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PROTECTIVE ACTION OF DIETHYLDITHIOCARBAMATE AND CARBON DISULFIDE AGAINST RENAL INJURY INDUCED BY CHLOROFORM IN MICE

YASUSUKE MASUDA* and NOBUE NAKAYAMA
Department of Toxicology, Niigata College of Pharmacy, Niigata 950–21, Japan

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Abstract—Oral administration of diethyldithiocarbamate (DTC) and carbon disulfide (CS_2) protected mice against $CHCl_3$-induced kidney injury, as evidenced by normalization of delayed plasma phenolsulfonphthalein clearance, suppression of increased kidney calcium content and prevention of renal tubular necrosis. In CCl_4-treated mice, in which liver microsomal monooxygenase activities were decreased markedly, and kidney microsomal aniline hydroxylase and *p*-nitroanisole demethylase activities were increased to about twice those of the untreated mice, renal toxicity of $CHCl_3$ was greatly potentiated, and the latter effect was also blocked by both agents. DTC and CS_2 *per se* markedly decreased kidney microsomal aniline hydroxylase and *p*-nitroanisole demethylase activities at 1 hr after oral administration, accompanying a moderate loss of cytochrome P-450 content, in both normal and CCl_4-treated mice. The protection was not due to hypothermia, because pretreatment with DTC or CS_2 (p.o.) also prevented the hypothermia induced by $CHCl_3$. The mechanism of the protection may have involved inhibition of metabolic activation of $CHCl_3$ in the kidney rather than in the liver.

We reported previously that diethyldithiocarbamate (DTC) and carbon disulfide (CS_2) protect mice against hepatic injury induced by a variety of hepatotoxins that require metabolic activation, such as CCl_4, $CHCl_3$, $CBrCl_3$, thioacetamide, bromobenzene, acetaminophen, furosemide and dimethylnitrosamine, and that these protective agents suppress various drug-metabolizing enzyme activities of liver microsomes both *in vivo* and *in vitro* [1]. From these results and the reports of other investigators [2–6], it has been suggested that the protective action of DTC and CS_2 may be due to an inhibition of bioactivation of these hepatotoxins by the liver microsomal monooxygenase system.

Kidney microsomal fraction also contains cytochrome P-450 and drug biotransformation activity, though to a lesser extent than liver microsomes [7–9], and is considered to be involved in the onset of nephrotoxicity of some nephrotoxic substances including $CHCl_3$, bromo- and chlorobenzene, cephaloridine and furosemide [10–12].

We wished to know if DTC and CS_2 could protect against kidney injury induced by such nephrotoxins. In the present study, $CHCl_3$ was used as a nephrotoxin. Both DTC and CS_2 protected against renal injury induced by $CHCl_3$ in normal and CCl_4-treated mice, as evidenced by biochemical and histopathological examinations, and furthermore, suppressed drug-metabolizing enzyme activities in kidney microsomes.

It is postulated that the protective mechanism may involve inhibition of metabolic activation of $CHCl_3$ by CS_2 in the kidney.

* Correspondence should be sent to: Yasusuke Masuda, Department of Toxicology, Niigata College of Pharmacy, Kami-shin'ei cho, Niigata 950-21, Japan.

MATERIALS AND METHODS

Male mice of the ddY strain (SPF grade), weighing 28–32 g, were used. Animals were housed in an air-conditioned animal room (temperature 23 ± 1°, humidity 50–60%, and supplied with all fresh clean air) with food and water given *ad lib.* throughout the experiment.

Experiments on protective action: DTC (sodium diethyldithiocarbamate · trihydrate) or CS_2, dissolved in distilled water or olive oil, respectively, was given orally 30 min before i.p. administration of 0.25 ml/kg of $CHCl_3$ (dissolved in olive oil), and after 24 hr the animals were subjected to the measurements detailed below. In the experiment with CCl_4-poisoned mice (0.2 ml/kg, i.p., 24 hr), the dose of $CHCl_3$ was lowered to 0.05 ml/kg. Control animals received vehicles alone.

Renal excretion activity was assessed by measuring plasma phenolsulfonphthalein (PSP) concentration [13]. Fifty mg/kg of PSP was injected into the tail vein without anesthesia, the blood was rapidly collected into a heparinized syringe, after decapitation, at exactly 5 and 30 min, and the optical density of the plasma was read at 545 nm after dilution (1/60 for 5-min plasma and 1/30 for 30-min plasma) with 0.03 N NaOH. In normal mice, semilogarithmic plots of plasma PSP concentrations at 5, 15 and 30 min showed first-order kinetics; plasma half-time ($T_{1/2}$) was estimated, therefore, from the mean values at 5 and 30 min. Since hypothermia affects renal excretion rate, rectal temperature was measured prior to the PSP test by using a thermistor. A decrease in body temperature observed in some treated animals, however, remained within 1° below the normal range.

