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

GENERAL FEATURES OF THE SYSTEMS USED

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1. The general features of the reaction by which carbon tetrachloride stimulates lipid peroxidation have been elucidated in rat liver microsomal suspensions and in mixtures of microsomes plus cell sap. The production of lipid peroxides has been correlated with malonaldehyde production in the systems used. 2. The stimulation of malonaldehyde production by carbon tetrachloride requires a source of reduced $NADP^+$ and is dependent on the extent of the endogenous peroxidation of the microsomal membranes: if extensive endogenous peroxidation occurs during incubation then no stimulation by carbon tetrachloride is apparent. 3. The stimulation of malonaldehyde production by carbon tetrachloride has been shown to be proportional to the square root of the carbon tetrachloride concentration in the incubation mixture. It is concluded that the stimulation of malonaldehyde production by carbon tetrachloride results from an initiation process that is itself dependent on the homolytic dissociation of carbon tetrachloride to free-radical products. 4. The increased production of malonaldehyde due to carbon tetrachloride is accompanied by a decreased activity of glucose 6-phosphatase in rat liver microsomal suspensions. 5. The relative activities of bromotrichloromethane, fluorotrichloromethane and chloroform have been evaluated in comparison with the effects of carbon tetrachloride in increasing malonaldehyde production and in decreasing glucose 6-phosphatase activity. Bromotrichloromethane was more effective, and fluorotrichloromethane and chloroform were less effective, than carbon tetrachloride in producing these two effects. It is concluded that homolytic bond fission of the halogenomethanes is a requisite for the occurrence of the two effects observed in the endoplasmic reticulum.

The toxic action of carbon tetrachloride on rat liver, which is manifest as an extensive centrilobular necrosis, is dependent on the metabolism of carbon tetrachloride by an enzyme system located in the endoplasmic reticulum (see Slater, 1966*a*; Recknagel, 1967). It has been suggested that one likely route of the metabolism of carbon tetrachloride involves the formation of free-radical products (Hove, 1948; Butler, 1961; Wirtschafter & Cronyn, 1964; Recknagel & Ghoshal, 1966; Comporti, Saccocci & Dianzani, 1965; Slater, 1966*a*).

The formation of significant quantities of non-associative radicals such as the trichloromethyl radical, of transient existence but high chemical reactivity, can be expected to result in severe disturbances to enzyme activities and organized structure of the endoplasmic reticulum. In particular, the attack of free-radical intermediates on unsaturated lipids in intracellular membranes to result in lipid peroxidation has been stressed by several groups of workers to be of particular relevance to the necrogenic action of carbon tetrachloride (Ghoshal & Recknagel, 1965; Comporti *et al.* 1965; Slater, 1966*a*). Previous studies have shown that carbon tetrachloride will stimulate lipid peroxidation *in vitro* in suspensions of liver microsomes plus supernatant (Ghoshal & Recknagel, 1965; Comporti *et al.* 1965) and in microsomes alone provided that a source of NADPH is present (Slater, 1966*b*).

In view of the possible relationship between lipid peroxidation and the onset of necrosis (see Recknagel, 1967) it is of obvious importance to investigate the mechanisms by which carbon tetrachloride stimulates lipid peroxidation *in vitro*, and the relationship of such results *in vitro* to the situation *in vivo*. In this and following papers (Slater & Sawyer, 1971*a*,*b*) these points are considered.

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METHODS

General procedures and enzyme assays. The rats used were albino females of about 120g body wt.; they were fed on a modified diet M.R.C. 41 (Oxo Ltd., London S.E.1, U.K.) and water *ad libitum.* The rats used were obtained from four sources: (A) Twyford Laboratories Ltd.; (B) University College London; (C) Carworth (Europe) Ltd., Alconbury, Hunts., U.K.; (D) A. J. Tuck and Son, Rayleigh, Essex, U.K.; changes in the source of albino rats used were a consequence of external factors outside our control. Although the rate of endogenous peroxidation varied in liver suspensions prepared from rats from different sources, no significant differences were observed in the response to the addition of carbon tetrachloride to liver suspensions.

Rats were killed by cervical dislocation, and the livers were quickly removed, weighed and cooled in ice-cold 0.25M-sucrose. The suspensions were centrifuged at 11700$g_{av.}$ for 10min at 2°C; the supernatant solution was carefully removed and recentrifuged by using the same conditions. The supernatant solution so obtained was used as 'the microsomes-plus-supernatant mixture'. In experiments with microsome suspensions, the microsomes-plus-supernatant mixture described above was further centrifuged at 2°C for 40min at 157000$g_{av.}$ (Superspeed 50 centrifuge; MSE Ltd., Crawley, Sussex, U.K.). The microsomal pellet was rinsed until free from overlying supernatant with ice-cold 0.15M-KCl and was resuspended in ice-cold 0.15M-KCl such that 1ml of suspension contained microsomes equivalent to 1g wet wt. of original liver.

