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PROTECTIVE EFFECT OF DIETHYLDITHIOCARBAMATE AND CARBON DISULFIDE AGAINST LIVER INJURY INDUCED BY VARIOUS HEPATOTOXIC AGENTS

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Abstract—Diethyldithiocarbamate (DTC) and carbon disulfide (CS_2), at nearly equimolar oral dose levels, protected mice against liver damage induced by carbon tetrachloride, chloroform, bromotrichloromethane, thioacetamide, bromobenzene, furosemide, acetaminophen, dimethylnitrosamine and trichloroethylene, as evidenced by the suppression of elevations in plasma GPT activity and liver calcium content, and of histopathological alterations. Both agents also prolonged hexobarbital sleeping time and zoxazolamine paralysis time in mice. DTC and CS_2 alone, given orally, decreased microsomal metabolism of several substrates (aniline, *p*-nitroanisole, hexobarbital, zoxazolamine, aminopyrine and 3,4-benzpyrene), CCl_4-induced lipid peroxidation, and cytochrome P-450 content. The loss of microsomal drug-metabolizing enzyme activity was also observed in the experiments *in vitro* using liver slices and isolated microsomes. Since a characteristic common to such diverse hepatotoxins is that they require metabolic activation before exhibiting hepatotoxicity, the protective mechanisms of DTC and CS_2 may involve their interference with the process of metabolic activation of these hepatotoxins. The protective action of DTC may be mediated almost entirely through CS_2 when administered orally and at least partly with parenteral administration, since, in CCl_4-induced liver injury, DTC was most effective when given orally, while the action of CS_2 was less dependent on the route of administration. Thus, CS_2 and CS_2-producing agents *in vivo* such as dithiocarbamate derivatives and disulfiram may modify toxicological and pharmacological effects of foreign compounds by inhibiting microsomal drug-metabolizing enzyme activity in the liver.

A protective action of dithiocarbamate derivatives against CCl_4-induced liver injury was first reported in 1966 [1]. According to Masuda and colleagues, dithiocarbamates suppress very early biochemical changes produced by CCl_4 [2, 3] and this suppression is not due to a reduction of body temperature [2], suggesting that the protection concerns a fundamental process of CCl_4 hepatotoxicity. It is now generally accepted that, in CCl_4 hepatotoxicity, lipid peroxidation that has been triggered by active free-radical metabolites of CCl_4 may be one of the important causes of liver cell injury [4–6]. We have already shown that sodium diethyldithiocarbamate (DTC) inhibits CCl_4-induced lipid peroxidation both *in vivo* [3] and *in vitro* [7]. On a more fundamental level, however, the suppression of lipid peroxidation by DTC may be due to the inhibition of bioactivation of CCl_4 by the microsomal drug-metabolizing enzyme system. If so, DTC might prevent the hepatic injury produced by other hepatotoxins that require metabolic activation. Several reports that support this possibility have already been presented; e.g. Hunter and Neal [8] and Zemaitis and Green [9] reported loss of microsomal drug-metabolizing enzyme activities by DTC, and Strubelt *et al.* [10] and Siegers *et al.* [11] reported the protective effect of DTC against liver injury induced by thioacetamide, bromobenzene and acetaminophen.

Since DTC is chemically very unstable under acidic conditions, producing CS_2, and is partly converted to CS_2 *in vivo* [12, 13], there is also the possibility that the action of DTC might be mediated through CS_2, especially in the case of oral administration of DTC. Depression by CS_2 of liver microsomal drug-metabolizing enzyme activity both *in vivo* and *in vitro* has also been reported by several investigators [14–16].

In an attempt to confirm these points, the effects of DTC and CS_2 on liver injury induced by various hepatotoxins were compared in terms of plasma transaminase activity and histopathological examinations. Liver calcium content was determined simultaneously because its increase is considered to be associated with cell necrosis in some cases [17–19]. Effects on microsomal drug-metabolizing enzyme activity were also investigated *in vivo* and *in vitro*. The results obtained were generally consistent with the possibilities described above.

MATERIALS AND METHODS

Chemicals. Sources of the chemicals were as follows: sodium diethyldithiocarbamate (trihydrate), CS_2, CCl_4, $CHCl_3$, bromobenzene, dimethylnitrosamine, acetaminophen and allylformate (Wako Pure Chemical Ind. Ltd.); $CBrCl_3$ and furosemide (Tokyo Kasei Kogyo Co., Ltd.); thioacetamide and trichloroethylene (Nakarai Chemicals Ltd.); hexobarbital (Teikoku Chemical Industry Co., Ltd.); zoxazolamine (Aldrich Chemical Co., Inc.); aflatoxin

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B_1 (Makor Chemical Ltd.); and NADPH and isocitric dehydrogenase (Oriental Yeast Co., Ltd.). All reagents were of the highest purity available.

Animals and treatments. SPF male mice of the ddY strain, weighing 25–30 g, were used. Animals were housed in an air-conditioned room (temp. 23 ± 1°, humidity 50–60%, and supplied with all fresh clean air) and given food (a standard cube diet, MF-1, Oriental Yeast) and water *ad lib.* throughout the experiment. DTC (trihydrate) and CS_2, freshly dissolved in distilled water and olive oil, respectively, were given orally approximately 30 min before i.p. administration of hepatotoxins. Control animals received the vehicle alone. Doses and solutions of the hepatotoxins used were as follows: CCl_4 (0.2 ml/kg), $CHCl_3$ (1.0 ml/kg), $CBrCl_3$ (0.05 ml/kg), bromobenzene (1.0 ml/kg) and trichloroethylene (2.0 ml/kg) dissolved in olive oil; thioacetamide (100 mg/kg), furosemide (400 mg/kg) and dimethylnitrosamine (30 mg/kg) in saline; and acetaminophen (750 mg/kg) dissolved as described by Mitchell *et al.* [20].

Male Sprague–Dawley rats, weighing 180–200 g, were used for experiments with aflatoxin B_1 (1.0 mg/kg) and allylformate (0.75 ml/kg), which were dissolved in triethyleneglycol and saline respectively.

Biochemical and histological examinations. Animals were killed 24 hr after hepatotoxin challenge. In the case of acetaminophen, examinations were also conducted at 4 hr. Blood was obtained by cardiac puncture through a heparinized syringe under ether anesthesia, and plasma GPT activity was measured by the method of Reitman and Frankel [21]. Calcium content in the liver was determined by titration with EDTA using calcein as an indicator [22]. For microscopic observations, liver pieces were fixed in 10% formalin–saline, sectioned, and stained with hematoxylin and eosin by the usual method.

Hexobarbital sleeping time and zoxazolamine paralysis time. Hexobarbital (80 mg/kg) or zoxazolamine (100 mg/kg), dissolved in an equivalent amount of NaOH or HCl, respectively, was given to mice i.p. 30 min after oral administration of DTC or CS_2, and the duration of the loss of righting reflex was measured. In some experiments, the blood was collected by exsanguination in the presence of heparin at the time of recovery from the drug action, and plasma concentrations of hexobarbital [23] and zoxazolamine [24] were determined by the methods given in the references.

