The Effects of Carbon Disulphide on Rat Liver Microsomal Mixed-Function Oxidases, *in vivo* and *in vitro*

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(Received 13 September 1979)

An intraperitoneal dose of CS₂ (500 mg/kg) to male rats resulted in loss of liver microsomal mixed-function-oxidase activity (85% loss of biphenyl 4-hydroxylase), followed by denaturation of liver cytochrome *P*-450 to cytochrome *P*-420, and degradative loss of both cytochromes (50% loss). Losses of NADPH–cytochrome *c* reductase (20%) and cytochrome *b*₅ were considerably less. Intraperitoneal administration of CS₂ (100 mg/kg) to rats pretreated with phenobarbitone or 3-methylcholanthrene resulted in similar losses, but the rate of destruction was greater with cytochrome *P*-450 than with cytochrome *P*-448. At 12 h after intraperitoneal injection of CS₂ to non-pretreated rats, a new cytochrome (*P*-448) appeared. Rat liver microsomal preparations incubated with CS₂ in the presence of NADPH and O₂ resulted in loss of cytochrome *P*-450 and mixed-function-oxidase activity directly related to the concentration of CS₂ (10–100 μM) and to the period of incubation. Addition of EDTA (1 mM) completely inhibited this destruction of cytochrome *P*-450 by CS₂ *in vitro*. Addition of CS₂ to liver microsomal preparations resulted in moderate increases in the *K*_s values for type-I or type-II substrates, but these were insufficient to account for the inhibition of the mixed-function oxidases. We therefore suggest that desulphuration of CS₂ leads to binding of the S to cytochrome *P*-450, denaturation of cytochrome *P*-450 to cytochrome *P*-420, and ultimately to destruction of these cytochromes by autoxidation.

CS₂ is widely used as an intermediate in the production of viscose rayon, in the cold vulcanization of rubber, and in the manufacture of various fungicides and pesticides. Its high volatility and systemic toxicity make it a significant hazard in the industrial environment. In addition it is formed in the spontaneous decomposition of the dithiocarbamate fungicides and so may also constitute a health hazard in the agriculture environment (Fishbein, 1976). Chronic poisoning with CS₂ leads to muscular paralysis, hypertension, hepatic necrosis and encephalopathy (Davidson & Feinleib, 1972).

CS₂ is readily absorbed, and is extensively metabolized by oxidative desulphuration to carbonyl sulphide and CO₂ by the hepatic microsomal mixed-function oxidases (De Matteis & Seawright, 1973; De Matteis, 1974). Administration of CS₂ to rats also leads to inhibition of the microsomal mixed-function oxidases, and to a decrease of liver microsomal cytochrome *P*-450 (Bond & De Matteis,

1969; Freundt & Dreher, 1969). Chronic exposure of rats to low concentrations of CS₂ also decreased liver microsomal cytochrome *P*-450, but increased the NADH–cytochrome *c* reductase (Sokal, 1973). Using ¹⁴C- and ³⁵S-labelled carbon disulphide, De Matteis (1974) found that oxidative metabolism of the compound was accompanied by binding of ³⁵S to the liver microsomal fraction, which, he suggested, might be associated with the hepatotoxic effects.

The hepatotoxicity, and carcinogenicity, of many chemicals is known to be associated with their microsomal metabolism to highly reactive intermediates, which form ligand complexes with microsomal cytochrome *P*-450 with simultaneous loss of mixed-function-oxidase activity (Parke & Rahman, 1971; Lake & Parke, 1972; Buening & Franklin, 1976). The following studies were carried out to elucidate further the mechanism of CS₂ hepatotoxicity, and to ascertain if the accompanying loss of cytochrome *P*-450 and mixed-function-oxidase activity involve ligand-complex-formation with the cytochrome.

