Biochemical Pharmacology, Vol. 26, pp. 2275–2282. Pergamon Press, 1977. Printed in Great Britain.

CARBON TETRACHLORIDE-INDUCED LIPID PEROXIDATION OF RAT LIVER MICROSOMES *IN VITRO*

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(*Received* 8 *September* 1976; *accepted* 8 *March* 1977)

Abstract—Characteristics of carbon tetrachloride-induced lipid peroxidation of rat liver microsomes and effect on microsomal enzymes were studies *in vitro*. Microsomes isolated from well-perfused livers and washed with EDTA-containing medium exhibited low endogenous lipid peroxidation when incubated in a phosphate buffer (> 0.1 M) in the presence of NADPH, whereas carbon tetrachloride stimulated to a great extent the peroxidation under these conditions. The stimulation was dependent on the concentration of NADPH, neither NADH nor ascorbic acid being replaced. The stimulatory action by bromotrichloromethane was more marked than that by carbon tetrachloride, however chloroform had no stimulatory action. *N,N*-Diphenyl-*p*-phenylene diamine, diethyldithiocarbamate and disulfiram inhibited carbon tetrachloride-induced lipid peroxidation in low concentrations. Inhibitions by thiol compounds and EDTA were weaker. Ferricyanide, cytochrome *c* and vitamine K_3 inhibited the stimulation by carbon tetrachloride while no inhibition was seen with carbon monoxide. An increase in the degree of carbon tetrachloride-induced lipid peroxidation resulted in a coincidental decrease in microsomal cytochrome P-450 content accompanying a parallel loss in aminopyrine demethylase activity, while NADH-ferricyanide dehydrogenase and NAD(P)H-cytochrome *c* reductase activities, and cytochrome b_5 content remained unaffected. Similar results were obtained when microsomes were peroxidized with NADPH in combination with ferric chloride and pyrophosphate. Regarding the mechanism of hepatotoxic action of carbon tetrachloride, these results support the hypothesis of lipid peroxidation.

Lipid peroxidative damage of endoplasmic reticulum membranes has been postulated to play an important role in the onset and development of the liver injury produced by CCl_4 [1–3].

Experimental evidence supporting this hypothesis has accumulated: first, peroxidative breakdown of microsomal membranes *in vitro* produces not only chemical alterations in a lipid component [4] but also enzymic, physical and structural changes in microsomes [5, 6]; second, CCl_4 enhances microsomal lipid peroxidation both *in vitro* [7, 8] and *in vivo* [9–11]; and third, activities of microsomal membrane-bound enzymes such as glucose-6-phosphatase and drug oxidizing enzymes decrease early after administration of CCl_4 [11–15]. Details of the mechanisms involved in the process have not however been reported.

In vitro studies are particularly useful for this purpose. In earlier experiments, Comporti *et al.* [16] and Ghoshal and Recknagel [17] showed an increased malonic dialdehyde (MDA) production by CCl_4 with liver homogenates and microsome-supernatant fractions, respectively. Later, Glende and Recknagel [7] demonstrated the requirement of NADPH for the stimulation by CCl_4 using microsomes or microsome-supernatant fractions. Slater and Sawyer [8, 18, 19] also extensively studied characteristics of the stimulatory action of CCl_4, using similar fractions. In these experiments, however, endogenous MDA production, i.e., with NADPH but without CCl_4, was fairly high and the stimulatory effect of CCl_4 was not so marked. Moreover, the presence of a supernatant fraction may complicate the explanation of the results obtained.

We have developed a simple experimental method, in which microsomes are the only cellular fraction and endogenous MDA production is quite low as compared to the stimulation by CCl_4. Under these conditions, some characteristics of CCl_4-induced lipid peroxidation and the effects on microsomal enzymes were examined.

MATERIALS AND METHODS

Chemicals. Sources of the reagents are as follows: NADPH, NADH, isocitric dehydrogenase, P-L Biochemicals, Inc.; 2-thiobarbituric acid, ascorbic acid, E. Merck AG.; CCl_4, $CBrCl_3$, $CHCl_3$, CH_2Cl_2, malonaldehyde *Bis* (diethylacetal), Tokyo Kasei Kogyo Co. Ltd.; cytochrome *c*, Sankyo Co. Ltd. 2-Diethylaminoethyl-2,2-diphenylvalerate HCl (SKF-525A) was kindly provided by Smith Kleine & French Laboratories. Other reagents were of analytical grade.

Animals and isolation of microsomes. Healthy female rats of Sprague–Dawley strain, 7–8 weeks in age, were used throughout the experiments. After the animals had been decapitated, the livers were perfused thoroughly *in situ* through a portal vein with ice-cold 0.15 M KCl solution using a perfusion pump, and a 20% liver homogenate in 0.15 M KCl was prepared. Mitochondrial supernatant was obtained by centrifuging the homogenate at 15,000 *g* for 15 min. The supernatant containing no fluffy layer was centrifuged at 125,000 *g* for 30 min in order to precipitate microsomes, which were then floated off from the glycogen fraction at the bottom by shaking gently in a small amount of the fresh suspending medium. The microsomal fraction thus obtained was washed with 0.15 M KCl–1 mM EDTA (pH 7.5) and then with

0.15 M KCl–20 mM potassium phosphate (pH 7.5). The liver perfusion and the EDTA-washing were carried out in order to minimize contamination with iron compounds as they are known to enhance microsomal lipid peroxidation [20, 21]. Final microsomal precipitates were suspended in the latter medium at a concentration of 20–30 mg protein/ml, kept in an ice-cold Thunberg tube under nitrogen and used within 24 hr. All the media were cooled with ice and bubbled with nitrogen.

