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The Stimulatory Effects of Carbon Tetrachloride on Peroxidative Reactions in Rat Liver Fractions *in vitro*

INHIBITORY EFFECTS OF FREE-RADICAL SCAVENGERS AND OTHER AGENTS

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1. The effects of a number of free-radical scavengers and other agents on the stimulation of malonaldehyde production due to low concentrations of carbon tetrachloride have been studied in rat liver microsome suspensions. 2. Promethazine, propyl gallate and *NN'*-diphenyl-*p*-phenylenediamine were extremely active in inhibiting the stimulation of malonaldehyde production due to carbon tetrachloride; inhibitory effects were demonstrable with these agents at 0.1 μM. 3. Low concentrations (1–100nM) of vitamin E–polyethylene glycol 1000–succinate increased the stimulation of malonaldehyde production due to carbon tetrachloride, but higher concentrations of the vitamin E preparation decreased both the stimulation due to carbon tetrachloride and the endogenous peroxidation that occurs in the absence of carbon tetrachloride. 4. Other agents tested that were effective in the range 1–20 μM in decreasing the stimulation of malonaldehyde production due to carbon tetrachloride were inosine, desferrioxamine and EDTA. Agents tested that were not effective, except at very high concentrations (100 μM or greater), were Nupercaine, Cetab and sodium phenobarbitone. 5. The results are discussed in terms of the mechanisms responsible for the observed inhibitions of malonaldehyde production, and of the relevance of the *in vitro* system to the liver damage produced by carbon tetrachloride *in vivo*.

It has been shown by previous studies that carbon tetrachloride stimulates the production of malonaldehyde in rat liver microsomal suspensions *in vitro*; the stimulation involves an interaction of the halogenoalkane with the NADPH–cytochrome *P*-450-electron-transport chain present in liver endoplasmic reticulum whereby free-radical metabolites of carbon tetrachloride initiate lipid peroxidation of the lipoprotein-rich membranes (Slater & Sawyer, 1971*a*,*b*).

There have been several suggestions (for references see Slater, 1966; Recknagel, 1967) that the homolysis of carbon tetrachloride is an important step in the production of liver necrosis by that agent, and it is known that several free-radical scavengers retard the onset of necrosis when administered together with the carbon tetrachloride (see Slater, Sawyer & Sträuli, 1966). It was considered necessary, therefore, to study the effects of such free-radical scavengers on the interaction between carbon tetrachloride and the NADPH–flavoprotein in liver endoplasmic reticulum. In addition, since several surface-active agents retard the onset of liver necrosis *in vivo* (Bangham, Rees & Shotlander, 1962) it is also of importance to evaluate their action, if any, on the flavoprotein interaction *in vitro* that results in the production of free-radical metabolites of carbon tetrachloride. In the present paper we have examined the effects of such agents on the production of malonaldehyde by rat liver microsomes, or microsomes-plus-supernatant suspensions, in the presence of a source of NADPH, and with or without low concentrations of carbon tetrachloride.

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METHODS

The rats used were albino females, approx. 120g body wt.; they were fed a modified M.R.C. diet 41B (Oxo Ltd., London S.E.1, U.K.) and water *ad libitum*. The sources of rats and the preparation of the microsomes–stock and the microsomes-plus-supernatant–stock suspensions were as described by Slater & Sawyer (1971*a*).

Incubation of microsomal suspension with CCl_4 (2 μl of a mixture with liquid paraffin, 1:1, v/v) and the production of malonaldehyde in the incubation mixtures, measured by the thiobarbituric acid procedure, was as described by Slater & Sawyer (1971*a*). Agents to be tested were added in 0.5ml volumes to the mixtures. The effects of each substance on the stimulation of malonaldehyde

production due to the presence of carbon tetrachloride were studied over a wide range of concentrations. Tables 1–5 highlight the effects found with various concentrations of the agents used. In general, the lowest concentration required to produce an effect is given for each agent; the range of concentrations studied is given in the text. Several experiments were performed with every agent at each particular concentration to ensure that the results described were completely reproducible. The significance of difference between mean values was tested by Student's *t*-test. The standard errors of the mean values were always about 5% of the mean values given in the Tables; the variability in control mean values between experiments performed on different microsomes–stock or microsomes-plus-supernatant–stock suspensions has been discussed by Slater & Sawyer (1971*a*).