A standard stock solution contained 83.5mM-KCl; 37.2mM-tris-HCl buffer, pH8.0; 5.5mM-glucose 6-phosphate (sodium salt); 0.245mM-$NADP^+$ (sodium salt); 10mM-acetamide; and 8.4 i.u. of glucose 6-phosphate dehydrogenase in a total volume of 32.4ml. Standard stock solution (32.4ml) was mixed with either 4ml of microsome suspension or with 6ml of microsome-plus-supernatant suspension to constitute the microsome–stock suspensions or the microsomes-plus-supernatant–stock suspensions that were added immediately on preparation to the central compartments of the Warburg flasks. In a few instances the tris buffer was replaced by phosphate buffer (Na_2HPO_4–NaH_2PO_4, 0.1M, pH7.4) and the acetamide was replaced by 5mM-nicotinamide; no differences in experimental behaviour were observed in such cases.

Inorganic pyrophosphatase activity in the incubation mixtures was determined as described by Slater & Sawyer (1969). Glucose 6-phosphatase activity was determined by the method of Delaney & Slater (1970); incubations for both enzyme assays were for 30min at 37°C. Oxidized and reduced NADP were measured in tissue extracts by the method of Slater, Sawyer & Sträuli (1964).

Determination of malonaldehyde and lipid peroxide. To study the effects of CCl_4 and other halogenoalkanes on the production of material giving a positive reaction in the thiobarbituric acid reaction (see below), 2.5ml of a microsome–stock suspension, or of a microsomes-plus-supernatant–stock suspension, was placed in the central compartment of a Warburg flask. An appropriate quantity (1–10μl) of the halogenoalkane in liquid paraffin was added to the side arm of the flask with a Hamilton microsyringe to allow diffusion of the halogenoalkane during incubation. The flask was sealed and incubated at 37°C in the dark with gentle shaking (60 shakes/min). Incubation times were usually 10–15min for microsome–stock suspensions and 60min for microsomes-plus-supernatant–stock suspensions; the actual incubation time used and other individual details are given for each experiment at the appropriate position in the text.

At the end of the incubation periods 1ml samples of the suspension were removed from the central compartments of the Warburg flasks, mixed with 2ml of 10% (w/v) trichloroacetic acid and stood in ice for approx. 15min. The precipitates were separated by centrifugation and 2ml samples of the clear supernatant solution were mixed with 2ml of aq. 0.67% thiobarbituric acid and heated in a boiling-water bath for 10min. The solutions were then cooled in ice for 5min, after which the E_{535} was determined. The results are expressed in terms of malonaldehyde production; a calibration curve was constructed by using malonaldehyde bis-diethyl acetal (Schuchardt Chemische Fabrik, München, Germany) as a standard. The E_{535} was linear with increasing amounts of malonaldehyde added (range studied 0–25nmol). The molar extinction coefficient determined with the standard sample was 1.49×10^5 $l\cdot mol^{-1}\cdot cm^{-1}$; this was used in calculating results on a molar basis. Because the stimulations of malonaldehyde production due to CCl_4 were small in terms of the absolute amounts of malonaldehyde measured, incubations for each point described in the Results section were performed in triplicate and sometimes in pentuplicate. Thiobarbituric acid determinations on the incubated mixtures were carried out on each incubation mixture in duplicate; each value described in the Results section is thus the mean of six to ten malonaldehyde determinations.

Gas–liquid chromatography of the thiobarbituric acid reaction mixture (Fränz & Cole, 1962) has indicated the presence of several reaction products. This and other evidence (Saslow, Corwin & Waravdekar, 1966) suggests that the material(s) measured in tissue extracts by the thiobarbituric acid reaction may be more complex than malonaldehyde alone. Bearing in mind such reservations about the precise identity of the component reacting with thiobarbituric acid, we shall use 'malonaldehyde' synonymously in this and the following papers (Slater & Sawyer, 1971*a*,*b*) with 'material giving a positive reaction with thiobarbituric acid'.

Malonaldehyde production that occurred in microsome suspensions (or in microsomes-plus-supernatant suspensions) mixed with stock solution containing NADPH is described as endogenous peroxidation to distinguish it from the additional peroxidation that results from incubation in the presence of CCl_4. In a few instances experiments were performed with tissue suspensions in the absence of NADPH; this is noted at the relevant position in the text.

Lipid peroxide was measured in samples of incubation mixture by the procedure of Swoboda & Lea (1958). A calibration curve was prepared with a solution of lauryl peroxide in acetic acid–chloroform (3:2, v/v); the E_{350} of the final cadmium acetate aqueous phase was linear with increasing amounts of lauryl peroxide over the range 0–1.0μmol.

When different concentrations of microsomes-plus-supernatant in 0.25M-sucrose were incubated for 2h at

37°C there was a very good correlation between lipid peroxide concentration (measured iodometrically) and malonaldehyde concentration (measured by the thiobarbituric acid reaction). The relationship was linear over the corresponding ranges studied (malonaldehyde, 0–16nmol/ml of suspension; lipid peroxide, 0–400nmol/ml of suspension). It can be seen that malonaldehyde production is much lower on a molar basis than the corresponding change in lipid peroxide. This is a well-recognized hazard of using malonaldehyde determinations to follow the course of lipid peroxidation and it is important to check the basic correspondence between the two parameters in the particular system under study. Provided that the incubation time was kept constant then malonaldehyde concentration was linearly related to the concentration of lipid peroxide in the systems studied here.