Measurement of drug-metabolizing enzyme activities. Mice were killed by exsanguination 1 hr after oral administration of DTC or CS_2, the liver was

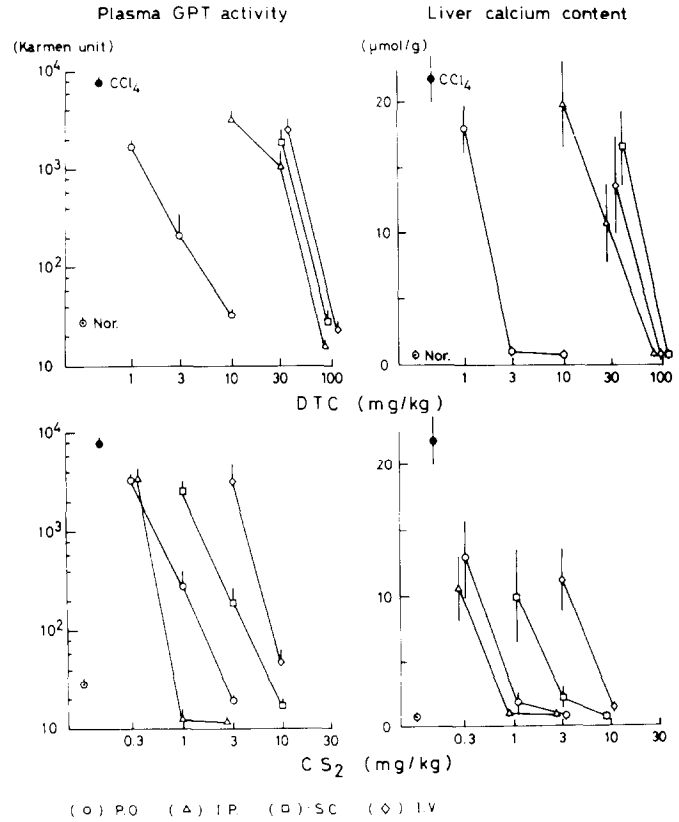


Fig. 1. Effect of route of administration on the protective action of DTC and CS_2 against CCl_4-induced elevation of plasma GPT activity and liver calcium content in mice. DTC and CS_2 were freshly dissolved in isotonic saline and olive oil, respectively, and were given approximately 30 min prior to the administration of CCl_4 (0.2 ml/kg, i.p.). In the case of i.v. administration of CS_2, an ethanol solution of CS_2 was injected at a volume of 3 μl/10 g of body weight via the tail vein by using a microsyringe. Each point is the mean ± S.E.M. of five to ten mice in the experimental group. Normal group: N = 51 and 36; CCl_4-group: N = 23 and 16 for GPT activity and calcium content respectively. Note that doses are plotted on the same molar scale.

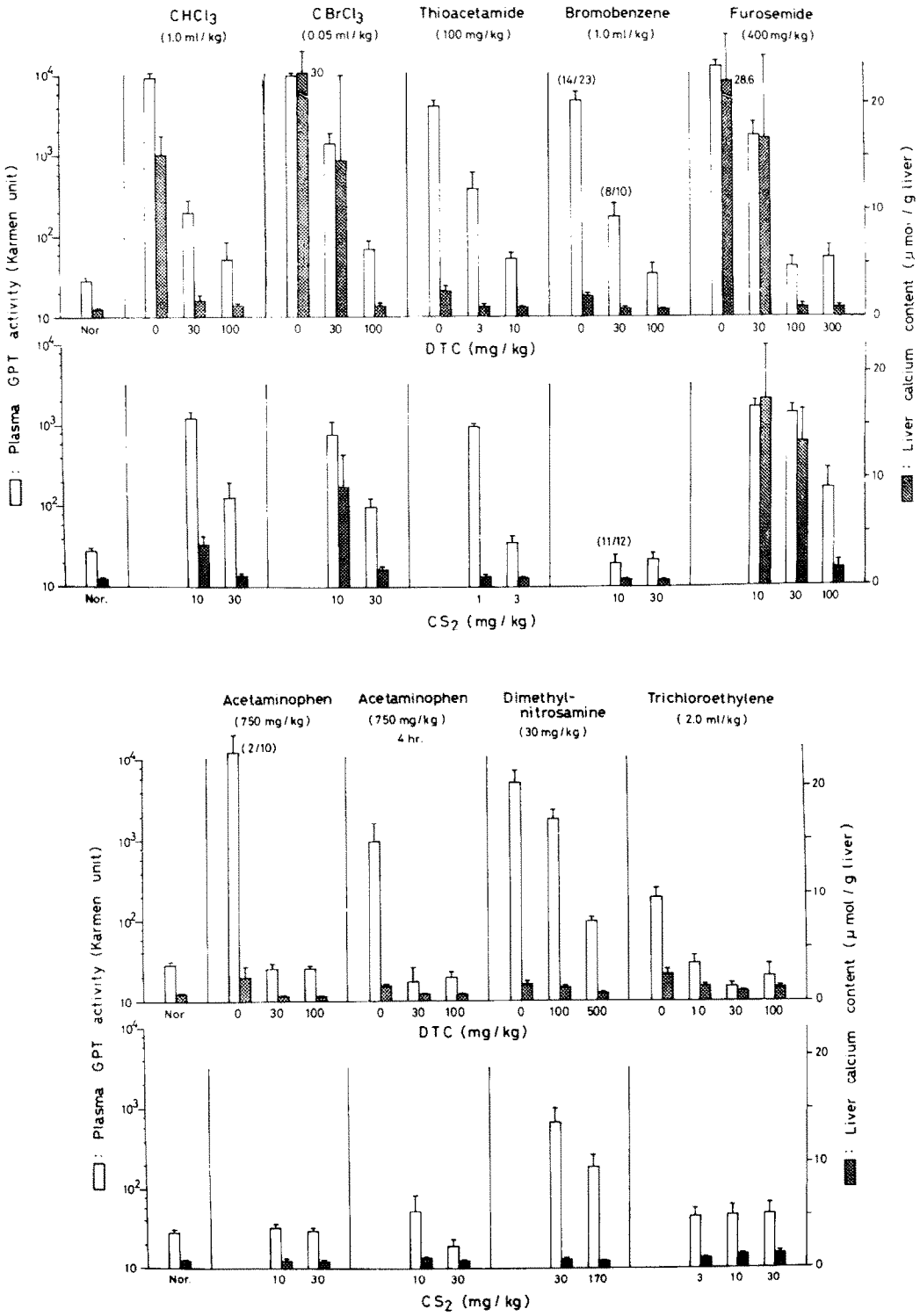


Fig. 2. Protective action of DTC against elevation of plasma GPT activity and liver calcium content induced by various hepatotoxins in mice. DTC and CS_2 were given orally approximately 30 min before i.p. administration of hepatotoxins, and plasma GPT activity and liver calcium content were determined after 24 hr as described in Materials and Methods. Each column represents a mean ± S.E.M. of N = 5–23 in the experimental group. Normal group: N = 51 and 36 for GPT activity and calcium content respectively.