For determination of tissue calcium content, the

Table 1. Effects of DTC and CS_2 on the delayed plasma disappearance of phenolsulfonphthalein (PSP) induced by $CHCl_3$ in normal mice*

Treatment	Plasma PSP concentration (μg/ml) 5 min	30 min	$T_{1/2}$ (hr)
Control	91.3 ± 16.7(4)	15.5 ± 2.9(4)	0.16
$CHCl_3$ alone	103.0 ± 7.4(8)	34.3 ± 5.8†(8)	0.26
DTC (30 mg/kg) + $CHCl_3$	113.6 ± 11.8(4)	24.8 ± 4.1‡(4)	0.19
(100 mg/kg) + $CHCl_3$	105.2 ± 8.3(4)	25.0 ± 5.5‡(4)	0.20
(300 mg/kg) + $CHCl_3$	91.0 ± 6.9(4)	16.2 ± 2.3§(4)	0.17
CS_2 (100 mg/kg) + $CHCl_3$	94.8 ± 22.3(4)	16.2 ± 0.7§(4)	0.16
DTC (300 mg/kg) alone	88.3 ± 5.1(4)	16.7 ± 3.3(4)	0.17

* DTC or CS_2 was given orally 30 min before administration of $CHCl_3$ (0.25 ml/kg, i.p.), and 24 hr later PSP clearance was determined as described in Materials and Methods. Each value represents mean ± S.D. (N).
† Significantly higher than the control ($P < 0.01$).
‡ Significantly lower than the group given $CHCl_3$ alone ($P < 0.05$).
§ Significantly lower than the group given $CHCl_3$ alone ($P < 0.01$).

left whole kidney was completely sonicated in 8% trichloroacetic acid (TCA), and the TCA extract was titrated with EDTA, using calcein as an indicator [14].

Specimens for histopathological examination were prepared by the regular method. The right kidney was fixed with 10% buffered formalin, dehydrated with ethanol, imbedded in paraffin and cut at 4 μm thickness; then the sections were stained with hematoxylin and eosin.

Assays of microsomal drug-metabolizing enzyme activities. One hour after oral administration of DTC or CS_2, mice were exsanguinated and microsomes were isolated from the whole kidney as follows. In each separate preparation, kidneys from four mice were pooled and a 20% homogenate was prepared using 0.15 M KCl–20 mM Tris–HCl (pH 7.4). The homogenate was centrifuged at 5000 *g* (at the center of the tube) for 20 min, and the supernatant fraction, without the fluffy layer, was further centrifuged at 105,000 *g* for 60 min. The microsomal pellet was resuspended in the same medium at a protein concentration of 20 mg/ml and used for the enzyme assays. Protein was determined by the method of Lowry *et al.* [15].

Cytochrome P-450 content was determined from dithionite difference spectra of carbon monoxide-saturated microsomal suspensions containing microsomes (2 mg protein/ml), 10 mM succinate and 0.1 M potassium phosphate buffer (pH 7.5)–20% glycerol, as reported by Orrenius *et al.* [7]. An extinction coefficient of 91 $mM^{-1} cm^{-1}$ was used for the calculations.

Incubation mixtures for drug-metabolizing enzyme assays consisted of microsomes (1 mg protein/ml), and NADPH-generating system (1 mM NADPH, 5 mM $MgCl_2$, 2.5 mM nicotinamide, 10 mM isocitrate and 0.4 units of isocitrate dehydrogenase), 0.1 mM EDTA, 0.1 M Tris–HCl buffer (pH 7.4) oxygenated previously, and either 25 mM aminopyrine, 5 mM aniline or 2 mM *p*-nitroanisole in a final volume of 2.0 ml. Appropriate blanks were always run in parallel. The mixture prepared in a 20 ml-flask was flushed with oxygen for 30 sec, capped, and incubated at 37° for 40 min with shaking. The reaction was linear within this time. Aminopyrine *N*-demethylase activity was assayed by measuring formaldehyde production by the Nash reaction according to the procedure described by Mazel [16]. Aniline hydroxylase activity was measured by following the production of *p*-aminophenol by the method of Imai *et al.* [17]. *p*-Nitroanisole *O*-demethylase activity was assayed by measuring *p*-nitrophenol production according to the method of Arfwidsson *et al.* [18]. Because of the fairly low activities, the optical density was read on a magnified scale (× 5) using a recorder.

Liver microsomal fraction was prepared similarly, and its drug-metabolizing enzyme activities and cytochrome P-450 content were determined as described previously [1].

