate assays, in which variations were kept within 5%. Key: (●) cytochrome P-450; (○) 7-ethoxyresorufin O-deethylation; (□) 7-ethoxycoumarin O-deethylation; (△) aniline hydroxylation; and (▽) aminopyrine N-demethylation. Control values of these items for PB- and 3-MC-microsomes were 1.62 and 1.77 nmol cytochromne P-450/mg protein, 0.23 and 2.14 nmol resorufin/mg protein/min, 6.33 and 7.46 nmol umbelliferone/mg protein/min, 158 and 157 nmol *p*-aminophenol/mg protein/hr, and 1.77 and 0.69 μmol formaldehyde/mg protein/hr respectively.

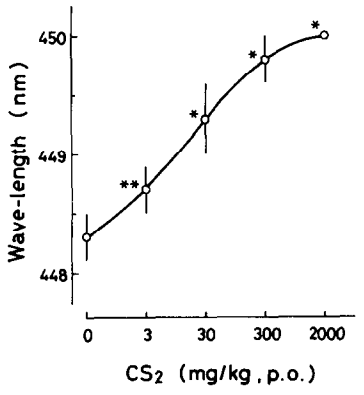

Fig. 4. Shift of the peak wavelength of CO difference spectrum of dithionite-reduced microsomes following administration of CS_2 in 3-MC-pretreated BL mice. The data were obtained from spectral drawings used for the determination of cytochrome P-450 shown in Figs. 2 and 5. Each point represents the mean SD (N = 3 at 0–300 mg/kg and N = 2 at 2 g/kg). A single or double asterisk indicates values significantly different from the control value at $^*P < 0.01$ and $^{**}P < 0.05$.

reduced the SKF-525A-induced change. CS_2 administration considerably reduced the former spectral change but only slightly the latter (Fig. 5).

In *n*-octylamine difference spectra (Fig. 6), about a 3-fold increase of ΔA (500–392 nm) in 3-MC-microsomes and its marked decrease after CS_2 administration were noticed. This resulted in a decrease of the ΔA (500–410):ΔA(500–392) ratio of 3-MC-microsomes and its considerable increase after CS_2. On the other hand, PB pretreatment alone increased the ratio, which was further increased after CS_2 administration due to a greater decrease in ΔA(500–392). Decrease in ΔA(peak–trough) following CS_2 administration was greater in 3-MC-microsomes.

SDS–PAGE analysis. Pooled microsomes used in the spectral assays were subjected to electrophoresis and densitometry (Fig. 7). In untreated microsomes, the effect of CS_2 administration on the protein bands (peaks "a", "b", "c" and "d") located at cytochrome P-450 hemoprotein region was slight. In PB-microsomes, proteins at bands "b" and "c" increased and a new band "e" appeared on the higher molecular weight side of band "a". Among them, bands "b" and "c" were especially decreased by CS_2. 3-MC