Chemicals were obtained as follows. Promethazine was a generous gift of May & Baker Ltd., Dagenham, Essex, U.K.; aqueous solutions were prepared immediately before use and were protected from light. Desferrioxamine was kindly provided by CIBA Ltd., Horsham, Sussex, U.K. Vitamin E–polyethylene glycol 1000–succinate was generously donated by Eastman Kodak Ltd., Rochester, N.Y., U.S.A. Pure samples of 2-butoxy-*N*-(2-diethylaminoethyl)cinchoninamide hydrochloride (Nupercaine) and cetyltrimethylammonium bromide (Cetab) were kindly provided by Mr G. Williams, University College Hospital, London W.1, U.K.

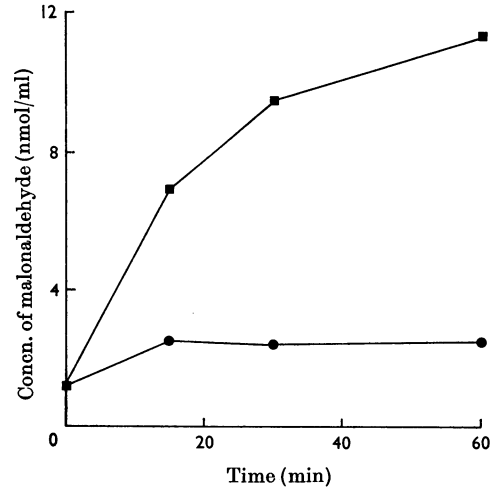

Fig. 1. Effect of promethazine (5 μM) on endogenous production of malonaldehyde in a suspension of microsomes in standard stock at 37°C. Samples (8 ml) of microsomes–standard stock suspensions with and without 5 μM-promethazine were incubated in conical flasks that were shaking at 60 cycles/min; 1 ml samples were withdrawn at various times for analysis of malonaldehyde. ■, Control; ●, in the presence of promethazine.

RESULTS

Promethazine. This agent was studied over the concentration range 0.001–156 μM. Table 1 shows that promethazine *in vitro* is exceptionally effective in preventing the stimulation of malonaldehyde production due to carbon tetrachloride; an effect of promethazine is apparent even at 0.1 μM (Table 1). Promethazine was also effective in decreasing the stimulation in malonaldehyde production due to carbon tetrachloride in microsomes-plus-supernatant–stock suspensions: for example, 10 μM-promethazine inhibited the stimulation due to carbon tetrachloride by 85% during a 60 min incubation at 37°C. The results in Table 1 also show that carbon tetrachloride produced a more striking stimulation of malonaldehyde production in microsomes obtained from rats starved for 42 h before being killed than in normal fed rats. Promethazine was also effective in inhibiting the carbon tetrachloride stimulation in these preparations from starved rats. Endogenous peroxidation was not significantly affected by very low concentrations of promethazine (0.1 μM), but was progressively decreased with higher concentrations. The term 'endogenous peroxidation' refers to the production of malonaldehyde by liver suspensions in the presence of stock solution containing NADPH but in the absence of carbon tetrachloride; it must be distinguished from the additional amount of malonaldehyde produced in the presence of carbon tetrachloride (see Slater & Sawyer, 1971*b*). The inhibitory effect of promethazine on endogenous peroxidation was more pronounced as the incubation time at 37°C was prolonged (Fig. 1).

Vitamin E and inosine. Vitamin E–polyethylene glycol 1000–succinate was studied over the concentration range 0.005–10 μM and caused a small increase in the stimulation of malonaldehyde production due to carbon tetrachloride when present in concentrations less than 100 nM; higher concentrations of vitamin E (1–10 μM) decreased the carbon tetrachloride stimulation and also appreciably decreased the endogenous production of malonaldehyde (Table 2). Polyethylene glycol 1000 was not effective in decreasing the stimulation of malonaldehyde production due to carbon tetrachloride at concentrations where vitamin E–polyethylene glycol 1000–succinate was effective.

