THE HEPATOTOXIC ACTION OF CARBON TETRACHLORIDE STIMULATORY EFFECT OF CARBON TETRACHLORIDE ON LIPID PEROXIDATION IN MICROSOMAL SUSPENSIONS

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Carbon tetrachloride has been reported to stimulate lipid peroxidation in rat liver microsomal suspensions *in vitro* provided that a source of NADPH is present [1]; the dependence on NADPH suggests that the stimulatory action of carbon tetrachloride involves an interaction with the NADPH–cytochrome *P*-450 electron transport chain. Several suggestions have been made linking the stimulatory action of carbon tetrachloride on microsomal lipid peroxidation with its necrogenic activity *in vivo* [2]. In this paper we outline the major features of the stimulatory action of carbon tetrachloride on lipid peroxidation *in vitro* and discuss its relevance to the liver damage that occurs *in vivo* after administering carbon tetrachloride to rats.

Adult albino rats, body wt. approx. 130 g, were killed by cervical dislocation; liver homogenates (1:10, v/v, in ice-cold 0.25 M sucrose) were centrifuged twice at 11,700 $g_{av.}$ for 10 min at 2°; the pellets were discarded and the microsomes plus supernatant suspension was further centrifuged at 157,000 $g_{av.}$ for 40 min at 2°. The microsomal pellet was rinsed with ice-cold 0.15 M KCl and then resuspended in 0.15 M KCl so that the microsomes equivalent to 1 g wet wt. liver were contained in 1 ml suspension. A standard stock medium contained: 18 ml 0.15 M KCl (83.5 mM); 12 ml tris buffer, 0.1 M, pH 8.0 (37.2 mM); 1.8 ml of 0.1 M disodium glucose-6-phosphate dehydrogenase (8.4 I.U.); 8 μmoles sodium NADP (0.245 mM); 200 mg actamide (10 mM); final concentrations in parentheses. Either 4 ml microsomal suspension or 6 ml microsomes plus supernatant suspension were added to the stock medium (32.4 ml) to constitute the incubation mixture. Samples of incubation mixture (2.5 ml) were placed in the central compartments of Warburg flasks with side arms; 2 μl of a carbon tetrachloride–liquid paraffin solution (1:1, v/v) were placed in the side arms. The flasks were sealed and incubated at 37° in the dark and with gentle shaking at 60 cycles/min. Incubation times were 60 min for microsomes plus supernatant stock mixtures and 10–30 min for microsome stock mixtures. At the end of the incubation period samples were withdrawn and analysed for malonaldehyde concentration by the thiobarbituric acid reaction [3]. Under the above conditions the production of malonaldehyde was proportional to the lipid peroxide concentration as measured by the iodometric method of Swoboda and Lea [4].

Carbon tetrachloride stimulated the production of malonaldehyde in tissue suspensions at 37°; in microsomal stock suspensions the average stimulation ± S.E.M. of 64 separate experiments was 19 ± 1% of the control situation. In microsomes plus supernatant-stock suspensions the corresponding stimulation was 64 ± 13% (mean of 68 experiments). The stimulatory action of carbon tetrachloride on malonaldehyde production was destroyed by prior heating of the microsomal suspension at 70° for 5 min under anaerobic conditions. The stimulation in microsome stock suspensions was totally dependent on a source of NADPH, which was not replaceable with NADH.

When the concentration of carbon tetrachloride in the incubation mixture was varied (by altering the amount added to the side arms of the Warburg flasks)