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Experimental

Effect of carbon disulphide in vivo

Male Wistar albino rats (150 g body wt.), maintained on rodent breeding pelleted diet (Lab. Diet 1; Spratt's, Barking, Essex, U.K.) and tap water *ad lib.*, were given a single dose of CS₂ (500 mg/kg) in corn oil intraperitoneally, and killed at various times from 10 min to 24 h afterwards. Half the animals were starved for 15 h before dosing; control animals were given corn oil only. Other rats (200 g body wt.) were pretreated with sodium phenobarbitone (60 mg/kg per day) in 1.0 ml of aq. 0.9% NaCl intraperitoneally for 3 days, or 3-methylcholanthrene (20 mg/kg per day) in 1.0 ml of corn oil intraperitoneally for 3 or 5 days, 24 h before administration of CS₂ (100 mg/kg or 500 mg/kg) in 1 ml of corn oil intraperitoneally.

Rats were killed by cervical dislocation at 0.5–12 h after the administration of CS₂. Livers were removed, homogenized in cold aq. 1.15% (w/v) KCl (1 g of liver/4 ml) and centrifuged at 10 000 g for 20 min. The resulting 10 000 g-supernatant preparation was further centrifuged at 105 000 g for 1 h to yield the microsomal pellet, which was rehomogenized in 1.15% KCl, re-centrifuged and finally rehomogenized in 1.15% KCl buffered to pH 7.6 with 0.1 M-sodium/potassium phosphate to give the microsomal suspension (1 g of liver/ml). The 10 000 g supernatant was used for the determination of mixed-function-oxidase activities, and the microsomal suspension was used for the determination of cytochrome *P*-450 concentration, the wavelengths of CO-ligand spectra and substrate-binding spectra.

Biphenyl 2- and 4-hydroxylase activities (Creaven *et al.*, 1965), aniline hydroxylase (Guarino *et al.*, 1969), cytochrome *P*-450 (Omura & Sato, 1964a), cytochrome *P*-420 (Omura & Sato, 1964b), cytochrome *b*₅ (Schenkman *et al.*, 1967), NADPH-cytochrome *c* reductase (Masters *et al.*, 1967) and microsomal protein (Lowry *et al.*, 1951) were determined by standard methods. Substrate-binding spectra and the binding constant *K*_s were determined by the method of Schenkman *et al.* (1967). The wavelength of the u.v.-absorption maxima of the CO-reduced cytochrome *P*-450 complex was determined by using a model 219 Varian spectrophotometer with a reproducibility of 0.02 nm. Calculations of the concentration of cytochrome *P*-450 and cytochrome *P*-448 were made by using the absorption coefficient 91 mm⁻¹·cm⁻¹, and for cytochrome *P*-420 110 mm⁻¹·cm⁻¹.

Effect of carbon disulphide in vitro

Liver microsomal supernatant (equivalent to 125 mg of liver) from untreated male rats was preincubated at 37°C for 1 min in a shaking water bath with various concentrations of CS₂ [1 mM–

10 μM, added as 0.25 ml of a sonicated emulsion in 1.15% KCl containing 2% (v/v) Tween 80], plus 1.5 μmol of NADP⁺, 15 μmol of glucose 6-phosphate, 2 units of glucose 6-phosphate dehydrogenase, 10 μmol of MgCl₂, and 25 μmol of Tris/HCl buffer, pH 8.1, for the biphenyl hydroxylase assay, or 25 μmol of sodium/potassium phosphate buffer, pH 7.4, for the aniline hydroxylase assay, in a total volume of 2.0 ml. After preincubation, the microsomal supernatant preparations were incubated with biphenyl (3 μmol) or aniline (20 μmol) for a further period of 10 min for determination of mixed-function-oxidase activities.

Similar experiments, with the same concentration of cofactors, were carried out with appropriately diluted preparations of the microsomal suspensions for determination of the effects of CS₂ on cytochrome *P*-450 and cytochrome *b*₅ *in vitro*. The mixtures were incubated with CS₂ for 10 min, gassed with N₂ for 1 min to remove CS₂ that had not reacted, and then with O₂ for 1 min, before determination of the concentration of cytochromes.