Statistical analyses were done using Student's *t*-test; $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Experiments with normal mice. Figure 1 shows some examples of the histological specimens that

Table 2. Effects of DTC and CS_2 on kidney calcium accumulation induced by $CHCl_3$*

Treatment	Calcium content (μmoles/g kidney)
Control	1.31 ± 0.10(10)
$CHCl_3$ alone	3.98 ± 0.95†(10)
DTC (30 mg/kg) + $CHCl_3$	1.56 ± 0.54‡(5)
(100 mg/kg) + $CHCl_3$	1.32 ± 0.08‡(5)
CS_2 (10 mg/kg) + $CHCl_3$	2.55 ± 1.39§(5)
(30 mg/kg) + $CHCl_3$	1.30 ± 0.08‡(5)
DTC (100 mg/kg) alone	1.34 ± 0.05(5)
CS_2 (30 mg/kg) alone	1.37 ± 0.03(5)

* DTC or CS_2 was given orally 30 min before administration of $CHCl_3$ (0.25 ml/kg, i.p.) and 24 hr later kidney calcium content was determined as described in Materials and Methods. Each value represents mean ± S.D. (N).
† Significantly higher than the control ($P < 0.01$).
‡ Significantly lower than the $CHCl_3$ group ($P < 0.01$).
§ Significantly lower than the $CHCl_3$ group ($P < 0.05$).

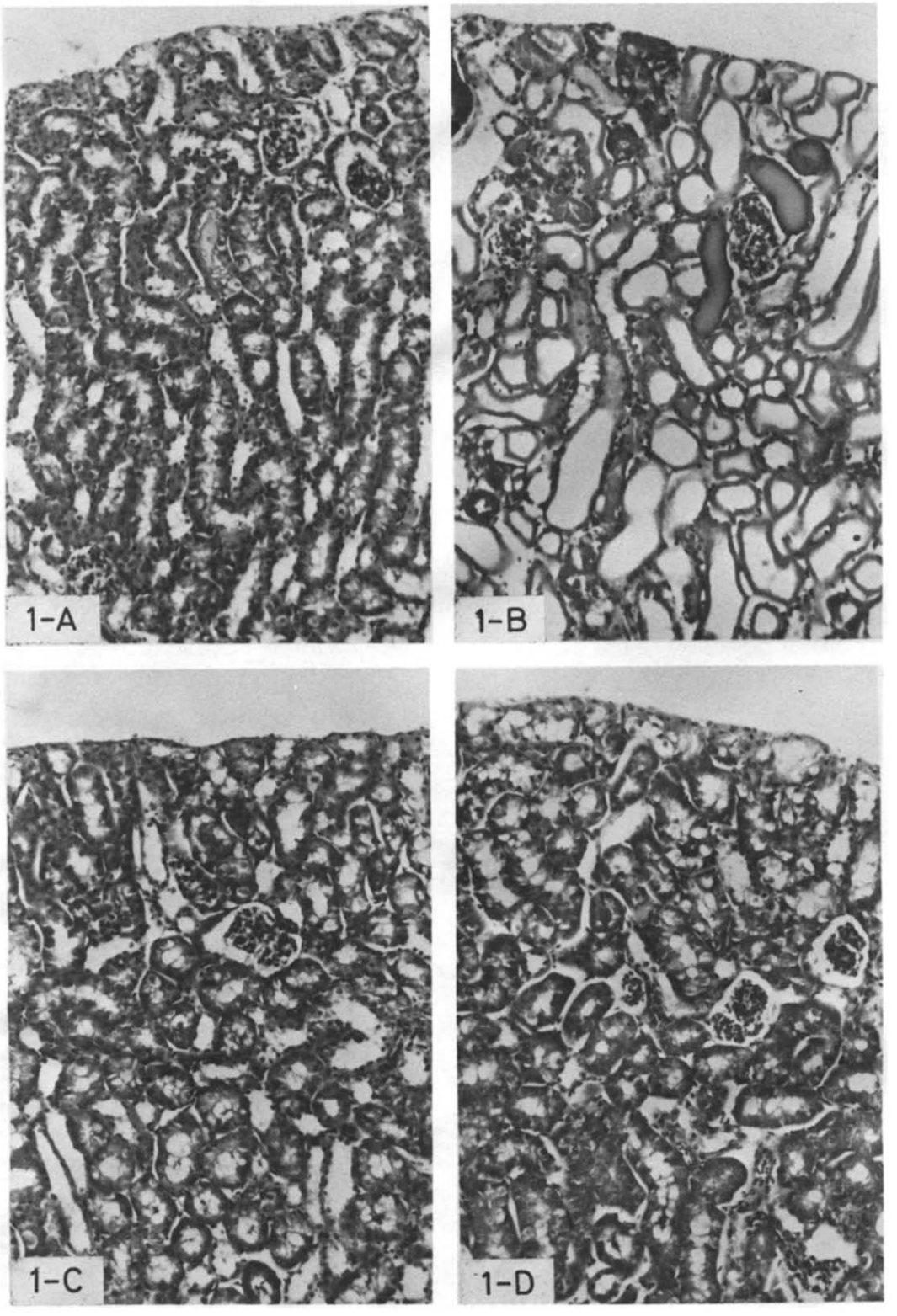


Fig. 1. Protection by DTC and CS_2 against $CHCl_3$-induced renal tubular necrosis. (A) Control kidney cortex. (B) Twenty-four hours after i.p. administration of $CHCl_3$ (0.25 ml/kg). (C) Pretreated with 300 mg/kg (p.o.) of DTC 30 min before administration of $CHCl_3$. (D) Pretreated with 100 mg/kg (p.o.) of CS_2 instead of DTC as in panel C. Magnification: × 150.