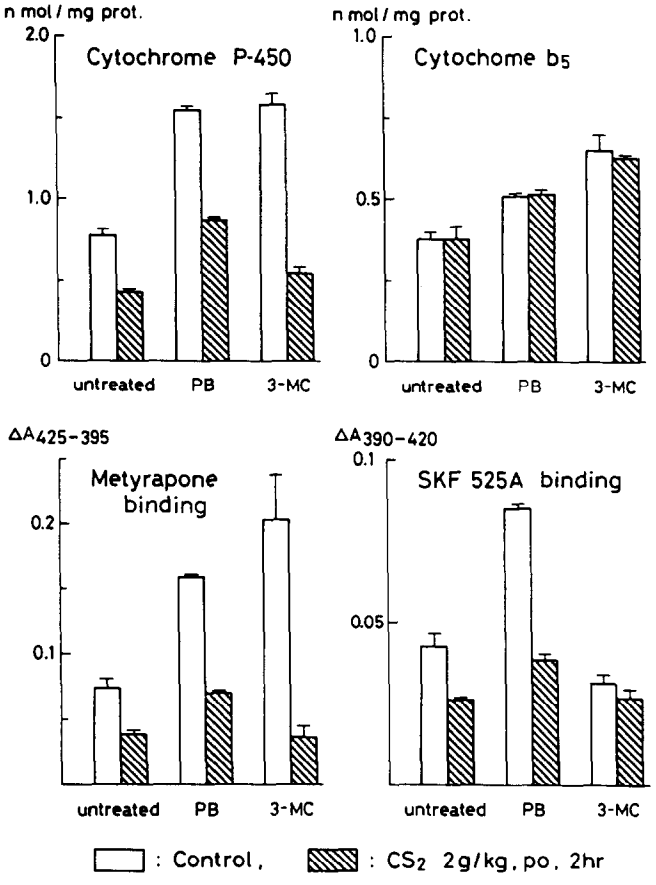

Fig. 5. Effects of a high dose of CS_2 on microsomal hemoprotein content and the magnitude of metyrapone- and SKF-525A-induced spectral changes in untreated, PB- and 3-MC-pretreated BL mice. Mice were killed 2 hr after administration of 2 g/kg, p.o., of CS_2. Each microsomal sample was prepared from pooled livers of four to five mice. Metyrapone and SKF-525A difference spectra of oxidized microsomes were measured at 2 mg protein/ml. Bars represent the means ± SD (N = 4 for untreated group and N = 2 for other groups).

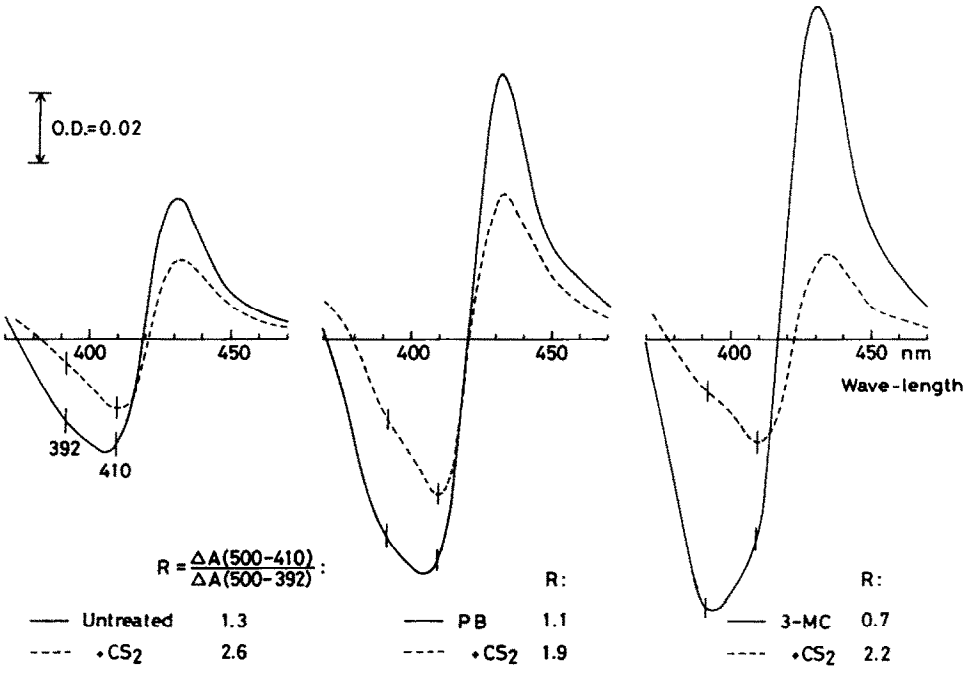

Fig. 6. *In vivo* effects of CS_2 on *n*-octylamine difference spectra of liver microsomes in untreated, PB- and 3-MC-pretreated BL mice. Microsomal samples were the same as described in the legend of Fig. 5. Typical spectral drawings are shown (protein concentration: 2 mg/ml). The *R* values are the means.

markedly and specifically induced the protein at band "a", which was located near the reported subunit molecular weight (55 kD) for purified cytochrome P-448 from 3-MC-treated BL mice [26]. This induced protein band "a" was more clearly decreased by CS_2.