perfused with cold 0.15 M KCl solution through the caudal vena cava *in situ*, and the microsomes were isolated by the calcium aggregation method of Cinti *et al.* [25]. Microsomal pellets were washed with 0.15 M KCl containing 20 mM potassium phosphate buffer (pH 7.5) and 1 mM EDTA, and, finally, suspended in 0.1 M potassium phosphate buffer (pH 7.5) containing 0.1 mM EDTA at a concentration of 20–30 mg/ml. Microsomal cytochrome P-450 content was determined by the method of Omura and Sato [26]. Aniline hydroxylase, *p*-nitroanisole demethylase and aminopyrine demethylase activities were assayed according to the method described by Mazel [23], with slight modifications. Complete incubation mixtures contained microsomes (2 mg protein/ml), 200 μM NADPH with generating system (2.5 mM nicotinamide, 10 mM sodium isocitrate, 5 mM $MgCl_2$ and 0.25 units/ml of isocitrate dehydrogenase) and a substrate (5 mM aminopyrine, 5 mM aniline hydrochloride or 1.2 mM *p*-nitroanisole) in 0.1 M potassium phosphate buffer (pH 7.5). The mixtures were incubated for 20 min at 37°. CCl_4-dependent malondialdehyde (MDA) production was measured by using the same incubation system in the absence, and presence, of 5 mM CCl_4 instead of drug substrate, as reported previously [7]. Metabolism of hexobarbital [23], zoxazolamine [24] and 3,4-benzpyrene [27] was measured essentially according to the methods described in the references. The standard reaction mixture contained 500 μM NADPH with the generating system, substrate (0.35 mM hexobarbital, 0.6 mM zoxazolamine or 66 μM 3,4-benzpyrene), 0.1 mM EDTA, 0.1 M potassium phosphate buffer (pH 7.5) and microsomes (1–2 mg protein/ml) in a final volume of 3.0 ml. After incubation at 37° for 30 min (benzpyrene) or for 1 hr (hexobarbital and zoxazolamine), the unchanged substrate was extracted from the medium and determined. Protein was determined by the method of Lowry *et al.* [28] using bovine serum albumin as standard.

Other experimental details are given in the legends.

RESULTS

Effect of route of administration. Since we had observed that administration of dithiocarbamate derivatives by the oral route was quite protective against CCl_4-induced liver injury [1–3], the influence of the route of administration on the protective effect of DTC was first examined. As shown in the upper dose–response curves of Fig. 1, orally administered DTC was most effective in preventing CCl_4-induced elevation of both plasma GPT activity and liver calcium content, i.e. all of the parenteral routes required approximately ten times as much as the oral protective dose. This finding suggested that the protective effect of DTC was mediated through CS_2 produced from DTC *in vivo*, especially via the oral route in which CS_2 could be easily produced under the acidic conditions in the stomach. This was verified by the following experiments with CS_2 (Fig. 1, lower panels).

CS_2 was also fully protective by the oral route at a dose which was nearly equimolar to the oral protective dose of DTC (note that the molecular weight ratio of DTC·trihydrate to CS_2 is 3:1) and, in this case, i.p. administration was as effective as the oral route. Moreover, with s.c. and i.v. routes, molar effective doses of CS_2 were lower than those of DTC. The variation in the protective activity of CS_2 observed with the different routes probably resulted from the difference in pharmacokinetic phases, particularly its distribution in the liver.

Thus, in addition to a protective action by itself, CS_2 might, therefore, mediate the protective action of DTC as well. In the following experiments, DTC and CS_2 were administered orally, unless indicated otherwise.

Effects of DTC and CS_2 on hepatic injury induced by various hepatotoxins (Figs. 2 and 3). $CHCl_3$ and $CBrCl_3$, like CCl_4, are well-known hepatotoxic halogenomethanes, which are different in potency but cause similar types of hepatic injury [3, 29]. Pretreatment with DTC or CS_2, at nearly equimolar doses, suppressed elevation of both plasma GPT activity and liver calcium content induced by $CHCl_3$ and $CBrCL_3$, although higher protective doses were necessary as compared with the case of CCl_4.

Thioacetamide also has long been known to produce liver necrosis [30]. Thioacetamide-induced elevation of GPT activity was completely blocked by low doses of DTC and CS_2. An increase in calcium content, though much less as compared with the case of the halogenomethanes, was also suppressed.

Bromobenzene-induced liver necrosis in rats was investigated by Brodie *et al.* [31]. In the present study with mice, a fairly large i.p. dose was needed to produce an increase in GPT activity, accompanied by a high mortality (9/23). Pretreatment with DTC and CS_2 protected mice against death and hepatic injury.

Furosemide, a diuretic drug, is also known to cause hepatic necrosis at a dose higher than that which produces diuresis [32]. At a dose of 400 mg/kg, it markedly increased both GPT activity and calcium content, which were suppressed by pretreatment with DTC or CS_2.

Acetaminophen-induced hepatic necrosis in mice was studied extensively by Mitchell *et al.* [20]. In our experiment, 750 mg/kg of acetaminophen (i.p.) caused a very high mortality (8/10) at 24 hr, but mice pretreated with DTC or CS_2 all survived with no increase in GPT activity. At 4 hr, all animals which received acetaminophen alone already exhibited severe hepatic congestion and a moderate increase in GPT activity, both of which were protected in DTC- or CS_2-pretreated mice. The severe hepatic congestion at this time may be a cause of high mortality at later hours. An increase in calcium content following acetaminophen was slight.

Dimethylnitrosamine, a carcinogen, is known to produce centrilobular hemorrhagic necrosis in an early stage after administration [33]. We also observed severe liver congestion accompanying a large volume of ascites and elevation of plasma GPT activity, but only a slight increase in calcium content. In this case, large doses of DTC (500 mg/kg) and CS_2 (170 mg/kg) were required to prevent hepatic congestion and to suppress the elevation of GPT activity.

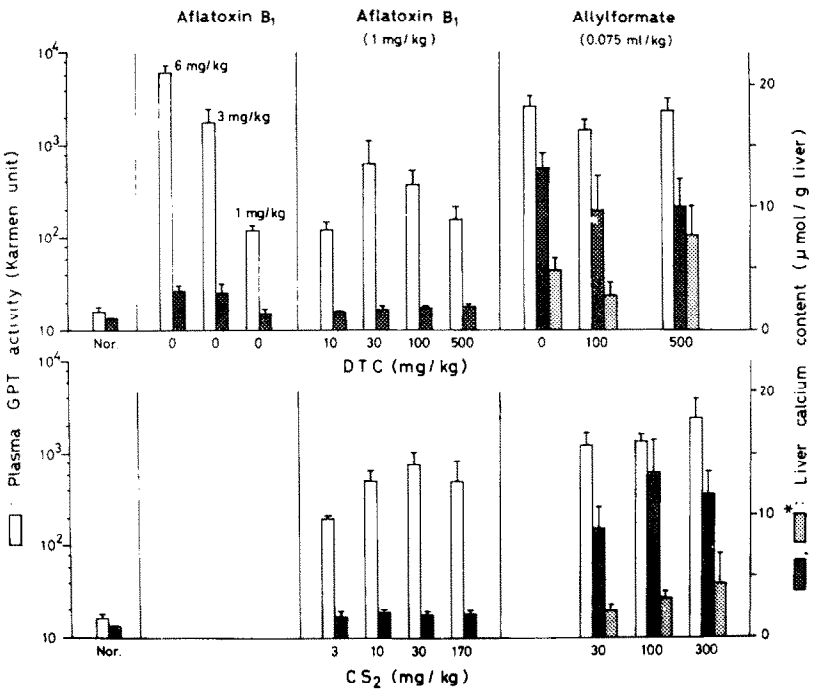


Fig. 3. Effects of DTC and CS_2 on elevation of plasma GPT activity and liver calcium content induced by aflatoxin B_1 and allylformate in rats. The experimental method is given in the legend of Fig. 2. Each column represents the mean ± S.E.M. of four to ten rats. Normal group: N = 8. Key: (*)Allylformate produced sporadic, localized liver damage. This column indicates the calcium content of the apparently undamaged liver portion.

Trichloroethylene produced only moderate increases in GPT activity and calcium content at a fairly high dose (2.0 ml/kg) in normal mice, though phenobarbital pretreatment is reported to enhance hepatotoxicity in rats [34]. DTC- and CS_2-pretreatment suppressed these biochemical changes.

In almost all cases described hitherto, the protective effect was apparent from the gross appearance of the liver at autopsy, and the protection by DTC and CS_2 occurred at nearly equimolar dose levels.