were obtained from the kidneys of ten mice for each group including the mice used for the biochemical assays. All mice that received $CHCl_3$ (0.25 ml/kg, i.p.) alone exhibited massive renal tubular necrosis at 24 hr, whereas no such tubular necrosis occurred in the mice pretreated with 300 mg/kg of DTC or an equimolar dose of CS_2 (100 mg/kg). With 100 mg/kg of DTC, the protection was also evident. In the group pretreated with 30 mg/kg of DTC, tubular dilation and some necrotic tubules were observed; however, the ameliorating effect was obvious. No histological alterations were observed with either DTC or CS_2 alone.

Biochemical evidence for protection is shown in Tables 1 and 2. The clearance of PSP from plasma was delayed by $CHCl_3$ administration; the delay was completely prevented by pretreatment with 300 mg/kg of DTC and an equimolar dose of CS_2 (Table 1). With 30 and 100 mg/kg of DTC, the prevention was imperfect but significant. Thus, kidney tubular function may have been normalized by pretreatment with DTC and CS_2.

As another biochemical index, calcium content of the kidney was measured, since an increase in tissue calcium content has been suggested to be associated with cell necrosis [19–21]. In the kidney, $CHCl_3$ produced about a 3-fold increase in calcium content, which also was blocked by pretreatment with DTC or CS_2 (Table 2). This provides further evidence for a protective action of DTC and CS_2 against $CHCl_3$-induced renal necrosis.

DTC and CS_2 are known to depress liver microsomal drug-metabolizing enzyme activities [1, 4–6]. Figure 2 shows the effects of various doses of DTC and CS_2 on kidney microsomal cytochrome P-450 content and drug-metabolizing enzyme activities at 1 hr after administration. Cytochrome P-450 content decreased gradually but significantly as the dose of DTC increased. *p*-Nitroanisole demethylase and aniline hydroxylase activities decreased more sharply even at a dose of 3 mg/kg of DTC, whereas aminopyrine demethylation appeared to be little affected. Similar patterns were observed with equimolar doses of CS_2. The lack of parallelism between the loss of the hemoprotein content and the decrease in drug-metabolizing enzyme activities was also noted in the liver [1]; however, the reason remains to be elucidated.

Thus, DTC and CS_2 inhibited the kidney microsomal monooxygenase system, in the range of doses that protected the kidney against morphological and biochemical impairments induced by $CHCl_3$.

Ilett *et al.* [10] demonstrated that the amount of covalent binding of the metabolite of $CHCl_3$ to kidney proteins paralleled the extent of renal necrosis in normal, phenobarbital- or piperonylbutoxide-pretreated mice. Similar observations were reported with bromobenzene [11] and furan derivatives [12]. When these reports are taken into consideration together, it suggests that DTC and CS_2 protect against $CHCl_3$-induced nephrotoxicity by inhibiting the metabolic activation of $CHCl_3$ in the kidney, although neither metabolism of $CHCl_3$ nor covalent binding of the metabolite was determined in the present study.

Experiments with CCl_4-poisoned mice. It is still controversial [11] whether bioactivation of nephrotoxins occurs directly in the kidney or if active metabolites formed in the liver are transferred to the kidney. A recent finding by Breen *et al.* [22] that totally