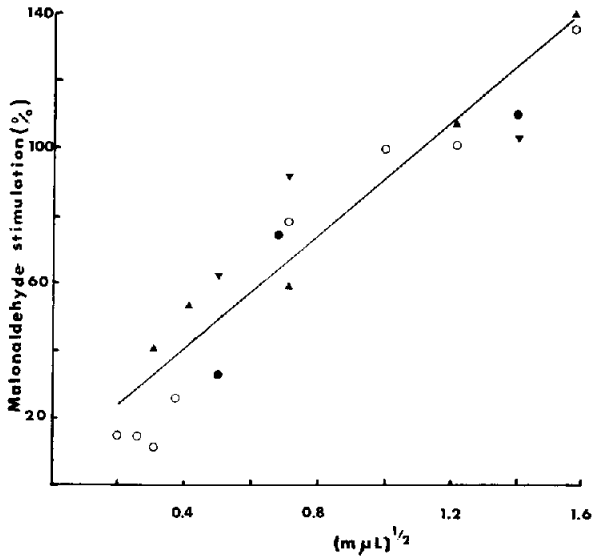

Fig. 1. The production of malonaldehyde in suspensions of microsomes plus supernatant in stock mixtures with varying amounts of carbon tetrachloride in the side arms of Warburg flasks used for the 60 min incubations at 37°. The four symbols shown represent the data obtained from four separate experiments. The data have been normalised to give a 100% stimulation of malonaldehyde production with 1 μl of carbon tetrachloride in the side arm. The abscissa gives the square root of the amount of carbon tetrachloride added to the side arms; the concentration of carbon tetrachloride in the incubation medium was shown to be linearly related to the amount added to the side arm. The regression line is drawn for which the correlation coefficient $r = 0.82$, $p \ll 0.001$.

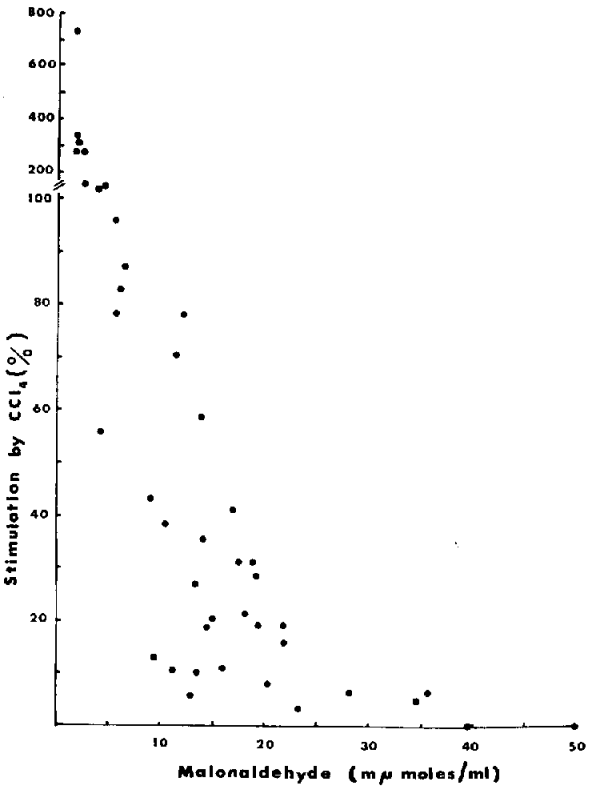

Fig. 2. The relationship between the stimulation of malonaldehyde production by carbon tetrachloride and endogenous malonaldehyde production (i.e. in the absence of carbon tetrachloride) is shown for suspensions of microsomes plus supernatant in standard stock mixtures. Incubation time was 60 min at 37°; 2 μl of a carbon tetrachloride–liquid paraffin mixture (1:1, v/v) in the side arms of Warburg flasks. Data from 42 separate experiments are shown.

it was found that the stimulation of malonaldehyde production was directly proportional to the square root of the carbon tetrachloride concentration in the incubation mixture (fig. 1). This result indicates that the stimulatory action of carbon tetrachloride is not a result simply of a lipophilic action on the membranes of the endoplasmic reticulum but that carbon tetrachloride itself is initiating the peroxidative process most probably through the prior formation of free radical metabolites such as trichloromethyl radicals (for a discussion of the kinetics of relevant situations see [5]).