To ascertain the effect of autoxidation/lipid peroxidation, EDTA (1 mM), an inhibitor of lipid peroxidation, was added to the microsomal suspensions, which were then incubated together with the NADPH-generating system and CS₂ (10 μM–1 mM) at 37°C in air for 10 min. The CS₂ was removed with N₂, then O₂ (at 4°C), and the cytochromes were determined as previously described.

To determine if CS₂ forms a ligand complex with cytochrome *P*-450, as occurs with saffrole or thiols (Mansuy *et al.*, 1974; Nastainczyk *et al.*, 1976), the liver microsomal suspension (5.0 ml, equivalent to 5.0 g of liver) from rats pretreated with sodium phenobarbitone was incubated aerobically or anaerobically with CS₂ (1 mM) in the presence of NADPH (3 mM) at 37°C for 10 min, and the u.v. difference absorption spectrum was measured against the same incubated microsomal suspension plus NADPH but without CS₂.

Results

Effect of carbon disulphide on liver microsomal enzymes in vivo

After intraperitoneal administration of high doses of CS₂ (500 mg/kg) to rats, maximum inhibition of biphenyl 2- and 4-hydroxylase occurred at 30 min, and the maximum loss of cytochrome *P*-450 occurred at 1–4 h after dosage (see Table 1). Even at 10 min after administration of CS₂, biphenyl 4-hydroxylase and cytochrome *P*-450 were only 20 and 66%, respectively, of controls. As cytochrome *P*-450 decreased so cytochrome *P*-420 increased, though as time progressed the sum of both cyto-

Table 1. *Effect of carbon disulphide in vivo on liver microsomal mixed-function oxidases of normal and starved rats* Male rats (150g body wt.) were given a single dose of CS₂ (500mg/kg in 1ml of corn oil, intraperitoneally). Half the rats were starved for 15h before administration of CS₂ and throughout the experiment. Animals from both fed and starved groups were killed in pairs (by cervical dislocation) 10, 30min and 1, 4, 12 and 24h after CS₂ administration, with the exception that only fed rats were killed 24h after CS₂ injection. Immediately after animals were killed liver homogenates were prepared and 10000g-supernatant and microsomal preparations were obtained and mixed-function oxygenases were determined as described in the text.

Time after CS ₂ administration	Period of starvation (h)	Cytochrome P-450 concn. (nmol/mg of protein)	Cytochrome P-420 (nmol/mg of protein)	NADPH-cytochrome c reductase (nmol/min per mg of protein)	Biphenyl 4-hydroxylase (μmol/h per g of liver)	Biphenyl 2-hydroxylase (μmol/h per g of liver)
No CS ₂	None	0.66	0.03	30	1.9	0.11
	15	0.79	0.04	10.5	2.0	0.07
10 min	None	0.44	0.07	25	0.39	0.05
	15	0.52	0.16	13	0.25	0.02
30 min	None	0.36	0.13	23	0.28	0.03
	15.5	0.40	0.22	16	0.31	0.02
60 min	None	0.33	0.15	26	0.31	0.03
	16	0.42	0.24	19	0.36	0.03
4 h	None	0.32	0.05	23	0.38	0.06
	19	0.41	0.18	13	0.53	0.06
12 h	None	0.46	0.03	22	0.91	0.17
	27	0.40	0.03	20	1.06	0.24
24 h	None	0.70	0.07	37	1.7	0.23

chromes diminished, indicating that further degradation had occurred.

The concentrations of cytochromes P-450 and P-420 were slightly higher in starved than in non-starved rats, at least for the first 4h after dosage. The NADPH-cytochrome c reductase activity was lower in the starved than in the fed rats, and CS₂ administration increased the NADPH-cytochrome c reductase activity of starved rats but decreased the reductase activity of fed animals.

In rats pretreated with phenobarbitone, administration of a single intraperitoneal dose of CS₂ (100mg/kg) again diminished the induced amount of cytochrome P-450 (by 40%) and activities of biphenyl 4-hydroxylase (by 50%), 2-hydroxylase (by 15%) and aniline hydroxylase (by 60%). After pretreatment of rats with methylcholanthrene, intraperitoneal administration of CS₂ gave a slightly smaller decrease in cytochrome P-448 (by 35%), although the decreases in biphenyl 4-hydroxylase (by 50%), biphenyl 2-hydroxylase (by 45%) and aniline hydroxylase (by 70%) activities were still high. Treatment with CS₂ did not change the wavelength maxima of the cytochrome P-450/P-448 species with which it interacted, within the 2h of the experiment.