Inosine (0.1 μM) had a strong inhibitory effect on the carbon tetrachloride stimulation but did not decrease endogenous malonaldehyde production at this concentration (Table 2); on the contrary, endogenous malonaldehyde production was increased by 0.1 μM-inosine ($P<0.001$). Inosine was studied over the range 0.1–10 μM.

*Propyl gallate and diphenyl-*p*-phenylenediamine.* Propyl gallate was studied over the concentration range 0.02–20 μM and was effective at micromolar concentration in decreasing the stimulation of malonaldehyde production due to carbon tetrachloride (Table 3). Large decreases in endogenous

Table 1. *Effect of promethazine on the increased production of malonaldehyde in microsomes–standard stock suspensions*

The suspensions were incubated for 30 min at 37°C in the dark with and without 2 μl of carbon tetrachloride–liquid paraffin (1:1, v/v) in the side arms of Warburg flasks. The significance of the stimulation of malonaldehyde production due to CCl_4 was tested by Student's procedure; the numbers of determinations performed for each value quoted are in parentheses. In Expts. (d)–(f) the rats had been pre-starved of food for 42 h before being killed but had free access to water. N.S., not significant. For differences between control stimulation and the stimulation in the presence of promethazine: † $P<0.01$; ** $P<0.001$.

Expt.	Addition	Concn. (μM)	Malonaldehyde production (nmol/ml)	Stimulation	P Treatment ± CCl_4
a	Control	—	4.9 (12)		
	Control + CCl_4	—	6.0 (12)	+1.1	<0.001
	Promethazine	0.1	4.6 (8)		
	Promethazine + CCl_4	0.1	5.1 (8)	+0.5**	<0.01
b	Control	—	4.7 (18)		
	Control + CCl_4	—	5.6 (18)	+0.9	<0.001
	Promethazine	1	2.6 (12)		
	Promethazine + CCl_4	1	3.0 (12)	+0.4**	<0.001
c	Control	—	4.8 (6)		
	Control + CCl_4	—	5.9 (6)	+1.1	<0.001
	Promethazine	156	1.2 (4)		
	Promethazine + CCl_4	156	1.3 (4)	+0.1	N.S.
d	Control	—	17.9 (6)		
	Control + CCl_4	—	21.7 (6)	+3.8	<0.001
	Promethazine	0.01	17.2 (4)		
	Promethazine + CCl_4	0.01	20.0 (4)	+2.8†	<0.001
e	Control	—	8.0 (6)		
	Control + CCl_4	—	11.7 (6)	+3.7	<0.001
	Promethazine	0.1	6.3 (6)		
	Promethazine + CCl_4	0.1	8.4 (6)	+2.1†	<0.001
f	Control	—	17.9 (6)		
	Control + CCl_4	—	21.7 (6)	+3.8	<0.001
	Promethazine	10	3.5 (4)		
	Promethazine + CCl_4	10	3.9 (4)	+0.4	<0.05

malonaldehyde production were observed with propyl gallate concentrations in excess of approx. 5 μM.

Low concentrations (0.1 μM) of *NN'*-diphenyl-*p*-phenylenediamine strongly inhibited both the endogenous production of malonaldehyde in microsomes–stock suspensions and the stimulation of malonaldehyde production due to carbon tetrachloride (Table 3). The range of concentrations studied for this agent was 0.1–10 μM.

EDTA and desferrioxamine. Low concentrations of EDTA (approx. 1 μM) and desferrioxamine (0.1 μM) increased the carbon tetrachloride effect on malonaldehyde production (Table 4), but higher concentrations of these agents (5–20 and 1 μM respectively), strongly inhibited both endogenous malonaldehyde production and the carbon tetrachloride stimulation.

Sodium phenobarbitone, sodium dodecyl sulphate, Nupercaine and Cetab. Sodium phenobarbitone was not effective in decreasing the stimulation of malonaldehyde production due to carbon tetrachloride when present in the concentration range 0.1–50 μM. At very high concentrations, however, sodium phenobarbitone had an inhibitory effect (Table 5). Nupercaine and Cetab behaved similarly to sodium phenobarbitone in having no significant effect on the production of malonaldehyde when present in low concentrations (0.02–20 μM); at high concentration (500 μM) both agents decreased the endogenous production of malonaldehyde as well as the additional stimulation due to carbon tetrachloride (Table 5). A high concentration of sodium dodecyl sulphate had the opposite effect; it stimulated both endogenous malonaldehyde production and the increased production due to carbon tetrachloride. Low concentrations (0.1–32 μM) of sodium dodecyl sulphate had no significant effect on the systems studied.