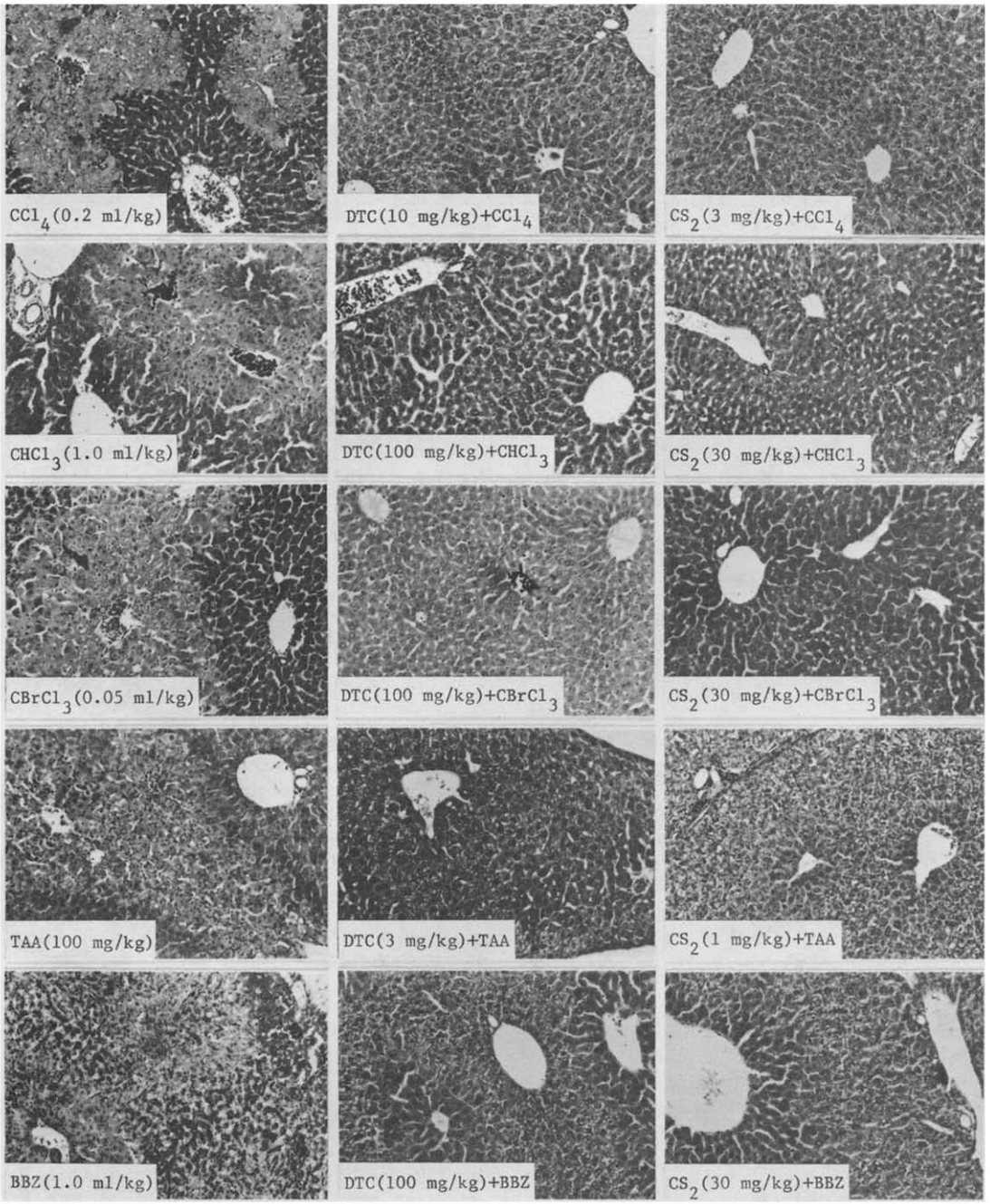
Aflatoxin B_1 is a hepatotoxic, as well as a hepatocarcinogenic, agent and produces periportal parenchymal cell necrosis [35]. Since mice were resistant to this hepatotoxin, male rats were used. Aflatoxin B_1 produced a marked dose-dependent increase in GPT activity and a 2- to 3-fold increase in calcium content (Fig. 3). Preliminary experiments with DTC and CS_2 showed no protection against a high dose (6 mg/kg) of the hepatotoxin and, therefore, a lower dose (1 mg/kg) was chosen. From the data, DTC and CS_2 appear to have had no protective action against aflatoxin B_1-induced hepatotoxicity.

Allylformate is known to produce hepatic periportal necrosis [36]. In our experiment, mice treated with higher doses of this hepatotoxin died of its central action soon after administration and with lower doses no hepatic injury was produced. In rats, it produced sporadic liver damage on gross appearance accompanied by a marked elevation of GPT activity and calcium content, against which DTC or CS_2 was not preventive.

Histological examinations. As shown in Fig. 4, centrilobular necrosis induced by CCl_4, $CHCl_3$, $CBrCl_3$, thioacetamide, bromobenzene, furosemide, acetaminophen and dimethylnitrosamine was prevented or greatly ameliorated by pretreatment with DTC or CS_2 at the doses that prevented elevation of plasma GPT activity. Bromobenzene, acetaminophen and dimethylnitrosamine caused severe congestion in sinusoids also, which was completely blocked by DTC or CS_2. Trichloroethylene and a lower dose (1 mg/kg) of aflatoxin B_1 produced no marked pathological alterations (not shown). Allylformate produced severe periportal necrosis, which was not ameliorated by DTC and CS_2.

Effect on the duration of action of hexobarbital and zoxazolamine. As shown in Table 1, oral administration of DTC and CS_2 prolonged hexobarbital sleeping time and zoxazolamine paralysis time. As in the case of CCl_4-induced hepatic injury (Fig. 1), DTC was more effective by the oral route than by the i.p. route, whereas CS_2 was equally effective by both routes. Furthermore, plasma hexobarbital and zoxazolamine levels at the time of recovering the righting reflex were even higher in DTC-pretreated mice (Table 2), indicating that the prolongation of the drug action may not be due to an increased sensitivity of the central nervous system to both drugs, but to a delayed clearance of the drugs from the blood.

Effects on microsomal drug-metabolizing enzyme activities. Drug-metabolizing enzyme activities of the


CCl_4(0.2 ml/kg)
DTC(10 mg/kg)+CCl_4
CS_2(3 mg/kg)+CCl_4
$CHCl_3$(1.0 ml/kg)
DTC(100 mg/kg)+$CHCl_3$
CS_2(30 mg/kg)+$CHCl_3$
$CBrCl_3$(0.05 ml/kg)
DTC(100 mg/kg)+$CBrCl_3$
CS_2(30 mg/kg)+$CBrCl_3$
TAA(100 mg/kg)
DTC(3 mg/kg)+TAA
CS_2(1 mg/kg)+TAA
BBZ(1.0 ml/kg)
DTC(100 mg/kg)+BBZ
CS_2(30 mg/kg)+BBZ