Action of CS_2 in the reconstituted monooxygenase system. For convenience, cytochrome P-450 preparations used in this study, P-450 (PB) and P-448 (3-MC), were obtained from PB- and 3-MC-treated rats. Phospholipid vesicles embedded with NADPH-

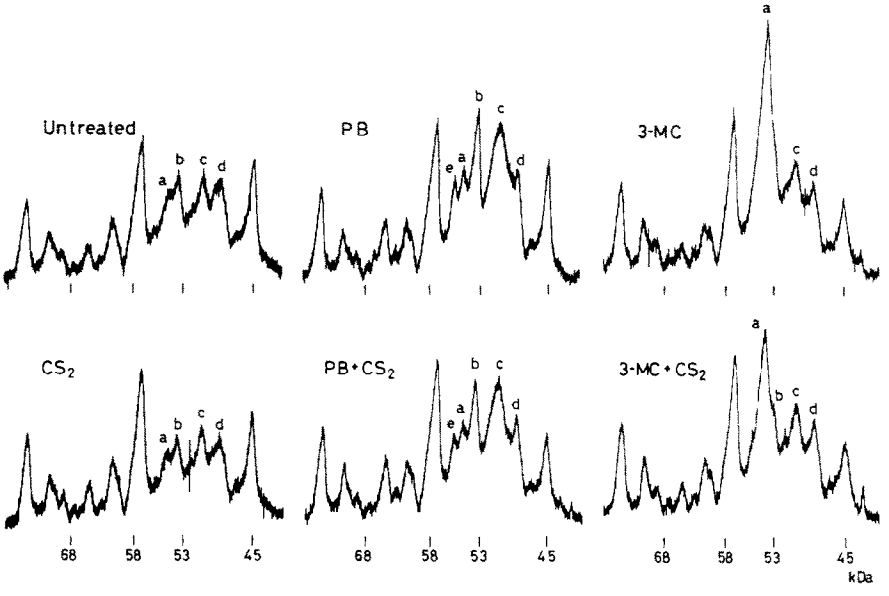

Fig. 7. Effects of CS_2 administration on liver microsomal protein patterns of untreated, PB- and 3-MC-pretreated BL mice. Pooled microsomes described in the legend of Fig. 5 were used. Microsomes (40 μg protein applied on 1-cm wide wells) were subjected to SDS-PAGE, stained, and scanned by densitometer. For details, see text.

Table 1. Effects of CS_2 on some drug-metabolizing activities in the reconstituted monooxygenase system

Cytochrome P-450 preparation	NADPH	CS_2	7-Ethoxyresorufin O-deethylation (nmol resorufin/min)	7-Ethoxycoumarin O-deethylation (nmol 7-hydroxycoumarin/min)	Aniline hydroxylation (nmol *p*-aminophenol/hr)	Aminopyrine N-demethylation (μmol HCHO/hr)
P-450(PB)	−	−	—*	0.262 ± 0.004 (100)	16.2 ± 0.9 (100)	0.146 ± 0 (100)
	−	+	—	0.258 ± 0.004 (99)	16.9 ± 0 (104)	0.147 ± 0.001 (101)
	+	−	—	0.267 ± 0.001 (102)	14.2 ± 0.2 (88)	0.157 ± 0.003 (108)
	+	+	—	0.254 ± 0.006 (97)	11.9 ± 0 (74)	0.145 ± 0 (99)
P-448(3-MC)	−	−	0.296 ± 0.018 (100)	1.85 ± 0.01 (100)	18.0 ± 0.3 (100)	0.093 ± 0 (100)
	−	+	0.288 ± 0.006 (97)	1.91 ± 0.05 (103)	19.6 ± 0.4 (109)	0.094 ± 0.001 (101)
	+	−	0.259 ± 0.009 (88)	1.87 ± 0.04 (101)	17.8 ± 0.8 (99)	0.091 ± 0.002 (98)
	+	+	0.171 ± 0.006 (58)	1.50 ± 0.02 (81)	10.2 ± 0.2 (57)	0.053 ± 0.002 (56)