Determination of halogenoalkanes in tissue suspensions. The concentrations of halogenoalkanes present in the incubation mixtures contained in the central compartments of Warburg flasks, and resulting from the diffusion of the halogenoalkanes from the side arms of the flasks, were determined by three methods: colorimetry, radioisotope dilution, and g.l.c.

CCl_4 was determined colorimetrically by the method of Recknagel & Litteria (1960). A calibration curve was constructed by using a CCl_4–toluene mixture; the E_{530} of the final heated alkaline pyridine mixture was linear with increasing amounts of CCl_4 added (range studied 0–3μmol of CCl_4/ml of toluene).

The concentration of CCl_4 in incubation mixtures was also determined by radioisotope dilution. $^{14}CCl_4$ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and was accurately diluted with $^{12}CCl_4$. Samples (2μl) of various dilutions of the labelled $^{14}CCl_4$ solution were added to a mixture of 15ml of scintillation fluid and 0.5ml of microsome-plus-supernatant–stock suspensions to provide a calibration curve. Microsomes-plus-supernatant–stock suspensions (3.0ml) were incubated for 60min at 37°C in the central compartments of Warburg flasks containing known amounts of the $^{14}CCl_4$ solutions in the side arms after which samples (0.5ml) of the suspensions in the central compartment were added to 15ml of scintillation fluid. The scintillation fluid was the thixotropic mixture described by Bray (1960) and Gordon & Wolfe (1960). The radioactive content of the mixtures was determined by using a Packard Tri-Carb spectrometer on the ^{14}C-mode, set for 10000c.p.m. or 10min.

Halothane, bromotrichloromethane, fluorotrichloromethane and chloroform were measured in tissue suspensions by g.l.c. by using a Pye model 104 gas chromatograph with a ^{63}Ni electron-capture detector.

RESULTS

Coenzyme dependence and incubation time. The stimulation of malonaldehyde production in liver microsomes–stock suspensions by carbon tetrachloride is dependent on a source of NADPH that cannot be replaced by NADH (Table 1). The stimulation of malonaldehyde production in microsomes–stock suspensions increased linearly with the time of incubation at 37°C up to approx. 15min but then decreased; with microsomes-plus-supernatant–stock suspensions the stimulation by carbon tetrachloride increased linearly with incubation time up to at least 90min at 37°C (Fig. 1). Measurements of malonaldehyde concentration during the linear regions of the two curves in Fig. 1 may thus be equated in a proportional manner with the rate of production of malonaldehyde.

This conclusion is further favoured by the finding that no significant destruction of added malonaldehyde (0–10nmol/ml of suspension) could be detected in a microsomes–stock suspension during an incubation period of 15min at 37°C.

The decrease in the stimulation produced by carbon tetrachloride in microsomes–stock suspensions with incubation times greater than 15min (Fig. 1) possibly reflects the rapid destruction of $NADP^+$+NADPH in the microsomes–stock suspension. There was a rapid destruction of $NADP^+$+ NADPH during the incubations at 37°C, as shown in Table 2 for microsomes-plus-supernatant–stock suspensions. The stimulation of malonaldehyde production by carbon tetrachloride was increased by high initial concentrations of $NADP^+$ in the stock solution; there was a similar rise in the endogenous production of malonaldehyde. Table 3 shows the results obtained by using a microsome suspension prepared from female rats prestarved of food for 24h before death; a similar correlation of stimulation with the concentration of $NADP^+$ was found by using microsomes isolated from normally fed rats.

No stimulation of malonaldehyde production by carbon tetrachloride was observed with microsomes–stock suspensions in which the microsome sample had first been heated under anaerobic conditions for 5min at 70°C or 5min at 100°C. Similarly, no stimulation was observed when glucose 6-phosphate was omitted from the standard stock solution. These results indicate that the stimulatory effect of carbon tetrachloride involves an enzymic interaction with the microsomes and requires the presence of NADPH.

The mean stimulation of malonaldehyde production by carbon tetrachloride (2μl of mixture with liquid paraffin, 1:1, v/v, in the side arms of Warburg flasks) in microsomes–stock suspensions after incubation times of 10–15min, was 19±1% (mean of 64 separate experiments ±S.E.M.) expressed as a percentage of the endogenous peroxidation measured in the absence of carbon tetrachloride. In corresponding experiments with microsomes-plus-supernatant–stock suspensions and an incubation time of 60min at 37°C, the mean stimulation in malonaldehyde production due to carbon tetrachloride was 64±13% (mean of 68 experiments ±S.E.M.). The wide variability in the stimulation produced by carbon tetrachloride in microsomes-plus-supernatant–stock suspensions is discussed below.