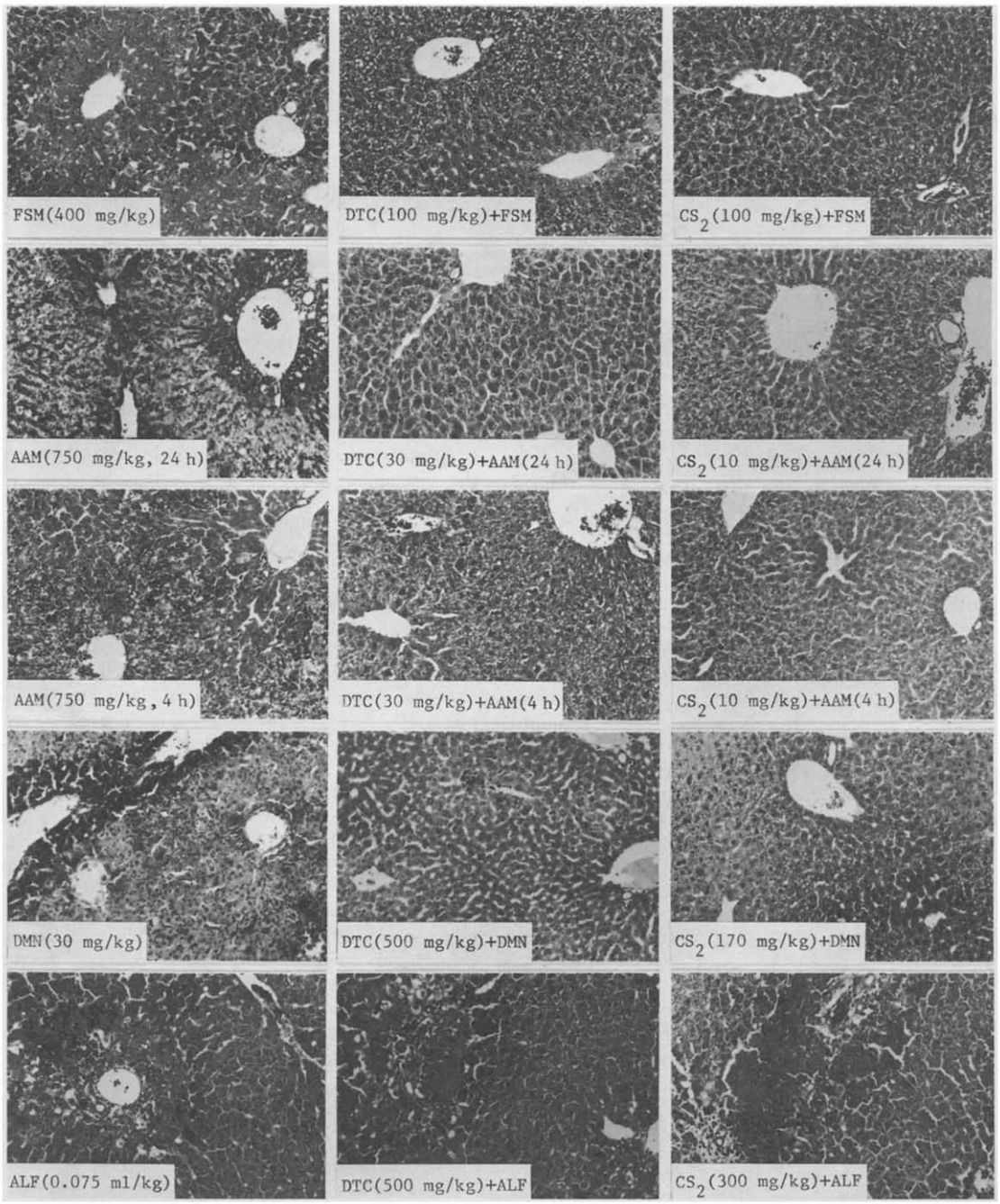


Fig. 4. Histopathological examination of protective effect of DTC and CS_2 against various hepatotoxins. Magnification: × 70. Abbreviations: TAA, thioacetamide; BBZ, bromobenzene; FSM, furosemide; AAM, acetaminophen; DMN, dimethylnitrosamine; and ALF, allylformate.

Table 1. Effects of DTC and CS_2 on the duration of action of hexobarbital and zoxazolamine in mice*

Group	Hexobarbital sleeping time (min)	Zoxazolamine paralysis time (min)
Control	40 ± 4	37 ± 2
DTC, 10 mg/kg, p.o.	60 ± 4†	48 ± 2†
30 mg/kg, p.o.	90 ± 6†	55 ± 4†
100 mg/kg, p.o.	146 ± 13†	101 ± 6†
100 mg/kg, i.p.	70 ± 2†	60 ± 4†
Control	44 ± 4	36 ± 5
CS_2, 3 mg/kg, p.o.	45 ± 3	37 ± 3
10 mg/kg, p.o.	48 ± 3	42 ± 3
30 mg/kg, p.o.	133 ± 12†	87 ± 9†
30 mg/kg, i.p.	135 ± 18†	114 ± 5†

* DTC or CS_2 was given 30 min before administration of hexobarbital (80 mg/kg, i.p.) or zoxazolamine (100 mg/kg, i.p.). Experimental details are given in Materials and Methods. Values are means ± S.E.M.; N = 10.
† $P < 0.01$.

microsome isolated 1 hr after administration of various doses of DTC and CS_2 are shown in Fig. 5. Cytochrome P-450 content was least affected, yet significantly ($P < 0.01$) decreased at doses higher than 30 mg/kg of either DTC or CS_2. CCl_4-dependent MDA production was suppressed by lower doses of both agents, suggesting that CCl_4 metabolism *in vivo* was impaired. Among the substrates examined, aniline hydroxylation and *p*-nitroanisole demethylation were most sensitive. Significant suppression of the metabolism of hexobarbital and zoxazolamine occurred at dose ranges similar to those that prolonged the duration of pharmacological actions of these drugs (Tables 1 and 2). This strongly suggests that the prolonged drug action may have been due to an inhibition of the metabolism of these drugs. Suppression of aminopyrine and 3,4-benzpyrene metabolism was less, compared with that of other substrates. As a general tendency, the suppression occurred dose dependently and around the same molar dose levels of DTC and CS_2, even though the degree of the suppression was different with each substrate.

In vitro *experiments*. To confirm the inhibitory action of DTC and CS_2 on drug metabolism observed *in vivo*, the effects of these agents on microsomal drug-metabolizing enzyme activity were studied *vitro* using rat liver slices and isolated microsomes.

As shown in Table 3, liver slices that had been incubated in the presence of 10^{-4}–10^{-2} M DTC or CS_2 exhibited a decrease in microsomal cytochrome P-450 content and in aniline hydroxylase and aminopyrine demethylase activities.

When isolated microsomes were incubated with 10^{-5}–10^{-3} M DTC or CS_2 in the presence of NADPH, a greater decrease in cytochrome P-450 content and metabolism of aniline and aminopyrine was observed, whereas, in the absence of NADPH, no actual loss was observed (Table 4). These observations are consistent with the results reported by Hunter and Neal [8] and DeMatteis [37], also suggesting that DTC and CS_2 have to be metabolized in order to exert inhibitory action on the metabolism of other substrates.

In these experiments, both DTC and CS_2 appeared to have similar inhibitory potencies on microsomal drug-metabolizing enzyme activity *in vitro*; the differences in solubility and volatility of these agents, however, make a precise comparison of the results difficult, even though the incubation was conducted in a sealed vessel.

Table 2. Plasma levels of hexobarbital and zoxazolamine at the time of recovery from their pharmacological actions in DTC-pretreated mice*

	Hexobarbital		Zoxazolamine	
	Sleeping time (min)	Plasma concn (μg/ml)	Paralysis time (min)	Plasma concn (μg/ml)
Control	51 ± 3 (25)	22.9 ± 0.4 (5)	39 ± 2 (25)	27.2 ± 0.9 (5)
DTC (100 mg/kg)	135 ± 9† (25)	32.9 ± 0.7† (5)	96 ± 5† (25)	36.1 ± 1.1† (5)

* Mice were treated as described in Table 1. Blood was obtained from each mouse by cardiac puncture at the time of recovery of the righting reflex. Blood samples from five mice were pooled and 2 ml of the plasma was used for determination. Details are given in Materials and Methods. Values are means ± S.E.M. (N).
† $P < 0.01$.