For details, see text. Activities were expressed per 1 nmol of added cytochrome P-450. Values are given as means ± deviation of duplicate experiments. Values in parentheses are percentages to the controls.
* Activity was too low to be determined.

cytochrome P-450 reductase and P-450 (PB) or P-448 (3-MC) were preincubated in the presence of either or both NADPH and CS_2, and the drug metabolizing activities were measured. As shown in Table 1, when the P-448 (3-MC)-embedded vesicles were preincubated in the presence of both NADPH and CS_2, 19% of the EC O-deethylation activity and about 43% of the ER O-deethylation, AN hydroxylation and AP N-demethylation activities were suppressed. In the P-450 (PB)-reconstituted system, however, EC O-deethylation and AP N-demethylation were unaffected, although AN hydroxylation activity was somewhat decreased. ER O-deethylation was too low to be determined. In the absence of NADPH or CS_2, virtually no decrease in the activities was observed. The reason for a slight decrease in AN hydroxylation in the presence of NADPH alone is unknown.

Hepatotoxicity studies. In rats, PB pretreatment is reported to augment markedly CS_2 hepatotoxicity, producing severe centrilobular necrosis [6, 7]. The effects of PB- and 3-MC pretreatments on CS_2 hepatotoxicity were tested in mice. As shown in Table 2, judging from the plasma GOT and LDH activities

Table 2. Effects of CS_2 on some variables of hepatic necrosis in untreated, PB- and 3-MC-pretreated mice

	Dose of CS_2 (g/kg, i.p.)	Inducer treatment	N	Plasma GOT (KU)	Plasma LDH (units/l)	Liver calcium (μmol/g)
BL Mice	0	Untreated	8	97 ± 21	525 ± 74	0.62 ± 0.05
		PB	4	99 ± 24	486 ± 110	0.66 ± 0.08
		3-MC	4	84 ± 27	446 ± 81	0.67 ± 0.02
	0.5	Untreated	12	82 ± 11	444 ± 53	0.66 ± 0.05
		PB	6	94 ± 31	467 ± 109	0.67 ± 0.04
		3-MC	6	91 ± 20	480 ± 122	0.74 ± 0.11
	1.0	Untreated	12	161 ± 81	517 ± 144	0.77 ± 0.13
		PB	6	183 ± 36	587 ± 85	0.92 ± 0.29
		3-MC	6	131 ± 27	532 ± 149	0.75 ± 0.08
ddY Mice	0	Untreated	10	30 ± 11	514 ± 101	0.65 ± 0.04
	0.5	Untreated	10	32 ± 14	518 ± 152	0.55 ± 0.09
		PB	10	37 ± 11	412 ± 69	0.56 ± 0.07
	1.0	Untreated	10	49 ± 20	460 ± 96	0.88 ± 0.38
		PB	10	75 ± 25	402 ± 112	0.66 ± 0.09
ddY Mice (starved)*	1.5	Untreated	6/10†	299 ± 167	3820 ± 3550	1.12 ± 0.29
		PB	5/10†	237 ± 158	1810 ± 1530	1.15 ± 0.47

Values are given as means ± SD.
* Animals were deprived of food 24 hr before CS_2 administration.
† Number survived.

and liver calcium content, the necrogenic action of CS_2 was weak even at fairly high i.p. doses of CS_2 in normal mice. Neither PB nor 3-MC pretreatment augmented the CS_2 hepatotoxicity in BL mice. PB pretreatment also did not enhance the hepatotoxicity even at a lethal dose of CS_2 in the starved ddY mice.