Rats pretreated with phenobarbitone or 3-methylcholanthrene, or untreated, were given CS₂ (500mg/kg) intraperitoneally. Liver microsomal preparations obtained at 0, 0.5, 4 and 12h after dosage with CS₂ were studied spectrophotometrically to determine their binding spectra with a type-I substrate

(hexobarbitone) and a type-II substrate (aniline). With liver microsomal preparations from normal rats and from rats pretreated with phenobarbitone, type-I-substrate binding was slightly decreased by CS₂ treatment, giving K_s values 2–3-fold greater than were obtained without CS₂ treatment. With liver microsomal preparations from normal rats the K_s values for type-II substrate were decreased 2–3-fold. The maximum changes occurred at 0.5 and 4h after dosage of CS₂. With liver microsomal preparations from rats pretreated with 3-methylcholanthrene, the K_s value for the type-II substrate showed a 2-fold increase, but the K_s value for the type-I substrate showed no significant change. These relatively minor changes in K_s values were not sufficient to account for the rapid and extensive (80%) loss of mixed-function-oxidase activity.

The loss of cytochrome P-450 ($\lambda_{\max}$ of CO ligand, 450.0 ± 0.1 nm) in liver of normal rats treated intraperitoneally with CS₂ was 20 and 45% at 0.5 and 4h after administration; by 12h the loss of cytochrome P-450 was only 20%, indicating that new cytochrome had been synthesized. The wavelength of the CO-binding spectrum of this cytochrome P-450 from untreated rats 12h after dosage with CS₂ was 448.8 ± 0.1 nm, indicating that cytochrome P-448 was being formed preferentially. The rate of loss of cytochrome P-450 or P-448 was more rapid after pretreatment with phenobarbitone (37, 41 and 53% in 0.5, 4 and 12h) than after methylcholanthrene pretreatment (7, 24 and 41% in 0.5, 4 and 12h). This is not unexpected, since cytochrome

Table 2. *Effect of carbon disulphide concentration in vivo on rat liver microsomal cytochrome P-450 and mixed function-oxidase activity*

Liver microsomal preparations from untreated male rats, prepared as described in the Experimental section, were preincubated with various concentrations of CS₂ in the presence of the added cofactors for 1 min at 37°C. After addition of the substrates, biphenyl or aniline, incubation was continued for a further 10 min, and the mixed-function-oxidase activities were determined. For determination of cytochrome P-450 content the microsomal preparation plus cofactors was incubated with CS₂ at 37°C for 10 min, saturated with N₂ for 1 min and then with O₂ for 1 min at 4°C to remove CS₂ that had not reacted, and the cytochrome P-450 measured (Omura & Sato, 1964a) by using an absorption coefficient of 91 mm⁻¹ · cm⁻¹. Results are the mean values ± s.e.m. of three separate experiments.

Final concn. of CS ₂	Cytochrome P-450 (nmol/mg of protein)	Activity (μmol/h per g of liver)		
		Biphenyl 4-hydroxylase	Biphenyl 2-hydroxylase	Aniline hydroxylase
None	0.41 ± 0.09	1.34 ± 0.26	0.12 ± 0.03	0.45 ± 0.01
12.5 μM	0.34 ± 0.06	0.97 ± 0.04	0.08 ± 0.01	0.41 ± 0.06
50 μM	0.31 ± 0.03	0.68 ± 0.03	0.13 ± 0.04	0.34 ± 0.05
125 μM	0.23 ± 0.03	0.25 ± 0.10	0.10 ± 0.03	0.36 ± 0.06
250 μM	0.10 ± 0.03	0.18 ± 0.01	0.08 ± 0.01	0.28 ± 0.05
500 μM	0.08 ± 0.01	0.16 ± 0.02	0.16 ± 0.04	0.26 ± 0.02
1.25 mM	0.11 ± 0.01	0.19 ± 0.02		0.28 ± 0.05
5.00 mM	0.07 ± 0.01	0.17 ± 0.01		

P-450 is less stable than cytochrome P-448 and has a shorter half-life.