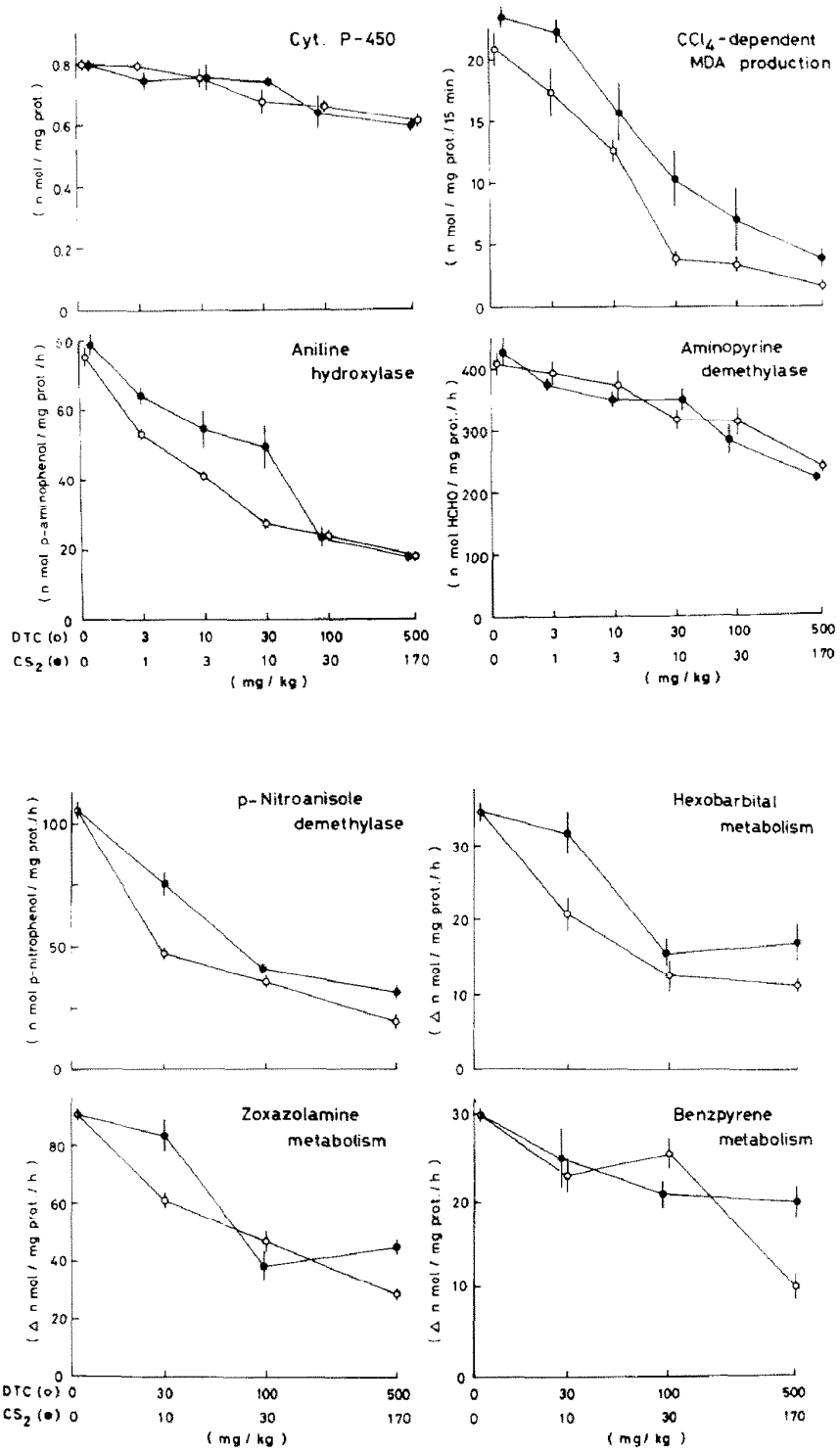


Fig. 5. Effects of DTC and CS_2 on liver microsomal cytochrome P-450 content, CCl_4-dependent MDA production, and drug-metabolizing enzyme activities in mice. Each assay was conducted 1 hr after oral administration of DTC or CS_2. Experimental details are given in Materials and Methods. Each point is the mean ± S.E.M. (N = 4 in experimental groups and N = 16 in control groups).

Table 3. Effects of DTC and CS_2 on microsomal cytochrome P-450 content and drug-metabolizing enzyme activities *in vitro* (slices)*

	Cytochrome P-450 (nmoles/mg protein)	Aniline hydroxylase [nmoles *p*-aminophenol·(mg protein)$^{-1}$·hr^{-1}]	Aminopyrine demethylase [nmoles HCHO·(mg protein)$^{-1}$·hr^{-1}]
Control (8)	0.75 ± 0.01	46.2 ± 2.4	413 ± 16
DTC, 10^{-4} M (2)	0.72 ± 0.02	32.5 ± 0.1	353 ± 0
10^{-3} (4)	0.48 ± 0.02	25.7 ± 2.2	300 ± 19
10^{-2} (2)	0.48 ± 0.02	23.8 ± 1.4	290 ± 7
CS_2, 10^{-4} M (2)	0.67 ± 0.01	36.7 ± 0.2	380 ± 3
10^{-3} (4)	0.56 ± 0.02	35.2 ± 0.3	346 ± 10
10^{-2} (2)	0.44 ± 0.05	19.9 ± 1.9	263 ± 17

* Approximately 500 mg of rat liver slices was placed in a 50-ml flask containing 10 ml of Krebs–Ringer phosphate solution and various concentrations of DTC or CS_2. CS_2 was added as 10 μl of ethanol solution. The flask was flashed with oxygen, capped, and incubated at 37° for 30 min with shaking. Microsomes were isolated from the slices by the calcium aggregation method, and cytochrome P-450 and drug-metabolizing enzyme activities were measured as given in Materials and Methods. No effect was observed with ethanol alone. Values are means ± S.E.M.

Table 4. Effects of DTC and CS_2 on microsomal cytochrome P-450 content and drug-metabolizing enzyme activities *in vitro* (isolated microsomes)*

	Cytochrome P-450 (nmoles/mg protein)		Aniline hydroxylase [nmoles *p*-aminophenol·(mg protein)$^{-1}$·hr^{-1}]		Aminopyrine demethylase [nmoles HCHO·(mg protein)$^{-1}$·hr^{-1}]	
	(−)	(+)	(−)	(+)	(−)	(+)
Control	0.90	0.91	57	56	543	510
DTC, 10^{-5} M	0.88	0.81	57	44	476	516
10^{-4}	0.90	0.64	54	23	536	356
10^{-3}	0.90	0.40	52	16	506	173
CS_2, 10^{-5} M	0.91	0.84	56	56	520	496
10^{-4}	0.86	0.70	50	27	480	386
10^{-3}	0.83	0.58	53	21	516	313

* Microsomes were isolated from rat liver by a calcium aggregation method. Mixtures containing 2 mg/ml of microsomes, 100 μM EDTA, and various concentrations of DTC or CS_2 in the absence (−) or presence (+) of 200 μM NADPH with generating system (as described in Materials and Methods), in a final volume of 10 ml of 0.1 M potassium phosphate buffer (pH 7.5), were prepared in 50-ml flasks, which were capped and incubated at 37° for 30 min with shaking. The mixture, after being cooled in ice, was centrifuged at 100,000 *g* for 60 min to precipitate microsomes. The pellets were washed once, suspended in 2.0 ml of the phosphate buffer, and assayed for cytochrome P-450 content and enzyme activities as described in Materials and Methods. The addition of EDTA and the use of a phosphate buffer protected against loss of enzyme activities from enhanced lipid peroxidation in the presence of NADPH. CS_2 was added as 10 μl of ethanol solution. Ethanol itself had no observable effect. Values are means of two experiments.