DISCUSSION

Rat liver microsomes readily undergo peroxidative changes during incubation at 37°C; the addition of NADPH increases the rate of reaction.

This 'endogenous' *in vitro* peroxidation is, to some extent, associated with the iron content of microsomal suspensions (Hochstein & Ernster, 1963; Wills, 1969). The additional peroxidation produced by the addition of low concentrations of carbon tetrachloride, although probably involving a smaller segment of the NADPH–cytochrome *P*-450 sequence than does the endogenous reaction (Slater & Sawyer, 1971*b*), may also involve the participation of metal ion as shown by the inhibitory action of EDTA and of desferrioxamine (Table 5). However, although promethazine is known to chelate iron (Borg & Cotzias, 1962) it is unlikely that the inhibitory action of this phenothiazine derivative is exerted through such a mechanism. Promethazine inhibits the stimulation of malonaldehyde production due to carbon tetrachloride at concentrations as low as 0.1 μM (Table 1); at such very low concentrations promethazine does not affect aminopyrine demethylation or NADPH–cytochrome *c* reductase (T. F. Slater & B. C. Sawyer, unpublished work), and is most probably acting as a free-radical scavenger. This is a well-known property of phenothiazine derivatives (see Murphy, Ravner & Smith, 1950).

Other substances that have been found to inhibit the stimulation of malonaldehyde production due to carbon tetrachloride *in vitro* at micromolar concentrations were: vitamin E–polyethylene glycol 1000–succinate, propyl gallate, inosine and *NN'*-diphenyl-*p*-phenylenediamine; at such low concentrations it is likely that these substances are likewise effective by a free-radical-scavenging function. The results with inosine are of particular note. Matsushita, Ibuki & Aoki (1963) showed that purine bases were as effective as α-tocopherol in inhibiting the autoxidation of linoleic acid; they found also that α-tocopherol acted as a pro-oxidant in low concentrations in contrast with its antioxidant action at higher concentrations, an effect similar to that described in Table 2. Inosine occurs in high concentrations in rat liver endoplasmic reticulum; its concentration is approx. 0.1 μmol/g equiv. of microsomes (Siekevitz, 1955); consequently, it is one of the major endogenous antioxidants of the microsome fraction.

In general the inhibitory effects of the free-radical scavengers studied here on endogenous peroxidation were noticeable only at considerably higher concentrations than required for severe inhibition of stimulatory action of carbon tetrachloride. This finding is consistent with the conclusion outlined by Slater & Sawyer (1971*b*) that endogenous malonaldehyde production involves a different sequence of electron carriers in the endoplasmic reticulum from that involved in the stimulatory action of carbon tetrachloride. Considerably higher concentrations of the free-radical scavengers, however, do inhibit the endogenous pathway, as is well known from previous studies with microsomes in the presence of ADP and iron (Hochstein & Ernster, 1963).

Table 2. *Effects of vitamin E–polyethylene glycol* 1000*–succinate (vitamin E) and inosine on the increased production of malonaldehyde due to carbon tetrachloride in microsomes-plus-supernatant–stock suspensions*

The suspensions were incubated for 60 min at 37°C with and without carbon tetrachloride in the side arms of Warburg flasks. Standard stock solution was used in these experiments. For other details see Table 1 and the Methods section. For difference between control stimulation and that in the presence of the drug: * Not significant; ‡ $P = 0.05$; § $P < 0.05$; ** $P < 0.001$.