DISCUSSION

First, the following findings and discussion suggest that the 3-MC-inducible form of cytochrome P-450 may be more susceptible to CS_2 than the PB-inducible form. (1) The decrease in microsomal cytochrome P-450 content after CS_2 administration was greater in 3-MC-pretreated mice than in PB-pretreated mice. The same tendency was observed *in vitro*. (2) In SDS–PAGE analysis, CS_2 administration decreased each protein band induced by 3-MC or PB to a greater extent with the protein band near 55 kD induced by 3-MC. (3) In 3-MC-pretreated mice, the peak wavelength of CO difference spectra of reduced microsomes shifted to the red region after CS_2 administration dose dependently. Administration of carbon tetrachloride, however, which is reported to specifically destroy the PB-inducible cytochrome P-450 form [27], did not produce such a peak shift in 3-MC-pretreated BL mice, even when the microsomal cytochrome P-450 content was decreased to less than half (our unpublished observation). (4) The profile of the suppression of the drug-metabolizing activities is not simple to explain, in general. However, the following point may be noteworthy. ER O-deethylation activity, which is reported to be specifically induced by 3-MC in rats [28], increased to about thirty times in BL mice after the inducer treatment. This activity was suppressed markedly following CS_2 administration in normal and PB-pretreated mice, whereas in 3-MC-pretreated mice the degree of the suppression was much less and almost comparable to the decrease in cytochrome P-450 content. This was also clear *in vitro*. (5) In the reconstituted monooxygenase system using partially purified P-450 (PB) and P-448 (3-MC), CS_2 suppressed P-448 (3-MC)-dependent drug-metabolizing activities, but had little or no effect on P-450(PB)-dependent activities. The validity of this experiment is supported by a similar experiment with carbon tetrachloride, in which the loss of drug-metabolizing activities occurred only with the P-450(PB)-reconstituted system as reported elsewhere [29].

Spectral studies with cytochrome P-450 inhibitors were done adjunctly, and a few points should be noted. (1) In *n*-octylamine difference spectra, the $\Delta A(500–410):\Delta A(500–392)$ value (R) is suggested to give an existing ratio of low spin to high spin forms of cytochrome P-450 in microsomal membranes [25, 30]. In BL mice, an increase in the $\Delta A(500–392)$ value by 3-MC pretreatment and its decrease following CS_2 administration, i.e. a considerable increase in the R value, were evident. This suggests that the high spin form of cytochrome P-450 induced by 3-MC is more sensitive to CS_2-induced destruction of the hemoprotein than the low spin form. And (2) SKF-525A and metyrapone have been suggested to react selectively with different fractions of microsomal cytochrome P-450 and the reactions to be independent of each other [31]. In the present study, 3-MC caused a greater increase in the magnitude of the metyrapone-induced spectral change, which was decreased considerably following CS_2 administration, whereas the magnitude of the SKF-525A-induced spectral change was only slightly affected by 3-MC alone or in combination with CS_2. These observations imply that metyrapone-binding cytochrome P-450 fractions induced by 3-MC may be more sensitive to CS_2.

Second, the present study also supports our previous view [15] that functional impairment of cytochrome P-450 may be greater than expected from a chemically determinable loss of this hemoprotein. For example, CS_2 produced a much greater decrease in AN (type-2 substrate) hydroxylation activity as compared with the loss of cytochrome P-450 content both *in vivo* and *in vitro* irrespective of the inducer pretreatment. This may be due partly to an alteration of the substrate binding site, or its vicinity, of cytochrome P-450 molecules, since the decrease in the magnitude of metyrapone- and *n*-octylamine-induced spectral changes (type 2) after CS_2 administration was greater as compared with the loss of cytochrome P-450.