Table 1. *Dependence on added $NADP^+$ of the stimulation in malonaldehyde production by carbon tetrachloride in microsomal suspension*

In Expt. 1 microsomes (equivalent to 1 g of liver/1 ml of 0.15M-KCl) were mixed with standard stock or with standard stock minus $NADP^+$. The mixtures were incubated for 15 min at 37°C in Warburg flasks in the dark with shaking at 60 shakes/min with and without 2 μl of CCl_4–liquid paraffin (1:1, v/v) in the side arms. In Expts. 2(a) and 2(b) microsomes were suspended in modified stock solutions (see below) and incubated with and without 2 μl of CCl_4–liquid paraffin (1:1, v/v) in the side arms for 10 min at 37°C. In Expt. 2(a) the standard stock solution was modified to include 5mM-nicotinamide in place of acetamide: in Expt. 2(b) the stock solution included ethanol (10mM), alcohol dehydrogenase (720 units), NAD^+ (0.25mM) and nicotinamide (5mM) in place of glucose 6-phosphate, glucose 6-phosphate dehydrogenase and acetamide.

	Expt. 1		Expt. 2	
			(a)	(b)
	$+NADP^+$	$-NADP^+$	$+NADP^+$	$+NAD^+$
Control	7.45	4.20	7.45	3.17
$+CCl_4$	9.30	3.95	8.35	3.22
Difference	+1.85*	−0.25†	+0.90‡	+0.05†

* $P<0.001$ for the difference between control and CCl_4 values.
† Difference not significant.
‡ $P<0.01$ for the difference between control and CCl_4 values.

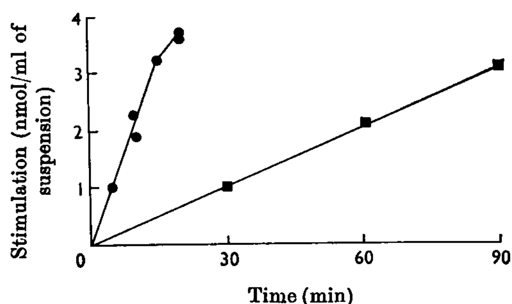

Fig. 1. Effect of incubation time at 37°C on the stimulation of malonaldehyde production due to carbon tetrachloride (2 μl of a mixture with liquid paraffin, 1:1, v/v) in microsomes–stock suspensions (●) and in microsomes-plus-supernatant–stock suspensions (■). The results are shown as the additional amount of malonaldehyde produced/ml of suspension in the presence of carbon tetrachloride. For other details see the Methods section.

Dependence on endogenous peroxidation. No significant stimulation of malonaldehyde production in microsomes–stock suspensions by carbon tetrachloride during a 15 min incubation at 37°C was obtained when the endogenous production of malonaldehyde was more than approx. 15 nmol/ml of suspension in the same period. A similar effect was seen also with microsomes-plus-supernatant–stock suspensions where, owing to differences in incubation conditions (i.e. longer incubation times), the stimulatory effect of carbon tetrachloride disappeared at a higher endogenous concentration of malonaldehyde production (25 nmol/ml of suspension; Fig. 2).

It can be seen that when a low endogenous production of malonaldehyde was obtained there was a correspondingly high stimulation by carbon tetrachloride (Fig. 2). The wide range of values for endogenous peroxidation shown in Fig. 2 was found without any additional experimental treatment of the microsomes-plus-supernatant–stock suspensions and probably results from a variety of factors. First, the experiments were performed over a time-period of some 3 years and albino rats from four sources were used. Secondly, endogenous peroxidation is markedly affected by the presence of Fe^{2+} and haem compounds (see Wills, 1969*b*); microsome suspensions contain variable quantities of adsorbed haemoglobin, and iron contamination of glucose 6-phosphate was found, in retrospect, to be severe in stocks from some suppliers. Thirdly, endogenous peroxidation and the stimulation of malonaldehyde production due to carbon tetrachloride are both markedly dependent on the dietary status of the rats from which liver samples are taken. An indication of the magnitude of this latter effect is seen after starving rats while allowing free access to water. Under such conditions endogenous peroxidation may be nearly trebled in extent and the stimulatory action of carbon tetrachloride is also greatly increased (Table 4). A combination of the effects outlined above is sufficient to account for the wide variation in endogenous peroxidation shown in Fig. 2.

Dependence on carbon tetrachloride concentration. The stimulation of malonaldehyde production by carbon tetrachloride at 37°C in microsomes-plus-supernatant–stock suspensions increased with an increasing concentration of carbon tetrachloride

Table 2. *Loss of NADPH during incubation of stock mixture at* 37°C

In Expt. (a) microsomes-plus-supernatant–stock suspensions were incubated for 10 or 20 min at 37°C in the presence or absence of CCl_4 (2 μl of a 1 : 1, v/v, mixture in liquid paraffin in the side arms of Warburg flasks). At the end of the incubation samples of the stock were mixed with 0.1 M-NaOH and placed in a boiling-water bath; NADPH was determined by the method of Slater *et al.* (1964). In Expt. (b) the conditions were similar to Expt. (a) but 5 μl of CCl_4 -liquid paraffin (1 : 1, v/v) was placed in the side arms of Warburg flasks. After 10 min incubation samples of the mixture were mixed with cold 1 M-$HClO_4$ or hot 0.1 M-NaOH. $NADP^+$ and NADPH were determined as described by Slater *et al.* (1964). Values are given as a percentage of the concentration present in the mixture before incubation at 37°C. The stock solution used in the experiments included 5 mM-nicotinamide in place of acetamide.