Effect of carbon disulphide on liver microsomal enzymes in vitro

Microsomal cytochrome P-450 and oxidation of the type-I and type-II substrates, biphenyl and aniline, were decreased after incubation of rat liver microsomal preparations with CS₂ (10 μM–1 mM) in the presence of NADPH and O₂ (see Table 2). Maximum decrease in the microsomal mixed-function oxidase activity was achieved after 10 min incubation with 0.5 mM-CS₂, when cytochrome P-450, biphenyl 4-hydroxylase and aniline 4-hydroxylase were decreased by 80, 88 and 40% respectively. Biphenyl 2-hydroxylase activity, which is an index of cytochrome P-448 and not cytochrome P-450 activity, shows no significant loss of activity (see Table 2). Inhibition of biphenyl hydroxylase and aniline hydroxylase activities and the decrease in cytochrome P-450 do not occur in the absence of NADPH. At lower concentrations of CS₂ (<0.1 mM), the loss of cytochrome P-450 and the extent of inhibition of mixed-function-oxidase activity are proportional to the period of preincubation of the rat liver microsomal preparation with CS₂.

Addition of EDTA (1 mM) to the microsomal incubation mixture prevented the decrease in cytochrome P-450 observed on incubation with CS₂ alone. Incubation with EDTA alone resulted in a 50% increase in the amount of cytochrome P-450, presumably owing to the inhibition of spontaneous destruction of the cytochrome by lipid peroxidation.

That no chemical interaction occurs between EDTA and CS₂ was established by u.v.-absorption spectra of the mixture, which showed a simple summation of the spectra of the two individual components.

The ligand complexes that cytochrome P-450 forms with thiols and other chemicals, after oxidative or reductive metabolic activation, exhibit characteristic oxidation/reduction difference spectra with maxima at about 455 nm. The product of incubation of liver cytochrome P-450 from phenobarbitone-pretreated rats with CS₂ in the presence of NADPH and the absence of O₂ showed no new maximum over the wavelength range 435–550 nm in the redox difference spectrum. Incubation with CS₂ in the presence of NADPH and O₂ gave a difference spectrum when read against the control microsomal preparation without CS₂ which had a slight maximum at 460 nm, suggestive of the formation of a ligand complex.

Discussion

The present studies confirm previous observations that CS₂ decreases hepatic microsomal cytochrome P-450 and the mixed-function oxidases concomitantly with its oxidative metabolism *in vivo* and *in vitro* (Bond & De Matteis, 1969; Freundt & Dreher, 1969; Sokal, 1973). Indeed our present findings *in vitro* show a direct relationship between the concentration of CS₂ from 10 to 100 μM and the decrease in hepatic cytochrome P-450 and mixed-function oxidation of both type-I and type-II substrates (see Table 2). The prevention of the loss of cytochrome P-450 by addition of EDTA is indicative that the destruction of cytochrome P-450

is due to lipid peroxidation, since Levin *et al.* (1973) showed that the catabolism of haem and destruction of cytochrome *P*-450 resulting from lipid peroxidation were prevented by the addition of EDTA. As it is common practice to add EDTA to liver microsomal incubation mixtures (De Matteis, 1974), this may account for some of the varied results observed with studies on CS₂.