Third, the loss of cytochrome P-450 following CS_2 administration also occurred in PB-pretreated mice, although less than in 3-MC-pretreated mice. SDS–PAGE analysis also revealed some loss of the PB-induced protein fractions at the hemoprotein region. It is inconceivable, therefore, that the loss of cytochrome P-450 can occur only with the 3-MC-inducible cytochrome P-448 form. Furthermore, it is reported that PB pretreatment of rats enhances not only the loss of cytochrome P-450 *in vitro* [1] but also microsomal bioactivation of CS_2 *in vitro* [10]. In our *in vitro* bioactivation system using partially purified rat liver P-450(PB), CS_2 showed only a slight or no effect, which could be due to the CS_2 concentration being still low. On the other hand, the cytochrome P-448-form-mediated monooxygenase activities were suppressed by CS_2, indicating that both bioactivation of CS_2 and the following suicidal destruction of the hemoprotein can also occur with the cytochrome P-448 form. Whether each form of cytochrome P-450 in the microsomal membranes is independently but variably responsible for both bioactivation of CS_2 and the ensuing destruction, or the cytochrome P-448 form is just more labile than the P-450 form to the attack by the reactive sulfur atom liberated through the bioactivation of CS_2, or both, remains to be determined.

Lastly, CS_2-induced hepatotoxicity is proposed to be mediated by the active metabolites of CS_2, reactive sulfur atoms, since PB pretreatment of rats markedly augmented hepatic necrosis induced by CS_2 and also increased covalent binding of sulfur atoms to microsomal membranes *in vitro* accompanying the enhanced loss of cytochrome P-450 [32]. There are, however, no related data available for 3-MC-pretreated rats. In the present study with mice, PB pretreatment did not potentiate the hepatonecrogenic action of CS_2 in BL and ddY mice, or in starved ddY mice. In addition, 3-MC pretreatment of BL mice, in which the loss of cytochrome P-450

by CS_2 was greatest, did not enhance the hepatic necrosis either. In our previous study with normal ddY mice [15], oral administration of CS_2 up to 2 g/kg did not produce hepatic necrosis as judged by plasma transaminase levels and liver calcium content, while, with i.p. administration, 1 g/kg produced very slight signs of hepatotoxicity and 1.5 to 2 g/kg was lethal, which was also seen in the present experiment. Thus, 1 g/kg of CS_2, i.p., is an almost maximum dose to examine the effects of the inducer on the hepatotoxicity. In rats, it is reported that an oral dose of 1 ml/kg of CS_2 produces marked hepatic necrosis in PB-pretreated and fasted rats [6], while an i.p. dose of 100–200 mg/kg is hepatotoxic under similar conditions [33, 34]. Taking all these facts into consideration, mice appear to be rather resistant to CS_2 hepatotoxicity even under a marked loss of cytochrome P-450. The reason for such a species difference is unknown. We speculate that the hepatonecrogenic action of CS_2 could not simply be explained by the bioactivation mechanism, as exemplified by the destruction of cytochrome P-450, alone. Other differences in the metabolic pathways of CS_2 and cellular defense mechanisms between both species also should be considered.

In conclusion, loss of liver microsomal cytochrome P-450 content and the related monooxygenase activities was observed after CS_2 administration not only in PB-pretreated ddY and BL mice but also in 3-MC-pretreated BL mice, and the cytochrome P-448 form induced by 3-MC seems to be more susceptible to CS_2. However, the roles of the different forms of cytochrome P-450 in bioactivation of CS_2 and its relationship to the impairment of the hemoprotein or development of hepatic necrosis remain to be studied.