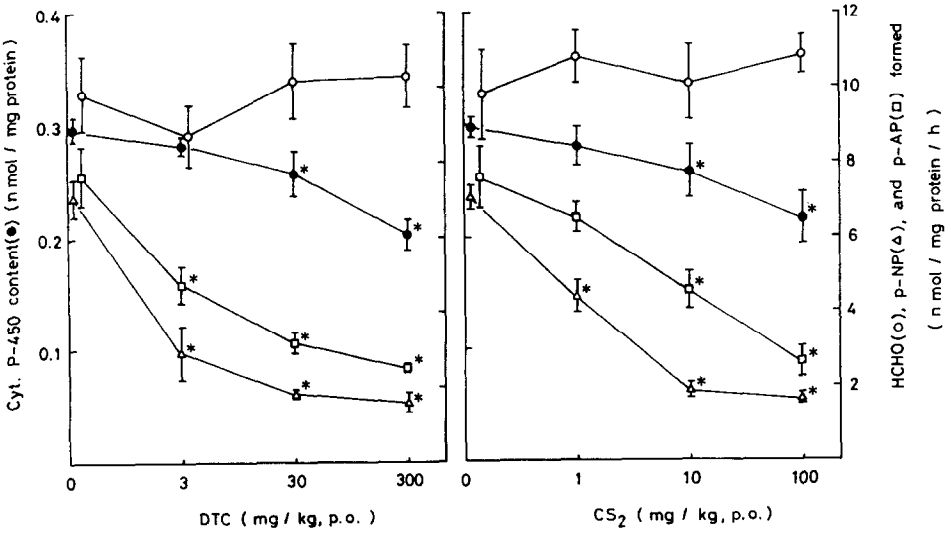


Fig. 2. Effects of DTC and CS_2 on kidney microsomal cytochrome P-450 content and drug-metabolizing enzyme activities in normal mice. Kidney microsomes were isolated at 1 hr after oral administration of various doses of DTC or CS_2. Cytochrome P-450 content (●) was determined by the dithionite difference spectrum of carbon monoxide-treated microsomes. Aminopyrine demethylase (○), *p*-nitroanisole demethylase (△) and aniline hydroxylase (□) activities were assayed by measuring the production of formaldehyde (HCHO). *p*-nitrophenol (p-NP) and *p*-aminophenol (p-AP) respectively. Details are given in Materials and Methods. Each point represents mean ± S.D. (N = 6 for the control group and N = 3 for the DTC- or CS_2-treated animals).(*) Key: significantly different from control at $P < 0.01$.

hepatectomized rats still form an acetaminophen metabolite that is covalently bound in the kidney, favors the former view for this drug.

To check this point, we used mice treated with 0.2 ml/kg of CCl_4, a dose that is followed by severe hepatic centrilobular necrosis at 24 hr [1], accompanied by a marked loss of liver microsomal drug-metabolizing enzyme activities (Table 3). The kidney, however, appeared normal histologically (Fig. 3A). Litterst *et al.* [9] also reported that the effect of CCl_4 on extrahepatic mixed function oxidation was less predictable. However, we observed about a 2-fold increase in kidney microsomal aniline hydroxylase and *p*-nitroanisole demethylase activities with a slight decrease in cytochrome P-450 content and aminopyrine demethylase activity (Table 3).

In CCl_4-poisoned mice, the renal toxicity of a lower dose of $CHCl_3$ (0.05 ml/kg) was greatly potentiated, as evidenced by the retention of a markedly high plasma PSP concentration, and this potentiation was blocked by 30–100 mg/kg of DTC and equimolar doses of CS_2 (Table 4). Histopathological examination of the kidneys of all these mice revealed a marked aggravation of $CHCl_3$-induced tubular necrosis in the CCl_4-poisoned group (Fig. 3B, C and D), which was also blocked by pretreatment with DTC and CS_2 (Fig. 3E and F).

Effects of DTC and CS_2 *per se* on kidney microsomal monooxygenase activity are shown in Fig. 4. The patterns of suppression were similar to those observed in normal mice, although the degree of suppression of *p*-nitroanisole demethylase and aniline hydroxylase activities was greater in the CCl_4-poisoned group.

These observations suggest (1) that suppression of the metabolism of $CHCl_3$ in damaged liver may increase the amount of $CHCl_3$ reaching the kidney, where the metabolism of some substrates would be evidently enhanced, as would be the formation of active metabolites of $CHCl_3$ there, resulting in augmented renal toxicity, i.e. the bioactivation of $CHCl_3$ responsible for renal injury may occur directly in the kidney, and (2) that the protective action of DTC and CS_2 against renal injury may be due to an inhibition of bioactivation of $CHCl_3$ in the kidney but not in the liver.

Effect on body temperature. Under the present experimental conditions, the protective action of DTC and CS_2 is not considered to be a result of decreased metabolic activity due to hypothermia, because both agents, when given orally, did not enhance the hypothermia induced by $CHCl_3$ but rather prevented it in normal and CCl_4-poisoned mice (Fig. 5). This observation, moreover, suggests the possibility that the hypothermic action of $CHCl_3$ may also have originated from bioactivation of $CHCl_3$.

On the other hand, when DTC was given i.p. simultaneously with or 30 min before $CHCl_3$ in normal mice, the hypothermia was not suppressed significantly (data not shown). This was probably because DTC alone lowered body temperature when given intraperitoneally, but not when given orally (unpublished data).

Finally, DTC is very unstable under acidic con-

Table 3. Microsomal drug-metabolizing enzyme activities and cytochrome P-450 contents of the liver and kidney in CCl_4-poisoned mice*