Expt.	Incubation time (min)	Addition	$NADP^+$	NADPH
(a)	0	—	—	100
	10	—	—	20
	10	CCl_4	—	14
	20	—	—	13
	20	CCl_4	—	10
(b)	0	—	100	100
	10	—	45	20
	10	CCl_4	50	20

Table 3. *Effect of $NADP^+$ concentration on the stimulation of malonaldehyde production due to carbon tetrachloride*

A microsomes–stock suspension was prepared as described in the Methods section by using a liver sample taken from a rat starved of food for 24 h before death. A standard stock suspension was prepared but without $NADP^+$ which was added separately to give the final concentrations shown. Incubation was for 30 min at 37°C in the dark with shaking at 60 cycles/min in Warburg flasks. Carbon tetrachloride (2 μl of a 1 : 1, v/v, solution in liquid paraffin) was added to the side arms where required. For other details see the Methods section. The significance of the differences between endogenous production and that in the presence of carbon tetrachloride are shown: * $P<0.05$; † $P<0.01$.

	Malonaldehyde production (nmol/ml of suspension)	
Concn. of $NADP^+$ (mM)	Endogenous	Stimulation by carbon tetrachloride
0.000	3.3	0.0
0.024	3.8	0.0
0.121	4.5	0.7*
0.242	6.1	4.3†
0.485	10.9	11.4†
0.975	12.1	20.6†
1.62	22.0	15.6†
3.24	34.0	17.4†
6.48	45.8	10.5†

(Fig. 3) but at very high concentrations of carbon tetrachloride (not shown in Fig. 3), both the stimulation by carbon tetrachloride and the endogenous peroxidation decreased. These latter effects are possibly due to a solvent-like action of carbon tetrachloride on the lipoprotein environment of the membrane-bound enzyme system in the microsomes. The results in Fig. 3 were obtained from four experiments; a similar non-linearity was observed in a further four experiments with microsome-plus-supernatant–stock mixtures and in the one experiment performed with a microsomes–stock mixture. Fig. 3 also gives the results relating the amount of carbon tetrachloride added to the side arms of Warburg flasks to the concentration in the stock medium in the central compartments: it can be seen that the relationship is linear. The results in Fig. 3 are replotted in Fig. 4 to show the relationship between the stimulation in malonaldehyde production and the square root of the concentration of carbon tetrachloride in the microsomes-plus-supernatant–stock mixtures: the relationship is linear.

For reasons already discussed, the concentration of malonaldehyde in microsomes-plus-supernatant–stock suspensions after 60 min incubation at 37°C is directly proportional to the rate of production of malonaldehyde during that time. The results in Fig. 4 thus show that in microsome-plus-supernatant–stock suspensions the rate of malonaldehyde production is proportional to the square root of the concentration of carbon tetrachloride in the suspension.

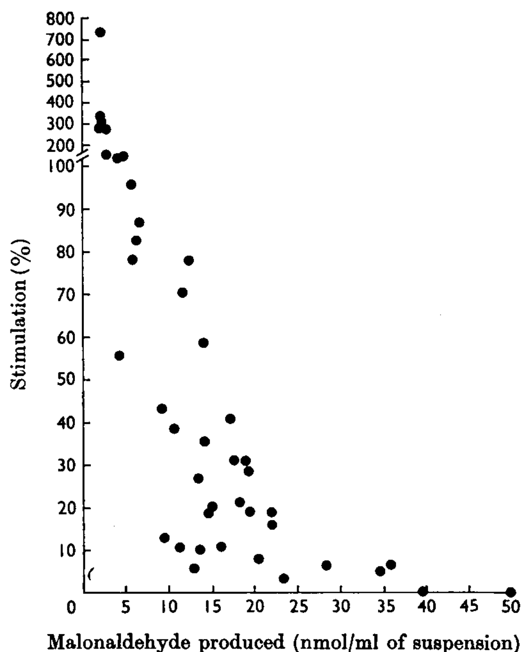

Fig. 2. Dependence of stimulation of malonaldehyde production on endogenous peroxidation. The ordinate shows the percentage stimulation of malonaldehyde production due to carbon tetrachloride (2 μl of CCl_4–liquid paraffin, 1:1, v/v) after an incubation time of 60 min at 37°C. The abscissa gives the corresponding amounts of malonaldehyde produced (nmol/ml of suspension) in the absence of carbon tetrachloride. Suspensions of microsomes plus supernatant in 0.25 M-sucrose (1 ml of suspension contained approximately 0.3 g wet wt. of liver) were mixed with standard stock solution; malonaldehyde was determined by the thiobarbituric acid reaction.

Effects of other halogenoalkanes. The results obtained for the concentration of various halogenoalkanes in stock medium containing microsomes plus supernatant after a 60 min incubation at 37°C and with 2 μl of halogenoalkane–liquid paraffin mixture (1:1, v/v) in the side arm of Warburg flasks are shown in Table 5. It can be seen that the colorimetric and radioisotope dilution methods for determination of carbon tetrachloride gave consistent results. The concentration found (approx. 0.2 mM) was considerably lower than the concentration reported for a saturated aqueous solution of carbon tetrachloride at 30°C (5.2 mM; International Critical Tables, 1928). The concentrations of the other halogenoalkanes in the microsomes-plus-supernatant–stock solution after 60 min at 37°C were: chloroform, 2.67 mM; halothane, 1.77 mM; fluorotrichloromethane, 0.066 mM; bromotrichloromethane, 0.0121 mM. Owing to the very high volatility of fluorotrichloromethane (b.p. 24°C) some doubt must exist as to the precision of the value quoted for its solubility in the stock mixture. The value given should be considered as a minimum one for its solubility in incubation mixture, so that its true concentration, in the absence of any loss by evaporation, is probably somewhat higher than given.