After intraperitoneal administration of CS₂, liver microsomal cytochrome *P*-450 is similarly decreased, as also are the rates of hydroxylation of both type-I and type-II substrates. The initial greater loss of biphenyl 4-hydroxylase activity than of cytochrome *P*-450 (see Table 1) indicates that inhibition of cytochrome *P*-450 occurs before its denaturation and degradation. The loss of cytochrome *P*-450 was shown to be the result first of denaturation to cytochrome *P*-420, which was formed parallel to the decrease in cytochrome *P*-450. This was subsequently followed by the degradation of both cytochromes (Table 1). If the degradation were the result of generalized lipid peroxidation, a loss of NADPH-cytochrome *c* reductase and other membrane components might be expected, and indeed a small loss was detected in the unstarved animals (Table 1). Because of the inhibition of hepatic microsomal mixed-function oxidases by CS₂ *in vitro*, other workers have concluded that CS₂ itself acts as inhibitor (Von Kromer & Freundt, 1976); they observed a 2-fold increase in the apparent *K_m* value for the inhibition by CS₂ of the *N*-demethylation of aminopyrine, a type-I substrate, which is comparable with the increases in the *K_s* values that we observed. Dalvi & Howell (1978) also found that CS₂ interfered with the binding of type-I substrates to microsomal cytochrome *P*-450, but not that of aniline, a type-II substrate. However, we find that CS₂ affects both type-I and type-II sites, though not enough to account for the full loss of mixed-function-oxidase activity, which is better explained by destruction of cytochrome *P*-450.

Although within 4 h of intraperitoneal administration of CS₂ the type of liver cytochrome *P*-450 of normal, phenobarbitone- or methylcholanthrene-pre-treated rats remained unchanged, at 12 h the wavelength of the spectral maximum of the CO ligand complex of the cytochrome had changed from 450.0 ± 0.1 to 448.8 ± 0.1 nm. At this time the concentration of cytochrome *P*-450 is increasing, presumably owing to synthesis *de novo*, so that the decrease in wavelength may be due to the preferential synthesis of cytochrome *P*-448, as occurs with carcinogens, or alternatively, to the preferred degradation of cytochrome *P*-450, which is known to be less stable than cytochrome *P*-448.

Using ³⁵S-labelled CS₂, De Matteis (1974) showed that on incubation with rat liver microsomal preparations the ³⁵S becomes covalently bound to the

microsomal protein simultaneously with the decrease in cytochrome *P*-450. Later work (Neal *et al.*, 1976; Savolainen *et al.*, 1977) has shown that the sulphur is bound principally to cytochrome *P*-450 itself, possibly to the apoprotein.

It is tempting to suggest that the sulphur from the desulphuration of CS₂ is involved in a ligand to the haem Fe of the cytochrome *P*-450, analogous to the hydrodisulphide bond with the cysteine sixth ligand proposed by Davis & Mende (1977) for the desulphuration of parathion. It is unlikely that the sulphur interacts primarily with the type-I substrate site or at the fifth ligand (type-II substrate site) of cytochrome *P*-450, as CS₂ does not sufficiently affect the *K_s* values for type-I or type-II substrates and does not readily show the u.v. difference absorption spectrum (455–460 nm) characteristic of ligand complexes (Nastainczyk *et al.*, 1975, 1976). If sulphur is transferred from CS₂ to cytochrome *P*-450 as the result of exchange with oxygen, it might be expected to become bound to the cytochrome *P*-450 near to the site of attachment of the molecular O₂, that is at the cysteine S of the sixth ligand (Chevion *et al.*, 1977). This would probably interfere with the subsequent binding of molecular O₂ to the cytochrome and would thus inhibit its mixed-function-oxidase activity. It might also account for the protective effects of cysteine, glutathione and dithiothreitol against CS₂ toxicity, observed by Morelli & Nakatsugawa (1978). CS₂, like CCl₄, is known to result in lipid peroxidation (Jarvisalo *et al.*, 1977). Inhibition of mixed-function-oxidase activity of cytochrome *P*-450 might be expected to lead to decoupling of the cytochrome from the flavoprotein reductase, and to autoxidation, lipid peroxidation, degradation of cytochrome *P*-450 and eventually centrilobular necrosis, as occurs with CCl₄ and certain other hepatotoxic chemicals (Parke, 1979).

We thank the World Health Organisation and Dr. J. Nofer, the former Director of the Institute of Occupational Medicine, Lodz, Poland, for supporting this work of M. J. O., carried out at the University of Surrey, and Dr. Teresa Nofer and Dr. C. Ioannides for their continued help and advice.

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