	Yield of microsomes $\left(\frac{\text{mg protein}}{\text{g tissue}}\right)$	Aminopyrine *N*-demethylase $\left(\frac{\text{nmoles HCHO}}{\text{mg protein} \cdot \text{hr}}\right)$	*p*-Nitroanisole *O*-demethylase $\left(\frac{\text{nmoles } p\text{-nitrophenol}}{\text{mg protein} \cdot \text{hr}}\right)$	Aniline hydroxylase $\left(\frac{\text{nmoles } p\text{-aminophenol}}{\text{mg protein} \cdot \text{hr}}\right)$	Cytochrome P-450 $\left(\frac{\text{nmoles}}{\text{mg protein}}\right)$
Liver					
Control	19.9 ± 1.9	466.7 ± 73.3	166.8 ± 15.7	56.8 ± 5.0	0.883 ± 0.091
CCl_4-treated	14.9 ± 1.2†	98.3 ± 44.4†	26.8 ± 12.8†	7.3 ± 3.8†	0.267 ± 0.066†
Kidney					
Control	15.3 ± 1.4	9.88 ± 0.67	5.53 ± 0.42	5.95 ± 0.59	0.243 ± 0.015
CCl_4-treated	16.7 ± 1.2	8.65 ± 0.66†	12.22 ± 1.05†	11.51 ± 0.83†	0.219 ± 0.017‡

* CCl_4 (0.2 ml/kg) was given i.p. 24 hr before sacrifice. Each value represents mean ± S.D. (N = 6 in each group).
† Significantly different from control ($P < 0.01$).
‡ Significantly different from control ($P < 0.05$).

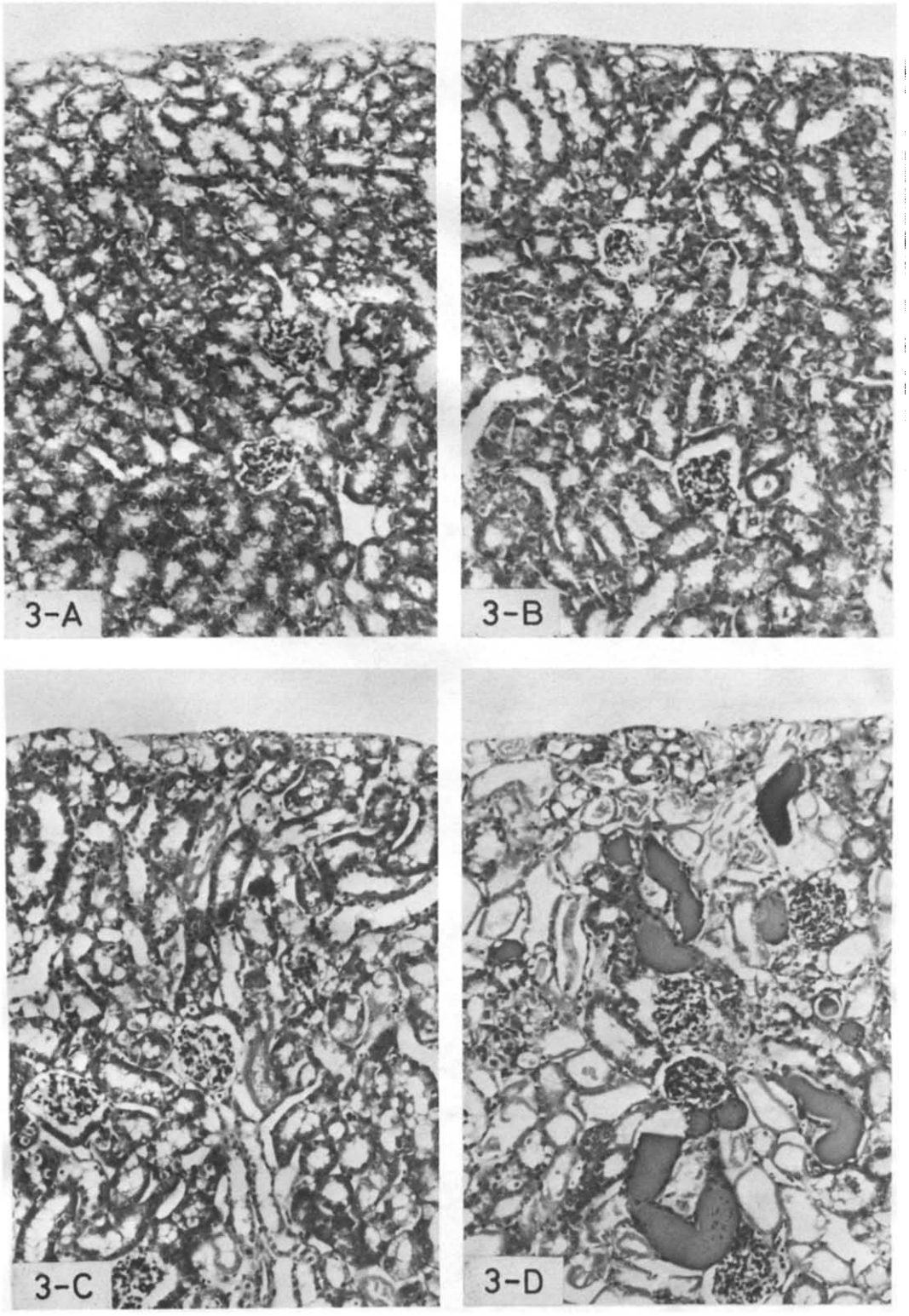
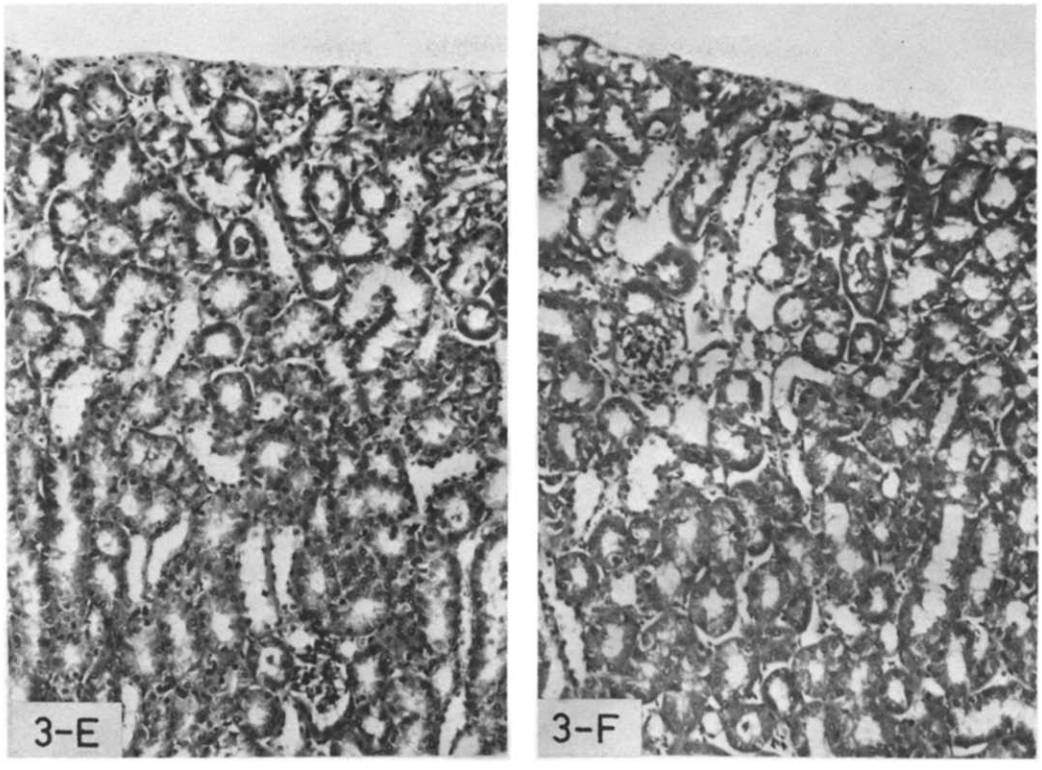