Whereas the stimulation of lipid peroxidation in microsomal suspensions by ADP plus ferrous ions (or by vitamin C plus ferrous ions) can proceed to complete oxidation of the unsaturated lipid [6] the stimulatory action of carbon tetrachloride seems limited to a particular and rather small proportion of the total microsomal unsaturated lipid. When the endogenous peroxidation rate during incubation was high the stimulation due to carbon tetrachloride was not detectable; however, when endogenous peroxidation was low then carbon tetrachloride had a pronounced stimulatory effect (fig. 2). These results suggests that the peroxidative effect of carbon tetrachloride is heavily damped by the presence of free radical scavengers. Prior starvation of the rats, which may be expected to decrease the concentration of such scavengers in the liver endoplasmic reticulum, markedly accentuates the stimulatory action of carbon tetrachloride; a 24 hr period of starvation increased the stimulatory effect of carbon tetrachloride by two-fold in microsomal stock suspensions.

Table 1
Effect of various free radical scavengers on the stimulation of malonaldehyde production due to carbon tetrachloride.

Drug	Concentration (μM)	Percentage change in the stimulation of malonaldehyde production
Promethazine	0.1	− 51[a,e]
	10	− 85[e]
PG	1	− 26[d]
	10	− 98[e]
DPPD	0.1	− 77[e]
	10	− 100[e]
Vit E	0.005	+ 62[c]
	5.5	− 92[e]
Inosine	0.1	− 40[d]
	2	− 85[d]
EDTA	0.1	+ 30[b]
	20	− 75[e]

Microsomes plus supernatant stock suspensions were incubated for 60 min at 37° in the central compartments of Warburg flasks with and without the free radical scavenger under study. The side arms of the flasks contained 2 μl of a carbon tetrachloride–liquid paraffin mixture (1:1, v/v) where necessary. The effect of the free radical scavengers on the stimulation of malonaldehyde production due to carbon tetrachloride are shown as percentage alterations compared to the stimulation obtained in the absence of the scavenger. *Abbreviations:* DPPD: *N, N'*-diphenyl-*p*-phenylene diamine; PG: propyl gallate; vit E: α-tocopherol polyethylene glycol 1000 succinate.

[a] With a microsome stock suspension, incubation time 30 min at 37°.
[b] $p < 0.1$; [c] $p = 0.05$; [d] $p < 0.01$; [e] $p < 0.001$.

Table 2
Stimulatory effects of halogenomethanes on malonaldehyde production in microsomes plus supernatant stock suspensions during incubation at 37° for 60 min.

Agent	Solubility (mM)	Bond dissociation energy (Kcal/mole)		Relative stimulation of malonaldehyde production	
CCl_3Br	0.012	C − Br	49	3650 ± 200	(4)
CCl_4	0.2	C − Cl	60	100	
CCl_3F	0.066[a]	C − F	75	34	(2)
CCl_3H	2.67	C − H	75	7 ± 2	(6)

Solubilities of the halogenomethanes in the incubation mixtures were measured by gas chromatography or radio-isotope dilution techniques [see 3]; bond dissociation energies are form Cotrell [12]. The stimulatory actions on malonaldehyde production are shown as percentages relative to the activity of CCl_4; mean values are given ± S.E.M. (no. of experiments in parenthesis).

[a] Possibly too low due to very high volatility of this material.

If the stimulatory action of carbon tetrachloride on malonaldehyde production involves the initiation of lipid peroxidation by trichloromethyl radicals then its stimulatory action should be decreased by the inclusion of low concentrations of free radical scavengers in the incubation mixture. Table 1 illustrates relevant data. A number of free radical scavengers were effective in decreasing the carbon tetrachloride stimulatory effect when present in concentrations of 0.1–1 μM. In contrast, sodium phenobarbitone, Cetab, Nupercaine, and Sodium dodecyl sulphate were ineffective at 1–10 μM final concentration.

The production of trichloromethyl radicals from carbon tetrachloride involves the homolysis of a

Table 3
The effects of various inhibitors of microsomal drug metabolism on the stimulation of malonaldehyde production caused by carbon tetrachloride.