DISCUSSION

Oral administration of DTC protected mice against liver injury induced not only by CCl_4 and related halogenomethanes [38, 39] but also by structurally quite different hepatotoxins such as thioacetamide [40], bromobenzene [31], furosemide [32], acetaminophen [41], dimethylnitrosamine [42] and trichloroethylene [34]. A characteristic common to these hepatotoxins is that they require metabolic activation by the microsomal monooxygenase system before producing hepatic injury, as described in the references. Aflatoxin B_1 is also considered to be metabolized to the active epoxide form in order to exhibit carcinogenicity and hepatotoxicity [43]. DTC, however, provided no protection against this hepatotoxin even at a dose of 500 mg/kg. Allylformate hepatotoxicity is postulated to be caused by the active metabolite, acrolein, formed in the presence of non-microsomal enzyme alcohol dehydrogenase [44]. It is conceivable from later discussion that DTC was ineffective in protecting against allylformate-induced liver injury.

Strubelt *et al.* [10] and Siegers *et al.* [11] reported the antihepatotoxic action of DTC (100 mg/kg, i.p.) in mice against liver injury induced by oral administration of CCl_4 (0.1 ml/kg), thioacetamide (50 mg/kg), bromobenzene (0.2 ml/kg) and acetaminophen (1.5 g/kg), but not by dimethylnitrosamine (100 mg/kg). Our present observations with DTC are compatible with their results except in the case of dimethylnitrosamine, against which we observed protection by a high dose (500 mg/kg) of DTC. A noticeable difference between both experimental conditions is the route of administration, i.e. they administered DTC i.p. and hepatotoxins p.o., while we administered them inversely, because DTC was most effective by the oral route in preventing CCl_4-induced hepatotoxicity. In our experiments, a protective oral dose of DTC against liver damage induced by i.p. administration of CCl_4 (0.2 ml/kg), thioacetamide (100 mg/kg), bromobenzene (1.0 ml/kg) and acetaminophen (750 mg/kg) ranged from 3 to 30 mg/kg. This dose range appears to be fairly low when both experimental conditions are compared.

On the other hand, DTC is very unstable at acidic pH, producing CS_2, and Merlevede and Casier [12] reported that 80% of orally administered DTC was expired as CS_2 within 7 hr in man. Moreover, according to the metabolic study by Strömme [13], about 10% of the DTC sulfur was recovered as CS_2 in expiratory air of rats, even after i.p. administration of DTC. Therefore, it was expected that the protective effect of DTC might be mediated through CS_2, especially when given orally. Our findings that nearly equimolar oral doses of DTC and CS_2 were effective without exception against various hepatotoxins, and that the oral protective dose of DTC was approximately one-tenth the parental protective dose in CCl_4-induced liver injury, while the protective dose of CS_2 was less affected by the route of administration, e.g. intraperitoneal administration was as effective as the oral route (Fig. 1 and Table 1), suggest that not only orally administered DTC but parenterally given DTC may also, at least in part, exert its action through CS_2 produced in the body. CS_2, because of the high solubility in lipids, may distribute more easily than DTC across cellular membrane systems.

The latter part of this study concerns the effects of DTC and CS_2 on microsomal drug-metabolizing enzyme activities. Depression of liver microsomal drug metabolism by CS_2 [14–16] and DTC [8, 9] has been reported by several investigators (as given in the references). The present results give confirmatory evidence with mice. Here, we have shown that prolongation of hexobarbital sleeping time and zoxazolamine paralysis time observed after pretreatment with DTC or CS_2 was caused by a delay in the clearance of the drugs from blood plasma that resulted from a reduction of microsomal metabolism of these drugs, and that metabolism of other substrates, such as aniline, *p*-nitroanisole, aminopyrine and 3,4-benzpyrene, and CCl_4-induced lipid peroxidation, which is triggered by the active free radical metabolite of CCl_4, were also reduced. The inhibition of drug metabolism was observed at early hours (1 hr) and at rather low oral dose levels as compared with the previous reports [8, 9, 15, 16]. Furthermore, the patterns of the inhibition by DTC and CS_2 were similar on a molar dose basis.

From these *in vivo* results, the protective action of CS_2 and DTC (p.o.) against such diverse hepatotoxins may be due to the interference of CS_2 with the bioactivation step of the hepatotoxins by the microsomal monooxygenase system.

The mechanisms involved may be as follows. First, the protective effect of DTC and CS_2 against various hepatotoxins cannot be simply explained by the loss of cytochrome P-450 content alone, since cytochrome P-450 content was least affected as compared with the reduction of individual drug-metabolizing activity and, in addition, the protection against some hepatotoxins such as CCl_4 or thioacetamide was observed at a dose of DTC or CS_2 that produced no significant loss of cytochrome P-450 content. Second, DTC and CS_2 may show some substrate-selective inhibition in microsomal hepatotoxin metabolism, since the extent of the reduction of drug-metabolizing activity varied with each substrate, i.e. they were increasingly less sensitive in the order of aniline, *p*-nitroanisole > hexobarbital, zoxazolamine > aminopyrine and 3,4-benzpyrene. This may be one of the conceivable reasons why aflatoxin B_1-induced hepatotoxicity was not blocked by DTC or CS_2 pretreatment. Further experiments are being conducted. And third, it has been demonstrated that CS_2 is also metabolized by the liver microsomal monooxygenase system followed by irreversible binding of the active sulfur to microsomal proteins [45], which may cause a reduction of drug-metabolizing activity and a degradation of cytochrome P-450. Therefore, at the level of the binding to cytochrome P-450 protein, competition of CS_2 with the hepatotoxins might occur; once CS_2 is metabolized, however, noncompetitive inhibition may proceed.

In the experiments *in vitro*, on the other hand, not only CS_2 but DTC itself suppressed cytochrome P-450 content and drug-metabolizing enzyme activities of liver slices and of isolated microsomes preincubated in the presence of NADPH. Hunter and Neal [8] also reported the loss of cytochrome P-450

content and benzphetamine-metabolizing activity in microsomes preincubated with DTC in the presence of NADPH, but not in the absence of NADPH, and Siegers and his colleagues [46–48] showed that DTC inhibited microsomal metabolism of CCl_4, acetaminophen and bromobenzene *in vitro*. These observations suggest that, if DTC could reach the endoplasmic reticulum in its form *in vivo*, it would act in a way similar to that postulated for CS_2. Thus, an interfering action of DTC itself at the site of bioactivation cannot be ruled out, especially in the case of parenteral administration of DTC, although we prefer the action through CS_2 originated from DTC as already described. The exact mechanism remains to be elucidated.

It is known that DTC, *in vivo* (i.p. administration in most cases) and/or *in vitro*, acts as a free radical scavenger, thus protecting animals against radiation [49] and inhibiting lipid peroxidation [7], and also as a copper chelator, inhibiting copper enzymes such as superoxide dismutase [50] and dopamine-β-hydroxylase [51]. These actions of DTC, however, do not appear to be mediated through CS_2 nor to require microsomal metabolism, but may be ascribed to the dithiocarbamyl group itself.