The stimulatory effects of the halogenoalkanes on the production of malonaldehyde in microsomes-plus-supernatant–stock suspensions are shown in Table 6 where the results have been calculated on an equimolar basis by using the solubilities given in Table 5. It can be seen that there is a definite order of reactivity with respect to stimulatory activity:

Table 4. *Effect of starvation on endogenous production of malonaldehyde and on the stimulatory action of carbon tetrachloride on malonaldehyde production in microsomes–stock suspensions*

Rats from source (D) were used in these experiments. Where necessary the rats were deprived of food for 24 h or 42 h but were allowed free access to water. Microsomes–stock suspensions were prepared as described in the Methods section and were incubated for 30 min at 37°C in the dark. Carbon tetrachloride (2 μl of a 1:1, v/v, solution in liquid paraffin) was added to the side arms of Warburg flasks. For other details see the Methods section. Mean values ±S.E.M. are shown with the number of rats used in parentheses.

	Malonaldehyde production (nmol/ml of suspension)	
Treatment	Endogenous	% stimulation by carbon tetrachloride
Normal feeding	4.9 ± 0.4 (6)	23 ± 4 (6)
Starvation, 24 h	7.0 ± 1.0 (5)	59 ± 9 (5)
Starvation, 42 h	12.2 ± 2.1 (4)	45 ± 9 (4)

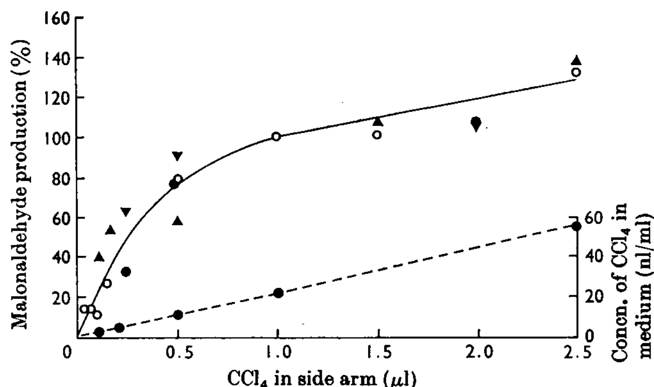

Fig. 3. Malonaldehyde production in microsomes-plus-supernatant–stock suspensions in the presence of various amounts of carbon tetrachloride in the side arms of Warburg flasks. The results from four separate experiments are shown. ● and ▼, The microsomes plus supernatant was mixed with standard stock solution; ○ and ▲, a modified stock in which acetamide was replaced by 5mM-nicotinamide was used. All incubations were for 60 min at 37°C; malonaldehyde was measured at the end of the experiments by the thiobarbituric acid reaction. The results have been normalized to a 100% value for malonaldehyde production with 1 μl of carbon tetrachloride in the side arm of Warburg flasks. The relationship between the amount of CCl_4 added to the side arm and the concentration in a microsomes-plus-supernatant–stock suspension in the central compartment is shown as the broken line. For details of determinations see the Methods section.

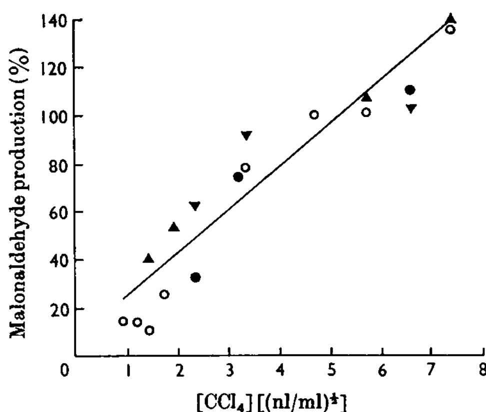

Fig. 4. Relationship between the square root of the concentration of carbon tetrachloride in the microsomes-plus-supernatant–stock suspensions in the central compartments of Warburg flasks and the production of malonaldehyde. Results were taken from Fig. 3. The symbols and other details are as in Fig. 3. The regression line is shown; correlation coefficient $r = 0.82$, $P \ll 0.001$.

bromotrichloromethane > carbon tetrachloride > fluorotrichloromethane, chloroform. A similar high reactivity of bromotrichloromethane compared with carbon tetrachloride was observed with microsomes–stock suspensions.

The effects of the halogenoalkanes on glucose 6-phosphatase activity in microsomes–stock suspensions and in microsomes-plus-supernatant–stock suspensions were also measured. The results with microsomes-plus-supernatant–stock suspensions are shown in Fig. 5 and are also corrected to an equimolar basis by using the solubilities given in Table 5. It can be seen that carbon tetrachloride is most active in decreasing glucose 6-phosphatase activity and is followed in relative effectiveness by chloroform. Halothane and fluorotrichloromethane were inactive over the concentration range where carbon tetrachloride was observed to be strongly inhibitory.