Addition	Concn. (μM)	Malonaldehyde production (nmol/ml)	Malonaldehyde production Stimulation	*P* Treatment ± CCl_4
Control	—	8.7 (6)		
Control + CCl_4	—	12.1 (6)	+3.4	<0.001
Vitamin E	0.005	7.0 (6)		
Vitamin E + CCl_4	0.005	12.5 (6)	+5.5‡	<0.001
Control	—	15.5 (6)		
Control + CCl_4	—	18.0 (6)	+2.5	<0.001
Vitamin E	0.055	15.7 (4)		
Vitamin E + CCl_4	0.055	18.7 (4)	+3.0*	<0.001
Vitamin E	5.5	9.0 (4)		
Vitamin E + CCl_4	5.5	9.2 (4)	+0.2**	N.S.
Control	—	4.4 (6)		
Control + CCl_4	—	10.7 (6)	+6.3	<0.001
Inosine	0.1	7.9 (4)		
Inosine + CCl_4	0.1	11.7 (4)	+3.8§	<0.001
Control	—	7.5 (6)		
Control + CCl_4	—	11.6 (6)	+4.1	<0.01
Inosine	10	7.5 (6)		
Inosine + CCl_4	10	8.5 (6)	+1.0§	<0.01

Table 3. *Effect of propyl gallate and diphenyl-p-phenylenediamine on the increased production of malonaldehyde due to carbon tetrachloride in microsomes-plus-supernatant–stock suspensions*

The suspensions were incubated for 60 min at 37°C with and without carbon tetrachloride in the side arms of Warburg flasks. The stock solution used was the tris–nicotinamide mixture (see the Methods section). For other details see Table 1 and the Methods section. For differences between control stimulation and that in the presence of the drugs: † $P = 0.10$; ** $P < 0.001$; ‡ $P = 0.01$; * $P = < 0.01$.

Addition	Concn. (μM)	Malonaldehyde production (nmol/ml)	Stimulation	P Treatment ± CCl_4
Control	—	10.7 (4)		
Control + CCl_4	—	14.7 (4)	+4.0	<0.01
Propyl gallate	0.09	11.6 (4)		
Propyl gallate + CCl_4	0.09	14.6 (4)	+3.0†	<0.01
Propyl gallate	1.88	10.5 (4)		
Propyl gallate + CCl_4	1.88	11.7 (4)	+1.2*	N.S.
Control	—	13.3 (4)		
Control + CCl_4	—	16.9 (4)	+3.6	<0.01
Propyl gallate	9.5	4.4 (4)		
Propyl gallate + CCl_4	9.5	4.5 (4)	+0.1‡	N.S.
Control	—	10.5 (6)		
Control + CCl_4	—	14.8 (6)	+4.3	<0.001
Diphenyl-*p*-phenylenediamine	0.1	6.5 (4)		
Diphenyl-*p*-phenylenediamine + CCl_4	0.1	7.5 (4)	+0.1**	<0.02
Control	—	9.1 (4)		
Control + CCl_4	—	10.4 (4)	+1.3	<0.001
Diphenyl-*p*-phenylenediamine	0.96	1.3 (4)		
Diphenyl-*p*-phenylenediamine + CCl_4	0.96	1.3 (4)	0.0**	N.S.
Control	—	8.8 (4)		
Control + CCl_4	—	10.4 (4)	+1.6	<0.05
Diphenyl-*p*-phenylenediamine	9.6	1.4 (4)		
Diphenyl-*p*-phenylenediamine + CCl_4	9.6	1.2 (4)	−0.2**	N.S.

Table 4. *Effects of EDTA and desferrioxamine on the increased production of malonaldehyde due to carbon tetrachloride in microsomes-plus-supernatant–standard stock suspensions*

The suspensions were incubated for 60 min at 37°C with or without CCl_4 in the side arms of Warburg flasks. For other details see Table 1 and the Methods section. For differences between control stimulation and that in the presence of the drug: † $P < 0.1$; ** $P < 0.001$.