Fig. 3. Protection by DTC and CS_2 against $CHCl_3$-induced renal tubular necrosis in CCl_4-poisoned mice. (A and B) CCl_4 (0.2 ml/kg, i.p.) alone, 24 and 48 hr respectively. (C) $CHCl_3$ (0.05 ml/kg, i.p.) alone, 24 hr. (D) $CHCl_3$ given 24 hr after CCl_4 treatment. (E) Pretreated with DTC (100 mg/kg, p.o.) 30 min before $CHCl_3$ in panel D. (F) Pretreated with CS_2 (30 mg/kg, p.o.) instead of DTC in panel E Magnification: × 150.



ditions, producing CS_2, therefore, the action of orally administered DTC may be mediated through CS_2 produced in the stomach, as discussed previously [1]. In the liver, the inhibitory effect of CS_2 on the microsomal monooxygenase system is thought to result from the metabolic activation of CS_2 in this system and the consequent covalent binding of the sulfur atom to microsomal proteins [23–25). Further experiments should be done to confirm this point in the kidney.

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Table 4. Effects of DTC and CS_2 on the delayed plasma disappearance of phenolsulfonphthalein (PSP) induced by $CHCl_3$ in CCl_4-poisoned mice*

Treatment	Plasma PSP concentration (μg/ml) (at 30 min)
Control	12.9 ± 2.2(8)
CCl_4 alone (48 hr)	16.1 ± 2.6(8)
$CHCl_3$ alone (24 hr)	24.5 ± 6.2(8)
CCl_4-treated (24 hr)	
+$CHCl_3$	80.5 ± 8.9†(8)
+DTC (30 mg/kg) + $CHCl_3$	21.9 ± 4.9(5)
(100 mg/kg) + $CHCl_3$	15.7 ± 4.9(5)
+CS_2 (10 mg/kg) + $CHCl_3$	24.9 ± 4.9(5)
(30 mg/kg) + $CHCl_3$	18.9 ± 3.3(5)

* Mice were treated with CCl_4 (0.2 ml/kg, i.p.) 24 hr before the administration of $CHCl_3$ (0.05 ml/kg, i.p.). DTC or CS_2 was given orally 30 min before $CHCl_3$ administration. PSP clearance was examined 24 hr after $CHCl_3$ administration, as described in Materials and Methods except that the PSP concentration was determined only at 30 min. Each value represents mean ± S.D. (N).