DISCUSSION

Many studies (see May & McCay, 1968; Wills, 1969*a*,*b*,*c*) have been concerned with endogenous lipid peroxidation in mixtures containing liver microsomes and a source of NADPH. Other reports (Hochstein & Ernster, 1963; Orrenius, Dallner & Ernster, 1964) have been concerned with the increase in endogenous peroxidation produced by the addition of ADP and Fe^{2+}. The production of malonaldehyde under such conditions (i.e. endogenous peroxidation or in the presence of added ADP and Fe^{2+}) is a combination of an enzymic pathway utilizing NADPH and a non-enzymic route (see Hochstein & Ernster, 1963). The enzymic route, at least in the presence of ADP and Fe^{2+} (Hochstein & Ernster, 1963), involves a major portion of the NADPH–cytochrome *P*-450 electron-transport chain. It is of importance to realize in

Table 5. *Concentrations of halogenoalkanes in microsomes-plus-supernatant–standard stock suspensions (2.5ml) in the central compartments of Warburg flasks*

The side arms of the flasks contained 1 μl of the halogenoalkane as a 1:1 (v/v) solution in liquid paraffin. Incubation time was 60min at 37°C. Details of the experimental procedures used are given in the Methods section.

Halogenoalkane	Method of determination	Concn. in stock solution (μg/ml)
CCl_4	Colorimetric	31
	Radioisotope dilution	34
$CHCl_3$	G.l.c.	266
$CFCl_3$	G.l.c.	9
$CBrCl_3$	G.l.c.	2.4
Halothane	G.l.c.	350

Table 6. *Comparative effects of halogenoalkanes on malonaldehyde production in rat liver microsomes-plus-supernatant–stock suspensions*

Incubations were for 60min at 37°C in the dark. The results are expressed relative to the stimulatory action of 2 μl of a carbon tetrachloride–liquid paraffin solution (1:1, v/v) in the side arms of Warburg flasks and are corrected to equimolar concentrations by using the results of Table 5. Mean values are given ±S.E.M. with the number of experiments in parentheses. For other details see the Methods section.

Halogenoalkane	Stimulatory effect on malonaldehyde production
CCl_4	100
$CHCl_3$	7±2 (7)
$CFCl_3$	34 (2)
$CBrCl_3$	3650±200 (4)
CF_3–CHClBr	5±2 (5)

advance of the discussion here, and in following papers (Slater & Sawyer, 1971*a*,*b*), that the stimulation of malonaldehyde production due to carbon tetrachloride probably involves a shorter segment of the NADPH–cytochrome *P*-450 chain than does the endogenous peroxidative route(s) dependent on NADPH. The two peroxidative pathways (endogenous peroxidation and the stimulation due to carbon tetrachloride) can, in fact, be varied independently of each other; evidence for this statement is given in the following paper (Slater & Sawyer, 1971*a*).

Stimulation of malonaldehyde production by carbon tetrachloride. The process of lipid peroxidation that is responsible for the production of malonaldehyde in tissue suspensions is a complex reaction involving a free-radical initiation stage. The stimulation of malonaldehyde production by carbon tetrachloride could represent the chemical involvement of carbon tetrachloride as an initiator of lipid peroxidation (this would entail the metabolism of carbon tetrachloride to free-radical intermediates) or an unspecific lipophilic action of carbon tetrachloride on the membranes of the endoplasmic reticulum. The latter possibility is unlikely since other halogenoalkanes with physical properties similar to carbon tetrachloride (e.g. chloroform, fluorotrichloromethane), but which undergo homolysis far less readily, are much less active in stimulating malonaldehyde production (see Table 6). Conversely, bromotrichloromethane, which enters homolytic reaction pathways more readily than carbon tetrachloride, is more active in stimulating the production of malonaldehyde in microsomes-plus-supernatant–stock suspensions. These results on the relative reactivities of the halogenomethanes not only rule out the possibility of the stimulatory actions on malonaldehyde production being unspecific in character but strongly indicate that a homolysis of the halogenomethane is necessary for its stimulatory action. The latter indication is substantiated, at least for carbon tetrachloride, by the results in Figs. 3 and 4. It was found that the rate of malonaldehyde production due to the presence of carbon tetrachloride was proportional to the square root of the concentration of carbon tetrachloride in the suspension. These results are strong evidence that initiation of the lipid-peroxidative reaction is by a free-radical product of the homolysis of carbon tetrachloride (for a general discussion of this point in relation to polymerization reactions see Walling, 1957). The most likely mechanism for the homolysis of carbon tetrachloride in the relatively nonpolar environment of the membranes of the endoplasmic reticulum is through an interaction between carbon tetrachloride and some endogenous 'frozen radical centre' in the membrane. A process of electron capture (Gregory, 1966) would thereby result in the formation of the highly reactive trichloromethyl radical:

$$CCl_4 + e^- \rightarrow CCl_3\cdot + Cl^-$$

the trichloromethyl radical then initiating peroxidation in neighbouring lipid as well as other and