In conclusion, CS_2 and CS_2-producing agents such as DTC and disulfirum [12, 18] may inhibit metabolism of foreign compounds at fairly low oral dose levels and, thus, not only protect against various hepatotoxins that require metabolic activation, but also affect efficacy and duration of action of many drugs. This may imply that DTC, if properly prescribed, may be useful in reducing the doses of other drugs as well as in preventing the toxicity arising from bioactivation of the drugs on the one hand. and an exposure to CS_2 or CS_2-producing agents may increase sensitivity to drug therapy and likely develop toxicity due to overdosage on the other.

Finally, an increase in the liver calcium content is considered to be associated with cell necrosis as demonstrated in the case of CCl_4- and D-galactosamine-induced liver injury [17, 18] and in ischemic liver injury [19]. In the present study, the halogenomethane group, furosemide, and allylformate produced a marked increase in calcium content, whereas other hepatotoxins such as thioacetamide, bromobenzene, acetaminophen, dimethylnitrosamine and aflatoxin B_1 showed a relatively small increase in liver calcium content despite a great increase in plasma transaminase activity and histopathological changes. Thus, an increase in liver calcium content appears to be specific to some hepatotoxins and is not always common to all types of cell necrosis.

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REFERENCES

1. T. Sakaguchi, H. Nishimura, Y. Masuda, I. Tsuge, K. Onishi and H. Tatsumi, *Biochem. Pharmac.* **15**, 756 (1966).
2. Y. Masuda, M. Kuchii, N. Okada and T. Murano, *Jap. J. Pharmac.* **23**, 773 (1973).
3. Y. Masuda, I. Yano and T. Murano, *J. Pharmacobio- Dynamics* **3**, 53 (1980).
4. T. F. Slater, *Nature, Lond.* **209**, 36 (1966).
5. R. O. Recknagel, *Pharmac. Rev.* **19**, 145 (1967).
6. E. A. Glende Jr., A. M. Hruszkewycz and R. O. Recknagel, *Biochem. Pharmac.* **25**, 2163 (1976).
7. Y. Masuda and T. Murano, *Biochem. Pharmac.* **26**, 2275 (1977).
8. A. L. Hunter and R. A. Neal, *Biochem. Pharmac.* **24**, 2199 (1975).
9. M. A. Zemaitis and F. E. Green, *Toxic. appl. Pharmac.* **48**, 343 (1979).
10. O. Strubelt, C. P. Siegers and A. Schütt, *Archs Toxic.* **33**, 55 (1974).
11. C. P. Siegers, O. Strubelt and M. Völpel, *Archs Toxic.* **41**, 79 (1978).
12. E. Merlevede and H. Casier, *Archs int. Pharmacodyn. Thér.* **132**, 427 (1961).
13. H. Strömme, *Biochem. Pharmac.* **14**, 393 (1965).
14. K. J. Freundt and W. Dreher. *Naunyn-Schmiedebergs Arch. Pharmak. exp. Path.* **263**, 208 (1969).
15. E. J. Bond and F. De Matteis, *Biochem. Pharmac.* **18**, 2531 (1969).
16. J. Järvisalo, H. Savolainen, E. Elovaara and H. Vainio, *Acta pharmac. tox.* **40**, 329 (1977).
17. E. S. Reynolds, R. E. Thiers and B. L. Vallee, *J. biol. Chem.* **237**, 3546 (1962).
18. J. L. Farber and S. K. El-Mofty, *Am. J. Path.* **81**, 237 (1975).
19. K. R. Chien, J. Abrams, R. G. Pfau and J. L. Farber, *Am. J. Path.* **88**, 539 (1977).
20. J. R. Mitchell, D. J. Jollow, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 185 (1973).
21. S. Reitman and S. Frankel, *Am. J. clin. Path.* **28**, 56 (1957).
22. L. Moore, G. R. Davenport and E. J. Landon, *J. biol. Chem.* **251**, 1197 (1976).
23. P. Mazel, in *Fundamentals of Drug Metabolism and Drug Disposition* (Eds. B. N. La Du, H. G. Mandel and E. L. Way), p. 546. Williams & Wilkins, Baltimore (1971).
24. A. Trevor, in *Fundamentals of Drug Metabolism and Drug Disposition* (Eds. B. N. La Du, H. G. Mandel and E. L. Way), p. 591. Williams & Wilkins, Baltimore (1971).
25. D. L. Cinti, P. Moldeus and J. B. Schenkman, *Biochem. Pharmac.* **21**, 3249 (1972).
26. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
27. A. H. Conney, E. C. Miller and J. A. Miller, *J. biol. Chem.* **228**, 753 (1957).
28. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
29. E. Burdino, E. Gravela and G. Ugazio, *Agents Actions* **4**, 244 (1973).
30. D. N. Gupta, *J. Path. Bact.* **72**, 183 (1956).
31. B. B. Brodie, W. D. Reid, A. K. Cho, G. Sipes, G. Krishna and J. R. Gillett, *Proc. natn. Acad. Sci. U.S.A.* **68**, 160 (1971).
32. J. R. Mitchell, W. Z. Potter, J. A. Hinson and D. J. Jollow, *Nature, Lond.* **251**, 508 (1974).
33. J. M. Barnes and P. N. Magee, *Br. J. ind. Med.* **11**, 167 (1954).
34. H. Allemand, D. Pessayre, V. Descatoire, C. Degott, G. Feldmann and J. P. Benhamou, *J. Pharmac. exp. Ther.* **204**, 714 (1978).
35. W. H. Butler, *Am. J. Path.* **49**, 113 (1966).
36. J. G. Piazza, *Z. exp. Path. Ther.* **17**, 318 (1915).
37. F. DeMatteis, *Molec. Pharmac.* **10**, 849 (1974).

38. I. G. Sipes, G. Krishna and J. R. Gillette, *Life Sci.* **20**, 1541 (1977).
39. Y. Masuda and T. Murano, *Biochem. Pharmac.* **27**, 1983 (1978).
40. R. A. Neal, in *Reviews in Biochemical Toxicology* (Eds. E. Hodgson, J. R. Bend and R. M. Philpot), Vol. 2, p. 131, Elsevier North Holland, New York (1980).
41. D. J. Jollow, J. R. Mitchell, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 195 (1973).
42. P. N. Magee and J. M. Barnes, *Adv. Cancer Res.* **10**, 163 (1967).
43. R. C. Garner, E. C. Miller and J. A. Miller, *Biochem. biophys. Res. Commun.* **45**, 774 (1971).
44. K. R. Rees and M. J. Tarlow, *Biochem. J.* **104**, 757 (1967).
45. R. R. Dalvi, A. L. Hunter and R. A. Neal, *Chem. Biol. Interact.* **10**, 347 (1975).
46. C. P. Siegers, J. G. Filser and H. M. Bolt, *Toxic. appl. Pharmac.* **46**, 709 (1978).
47. M. Younes and C. P. Siegers, *Archs Toxic.* **45**, 61 (1980).
48. M. Younes, C. P. Siegers and J. G. Filser, *Archs Toxic.* **42**, 289 (1979).
49. Z. M. Bacq, *Archs int. Physiol.* **61**, 433 (1953).
50. R. E. Heikkila, F. S. Cabbat and G. Cohen, *J. biol. Chem.* **251**, 2128 (1976).
51. M. Goldstein, E. Lauber and M. R. Mckereghan, *J. biol. Chem.* **240**, 2066 (1965).