† Significantly higher than the corresponding CCl_4-untreated group and also significantly suppressed by pretreatment with DTC or CS_2 ($P < 0.01$).

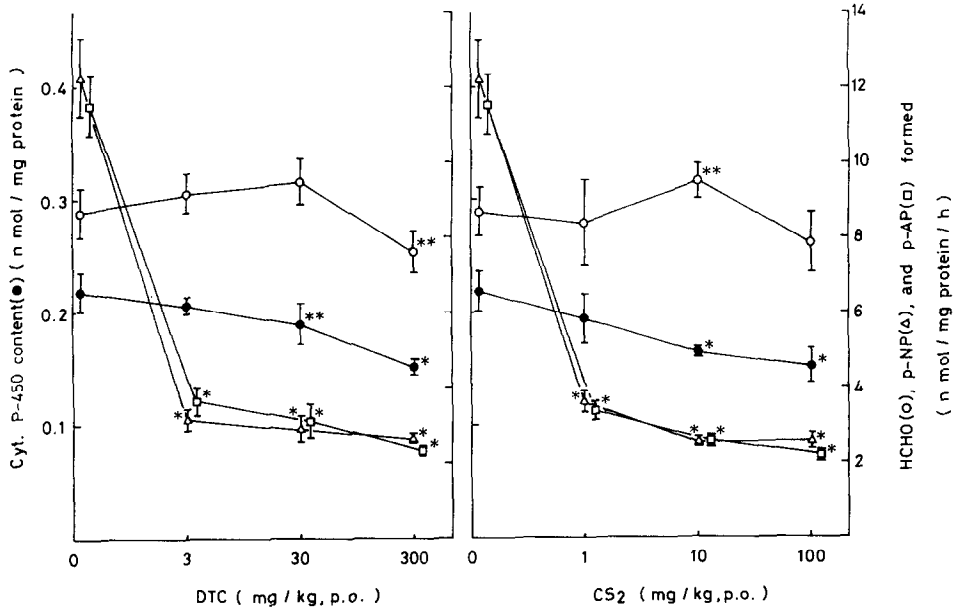

Fig. 4. Effects of DTC and CS_2 on kidney microsomal cytochrome P-450 content and drug-metabolizing enzyme activities in CCl_4-poisoned mice. Experimental methods were the same as those described in the legend of Fig. 2 except that CCl_4-treated (0.2 ml/kg, i.p., 24 hr) mice were used. Key: (●) cytochrome P-450; (○) aminopyrine demethylase; (△) *p*-nitroanisole demethylase; and (□) aniline hydroxylase. Each point represents mean ± S.D. (N = 6 for the CCl_4-treated control group and N = 3 for the DTC- and CS_2-treated animals). Values for CCl_4-nontreated controls examined in parallel are shown in Table 3. Key: significantly different from control at (*) $P < 0.01$ and (**) $P < 0.05$.

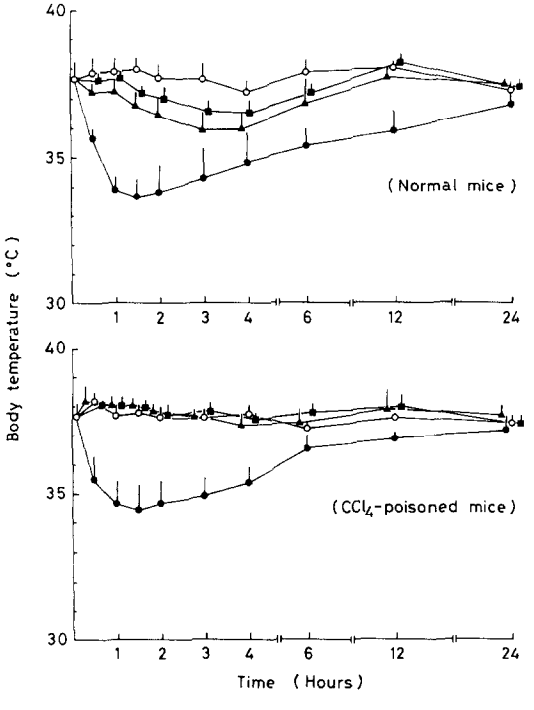

Fig. 5. Effects of DTC and CS_2 on $CHCl_3$-induced loss of body temperature in normal and CCl_4-poisoned mice. Doses of $CHCl_3$ were 0.25 and 0.05 ml/kg (i.p.) for normal and CCl_4-poisoned (0.2 ml/kg, i.p., 24 hr) groups respectively. DTC (100 mg/kg) and CS_2 (30 mg/kg) were given orally 30 min before administration of $CHCl_3$. Key: (○) control; (●) $CHCl_3$ alone; (▲) DTC plus $CHCl_3$; and (■) CS_2 plus $CHCl_3$. Each point represents mean ± S.D. (N = 6–8).

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