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REFERENCES

1. E. J. Bond and F. DeMatteis, *Biochem. Pharmac.* **18**, 2531 (1969).
2. K. J. Freundt and W. Dreher, *Naunyn-Schmiedeberg's Arch. Pharmak. exp. Path.* **263**, 208 (1969).
3. A. L. Hunter and R. A. Neal, *Biochem. Pharmac.* **24**, 2199 (1975).
4. K. J. Freundt, P. Kuttner and W. Dreher, *Arzneimittel-Forsch.* **26**, 793 (1976).
5. Y. Masuda and N. Nakayama, *Biochem. Pharmac.* **31**, 2713 (1982).
6. E. J. Bond, W. H. Butler, F. DeMatteis and J. M. Barnes, *Br. J. ind. Med.* **26**, 335 (1969).
7. L. Magos and W. H. Butler, *Br. J. ind. Med.* **29**, 95 (1972).
8. F. DeMatteis and A. L. Seawright, *Chem. Biol. Interact.* **7**, 375 (1973).
9. F. DeMatteis, *Molec. Pharmac.* **10**, 849 (1974).
10. R. R. Dalvi, R. E. Poore and R. A. Neal, *Life Sci.* **14**, 1785 (1974).
11. G. L. Catignani and R. A. Neal, *Biochem. biophys. Res. Commun.* **65**, 629 (1975).
12. R. R. Dalvi, A. L. Hunter and R. A. Neal, *Chem. Biol. Interact.* **10**, 347 (1975).
13. M. J. Obrebska, P. Kentish and D. V. Parke, *Biochem. J.* **188**, 107 (1980).
14. M. Torres, J. Järvisalo and J. Hakim, *Expl. molec. Path.* **35**, 36 (1981).
15. Y. Masuda, M. Yasoshima and N. Nakayama, *Biochem. Pharmac.* **35**, 3941 (1986).
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
17. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
18. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
19. F. P. Guengerich and M. V. Martin, *Archs. Biochem. Biophys.* **205**, 365 (1980).
20. A. Y. H. Lu and S. B. West, *Pharmac. Rev.* **31**, 277 (1980).
21. S. Reitman and S. Frankel, *Am. J. clin. Path.* **28**, 56 (1957).
22. J. A. Demetriou, P. A. Drewes and J. B. Gin, in *Clinical Chemistry: Principles and Techniques* (Eds. R. J. Henry, D. C. Cannon and J. W. Winkelman), 2nd Edn., p. 824. Harper & Row, New York (1974).
23. H. M. Von Hattingberg, H. M. Klaus, H. Lüllman and S. Zepb, *Experientia* **22**, 553 (1966).
24. S. Wallenstein, C. L. Zucker and J. L. Fleiss, *Circulation Res.* **47**, 1 (1980).
25. D. W. Nebert and J. E. Gielen, *Fedn. Proc.* **31**, 1315 (1972).
26. M. Negishi and D. W. Nebert, *J. biol. Chem.* **254**, 11015 (1979).
27. T. Noguchi, K-L. Fong, E. K. Lai, L. Olson and P. B. McCay, *Biochem. Pharmac.* **31**, 609 (1982).
28. M. D. Burke and R. T. Mayer, *Drug Metab. Dispos.* **2**, 583 (1974).
29. Y. Masuda and M. Yasoshima, *Jap. J. Pharmac.* **43**, 226 (1987).
30. C. R. E. Jefcoate, R. L. Calabrese and J. L. Gaylor, *Molec. Pharmac.* **6**, 391 (1970).
31. H. Grasdalen, D. Bäckström, G. L. E. Eriksson and A. Ehrenberg, *Fedn. Eur. Biochem. Soc. Lett.* **60**, 294 (1975).
32. F. DeMatteis and A. A. Seawright, *Panminerva med.* **18**, 364 (1976).
33. M. Torres, G. Feldmann, M. A. Perrault, J. Järvisalo and J. Hakim, *Expl. molec. Path.* **33**, 333 (1980).
34. S. P. Tucker, D. P. Lovell, A. A. Seawright and V. J. Cunningham, *Archs. Toxic.* **45**, 287 (1980).