Addition	Concn. (μM)	Malonaldehyde production (nmol/ml)	Stimulation	P Treatment ± CCl_4
Control	—	14.4 (6)		
Control + CCl_4	—	16.0 (6)	+1.6	0.05
EDTA	0.79	11.7 (4)		
EDTA + CCl_4	0.79	15.0 (4)	+3.3†	<0.001
EDTA	19.8	2.6 (4)		
EDTA + CCl_4	19.8	3.0 (4)	+0.4**	N.S.
Control	—	5.2 (6)		
Control + CCl_4	—	10.9 (6)	+5.7	<0.001
Desferrioxamine	0.1	4.6 (4)		
Desferrioxamine + CCl_4	0.1	12.7 (4)	+8.1**	<0.001
Desferrioxamine	1.0	4.3 (4)		
Desferrioxamine + CCl_4	1.0	7.7 (4)	+3.4**	<0.001

The inhibitory effects of the surface-active agents Nupercaine and Cetab at high concentrations on both endogenous peroxidation and on the stimulatory effect of carbon tetrachloride probably arise through alterations in the lipoprotein environment of the electron-transport chain in liver endoplasmic reticulum. It is known that by altering the conformation of the microsomal lipid (by heating to 60°C followed by solvent extraction) decreases its rate of peroxidation by the NADPH-linked system (May & McCay, 1968). The ionic charge on the detergent is important for this effect, however, for

Table 5. *Effects of sodium phenobarbitone, Nupercaine, Cetab and sodium dodecyl sulphate on the increased production of malonaldehyde due to carbon tetrachloride in microsomes-plus-supernatant–stock suspensions*

The suspensions were incubated for 60 min at 37°C with and without carbon tetrachloride in the side arms of Warburg flasks. For other details see Table 1 and the Methods sections. For difference between control stimulation and that in the presence of the drug: *, not significant; ‡ $P<0.01$; ** $P<0.001$.

Expt.	Addition	Concn. (μM)	Malonaldehyde production (nmol/ml)	Malonaldehyde production Stimulation	P Treatment $\pm CCl_4$
a	Control	—	10.5 (6)		
	Control + CCl_4	—	14.8 (6)	+4.3	<0.001
	Phenobarbitone	500	11.7 (4)		
	Phenobarbitone + CCl_4	500	14.5 (4)	+2.8‡	<0.001
b	Control	—	10.4 (6)		
	Control + CCl_4	—	12.9 (6)	+2.5	<0.001
	Nupercaine	500	4.1 (4)		
	Nupercaine + CCl_4	500	5.2 (4)	+1.1‡	<0.001
c	Control	—	10.5 (4)		
	Control + CCl_4	—	21.8 (4)	+11.3	<0.001
	Cetab	100	7.4 (4)		
	Cetab + CCl_4	100	8.3 (4)	+0.9‡	<0.05
	Cetab	500	3.7 (4)		
	Cetab + CCl_4	500	3.9 (4)	+0.2‡	N.S.
d	Control	—	10.5 (6)		
	Control + CCl_4	—	13.0 (6)	+2.5	<0.001
	Sodium dodecyl sulphate	500	22.8 (4)		
	Sodium dodecyl sulphate + CCl_4	500	28.1 (4)	+5.3**	<0.001

similar high concentrations of sodium dodecyl sulphate (an anionic detergent) increased both endogenous peroxidation and the stimulation due to carbon tetrachloride (Table 5).

Since both endogenous peroxidation utilizing NADPH, and the stimulation of malonaldehyde production due to carbon tetrachloride, involve the NADPH–flavoprotein (Slater & Sawyer, 1971*b*) it is apparent that inhibitors of the flavoenzyme will inhibit both of the peroxidative routes. An example of this behaviour is observed with relatively high concentrations of propyl gallate (Table 3). It has been found that propyl gallate will powerfully inhibit NADPH–cytochrome *c* reductase when added in a final concentration of greater than 10 μM (Torrielli & Slater, 1971); this property of propyl gallate should be distinguished from its free-radical scavenging action at much lower concentrations (e.g. 0.1 μM).

In this paper it has been shown that low concentrations of a number of free-radical scavengers decrease the stimulation of malonaldehyde production due to the presence of carbon tetrachloride in rat liver suspensions. The mechanism probably involves the removal of the highly reactive trichloromethyl radicals by reaction with the scavenger. This mechanism would account for the activity of such scavengers *in vivo* in attenuating the toxic effects of carbon tetrachloride. Other agents studied here (e.g. (Nupercaine, Cetab) are also active *in vivo* in decreasing the liver disturbances produced by carbon tetrachloride, and yet have no pronounced inhibitory action on the stimulatory effect of carbon tetrachloride on malonaldehyde production *in vitro*, except at very high concentrations.

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