Expt.	Inhibitor	Incubation time (min)	Concentration (μM)	Percentage change in malonaldehyde production
a	Proadifen	15	100	+ 33[c]
b	pCMB[a]	10	94	+ 85[d]
c	CO	15	–	+ 55[e]
d	Cytochrome *c*	10	2[b]	– 34
			50[b]	– 100

The data were obtained by incubating microsome stock suspensions at 37° with 2 μl of a carbon tetrachloride–liquid paraffin mixture (1:1, v/v) in the side arms of Warburg flasks. The various inhibitors were included with the microsome stock suspension in the central compartment. In experiment (c) samples of the microsome stock suspension were pregassed for 5 min at 0°with either 95% O_2–5% CO_2 or with carbon monoxide prior to the incubation at 37°. The results are shown as percentage changes in the stimulation of malonaldehyde production due to carbon tetrachloride.

[a] *p* - Chloromercuribenzoate; [b] mg/100 ml incubation mixture; [c] not significantly different from control; [d] $p < 0.05$; [e] $p < 0.01$.

carbon–chloride band. Trichloromethyl radical production should be more easy from trichlorobromomethane (CCl_3Br) and less easy from chloroform (CCl_3H) and trichlorofluoromethane (CCl_3F) than from carbon tetrachloride. Table 2 summarises the bond dissociation energies relevant to the production of trichloromethyl radicals from these halogenomethanes, and also lists their relative activities in stimulating malonaldehyde production in microsomes plus supernatant stock suspensions. It can be seen that ease of homolysis correlates with an increasing ability to stimulate malonaldehyde production.

Some data relating to the interaction site of carbon tetrachloride with the NADPH–cytochrome *P*-450 chain to produce a stimulation of malonaldehyde production is shown in table 3. It can be seen that inhibitors when added at concentrations that greatly decrease the rate of drug metabolism at the *P*-450 site (prodifen, carbon monoxide, or *p*-chloromercuribenzoate) actually increase the stimulatory action of carbon tetrachloride. These data suggest that the carbon tetrachloride interaction site is at or near the NADPH–flavoprotein which is known to oscillate between the fully reduced (FH_3) and the half-reduced ($FH_2^{\cdot}$) flavin semiquinone structures [6]. The interaction between carbon tetrachloride and the flavoprotein may involve a process of electron capture [7] in which a proton is released from the fully reduced flavin:

$$FH_3 + CCl_4 \longrightarrow FH_2^{\cdot} + CCl_3^{\cdot} + Cl^- + H^+$$

The proton release may be relevant to the decrease in glucose-6-phosphatase activity that occurs in conjunction with the increased production of malonaldehyde [8] and is an early manifestation of the *in vivo* toxicity of carbon tetrachloride; glucose-6-phosphatase is irreversibly inhibited by exposure to acid conditions [9]. The production of chemically reactive trichloromethyl radicals in the neighbourhood of the NADPH binding site of the flavoprotein is of considerable interest in view of the early destruction of NADPH *in vivo* during carbon tetrachloride intoxication [10]. It has been demonstrated *in vitro* that exposure of NADPH (but not NADP) to trichloromethyl radicals results in a loss of coenzyme activity. [11].

The results of this investigation suggest that the stimulation of lipid peroxidation by carbon tetrachloride involves an interaction on or near the NADPH flavoprotein leading to the production of trichloromethyl radicals. As a result there is not only a stimulation of lipid peroxidation but a decrease in both glucose-6-phosphatase activity and in NADPH content. These changes are all retarded to a greater or lesser degree by the inclusion of free radical scavengers in the system under study *in vitro* or *in vivo*. Since a number of such scavengers also retard the development of liver necrosis under *in vivo* conditions it may

be concluded that homolytic processes, of which lipid peroxidation is but one example, are an important feature of the necrogenic action of carbon tetrachloride in the rat.

Acknowledgement

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