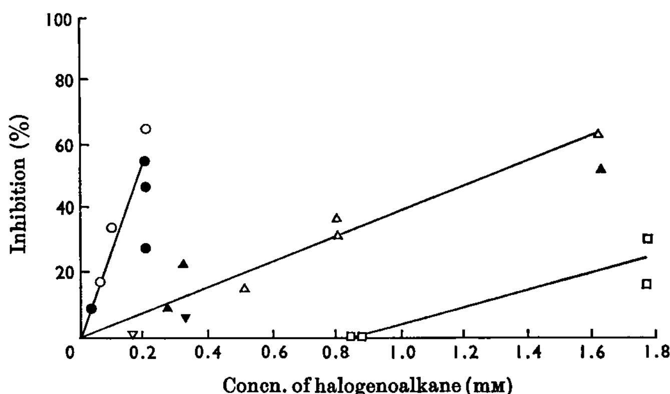

Fig. 5. Effect of four halogenoalkanes on glucose 6-phosphatase activity in microsomes-plus-supernatant–stock suspensions. The results were obtained from eight separate experiments. In four experiments (open symbols) microsomes plus supernatant (equivalent to 1g of liver in 5ml of suspension) in 0.25M-sucrose were mixed with a modified stock solution in which acetamide was replaced with 5mM-nicotinamide; incubations were for 60min at 37°C with or without the halogenoalkane in liquid paraffin in the side arms of Warburg flasks. In the remaining four experiments (closed symbols) microsomes plus supernatant (equivalent to 1g of liver in 3ml of suspension) in 0.25M-sucrose were mixed with standard stock and incubated as described above. After the 60min incubation period, 1ml samples were withdrawn and incubated for a further 30min period to determine the activity of either inorganic pyrophosphatase (open symbols) or glucose 6-phosphatase (closed symbols) as described in the Methods section. The concentration of halogenoalkane in the 60min incubation mixture has been calculated from the results given in the Methods section. ○, ●, Carbon tetrachloride; △, ▲, chloroform; □, ■, bromochlorotrifluoroethane; ▽, ▼, fluorotrichloromethane.

perhaps more important reactions such as destruction of neighbouring nucleotides and thiol-dependent enzymes. The production of significant quantities of trichloromethyl radicals would be expected to result in the formation of some hexachloroethane by self-annihilation; this material has been detected in rabbit liver after treatment with carbon tetrachloride (Fowler, 1969).

What might be considered to be an important difference between endogenous peroxidation in liver microsomes and the additional peroxidation due to the presence of carbon tetrachloride is shown up by the results in Fig. 2. Whereas endogenous peroxidation proceeds until most, if not all, of the unsaturated lipid in the microsomes is peroxidized (Hochstein & Ernster, 1963; May & McCay, 1968) the stimulation due to carbon tetrachloride is only apparent when the rate of endogenous peroxidation is low. This could be due to the effects of carbon tetrachloride on lipid peroxidation being restricted to a particular lipid-rich zone in the microsomes; once this had been destroyed by endogenous peroxidation then no additional effect of carbon tetrachloride would be evident. If so, then it would follow that the peroxidation produced by carbon tetrachloride is heavily damped by local concentrations of antioxidants and that the effective diffusion distance of trichloromethyl radicals is not sufficient to initiate homolytic initiation steps outside of this vicinity. In such a situation carbon tetrachloride would not cause a spreading wave of unspecific peroxidative damage throughout the endoplasmic reticulum but, instead, would result in a selective damage to a spatially confined zone. However, this conclusion is made unlikely by the results in Table 3, which show that the stimulatory action of carbon tetrachloride is increased about fivefold as the concentration of $NADP^+$ in the stock is raised to approximately five times that usually used. At the same time there is a smaller though still substantial increase (about twofold) in endogenous peroxidation. This suggests that the results shown in Fig. 2 were the consequence of a substantial destruction of $NADP^+$ during the incubation period (see Table 2) and that endogenous peroxidation has a lower requirement for $NADP^+$ than does the stimulatory action of carbon tetrachloride.

In this paper the general features of the process by which carbon tetrachloride stimulates the production of malonaldehyde in rat liver endoplasmic reticulum have been outlined. It has been concluded that carbon tetrachloride undergoes a homolytic cleavage, possibly by interaction with an endogenous radical component present in the endoplasmic reticulum, to initiate homolytic degradative reactions in neighbouring components. One

such degradative reaction is lipid peroxidation that is capable of ready demonstration by the measurement of malonaldehyde production. As such, malonaldehyde production is an indication of homolytic reactions involving carbon tetrachloride, of which lipid peroxidation is only one among several potentially degradative reactions that would ensue. The relative importance of such reactions to the necrogenic action of carbon tetrachloride *in vivo* is not known but remains a problem of considerable interest. It is of obvious importance to identify the site of interaction between carbon tetrachloride and the endoplasmic reticulum that is responsible for the homolytic consequences, and to investigate the effects of a number of free-radical scavengers on the deleterious homolytic reactions *in vitro* and *in vivo*. These studies are reported in the following papers (Slater & Sawyer, 1971*a*,*b*).

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