Lipid peroxidation. The degree of lipid peroxidation was estimated by measuring MDA formed in the incubation media. The regular procedure was as follows. The incubation mixtures containing 1 mg protein/ml of microsomes, 200 μM NADPH and 0.1 or 0.2 M potassium phosphate buffer (pH 7.5) in a final vol. of 2.0 ml, with or without CCl_4, were prepared in duplicate in the usual small test tubes (15 × 90 mm). CCl_4 was added directly or as a diluted solution (1 μl of CCl_4 per 10 ml of the above buffer). All the solutions or suspensions were handled in an ice bath. The tubes were capped with silicon rubber stoppers as soon as CCl_4 was added, mixed vigorously using a mixer, and then incubated at 37° for 20 min. The reaction was stopped by addition of 2.0 ml of 30% TCA and 0.2 ml of 5 M HCl. MDA was determined by the thiobarbituric acid method [22] using a molar extinction coefficient $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (at 535 mμ) for the colored product. Malonaldehyde *bis* (diethylacetal) was used as a routine standard. Under these conditions, microsomes alone or with CCl_4 produced only a negligible amount of MDA. MDA production due to NADPH and the stimulation of MDA production due to CCl_4 in the presence of NADPH are referred to as 'endogenous' and 'CCl_4-induced', respectively. Endogenous MDA production had a tendency to increase gradually with high concentrations of NADPH and also with longer incubation periods. Any changes of the procedure are noted in the results section.

Other assays. NAD(P)H-cytochrome *c* reductase and NADH-ferricyanide dehydrogenase activities were measured spectrophotometrically by recording the optical density change at 550 mμ for cytochrome c and at 420 mμ for ferricyanide as described elsewhere [23]. Aminopyrine demethylase activity was measured virtually according to the method described by Orrenius [24] except that potassium phosphate buffer (pH 7.5) was used. Formaldehyde was determined by the method of Nash [25]. Contents of cytochrome b_5 and P-450 were determined by the method of Omura and Sato [26] using a Hitachi 124 spectrophotometer attached with integrating spheres. Protein was determined by the method of Lowry *et al.* [27].

RESULTS

Effects of various suspending media on endogenous and CCl_4-induced lipid peroxidation of liver microsomes. Several investigators [7–9, 16–19] have reported enhancement of MDA production by CCl_4 in liver microsomal suspensions *in vitro*. However, endogenous MDA production is fairly high as compared with the stimulation by CCl_4. Therefore, we firstly examined whether or not the suspending media might affect endogenous MDA production as well as the stimulation by CCl_4, and if so to make attempts to reduce the former and increase the latter.

As shown in Fig. 1, both endogenous and CCl_4-induced lipid peroxidation were greatly influenced by using different suspending media. A high endogenous MDA production was observed with Tris–HCl or glycylglycine buffer as compared with 0.15 M KCl-containing media, while the stimulation by CCl_4 was not greatly changed. On the other hand, in potassium or sodium phosphate buffer endogenous peroxidation was kept quite low, although the stimulation also being depressed somewhat. Microsomes isolated from nonperfused livers and not washed with the EDTA-containing medium, however, showed a high endogenous MDA production even in the phosphate buffer. It appears roughly that the ratio of CCl_4-induced MDA production to the endogenous one decreases as the latter increases.

Effects of pH and concentration of phosphate buffer on MDA production are shown in Figs 2 and 3. Optimum pH for CCl_4-induced MDA production was about 7.0, whereas the endogenous was not much

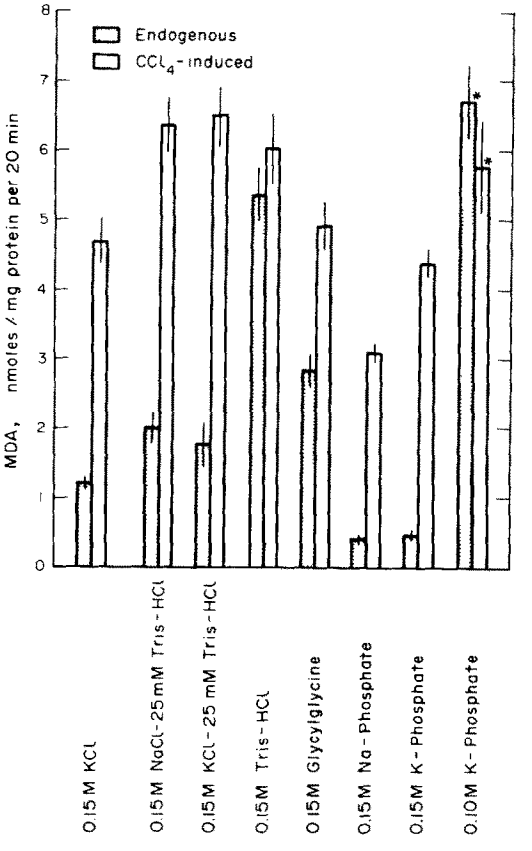

Fig. 1. Effect of suspending media on endogenous and CCl_4-induced malonic dialdehyde (MDA) production in liver microsomes. Microsomes (1 mg protein/ml) suspended in various buffers (pH 7.5) were incubated with or without CCl_4 (0.5 μl/ml) in the presence of 200 μM NADPH. Experimental details are given in the methods section. CCl_4-induced MDA production: MDA production in the absence of CCl_4 (endogenous) was subtracted from the MDA production in the presence of CCl_4. Each column represents the mean ± S.E.M. (bar) of 4 experiments. (*): Liver perfusion and EDTA-washing were omitted from the isolation procedure (2 experiments).

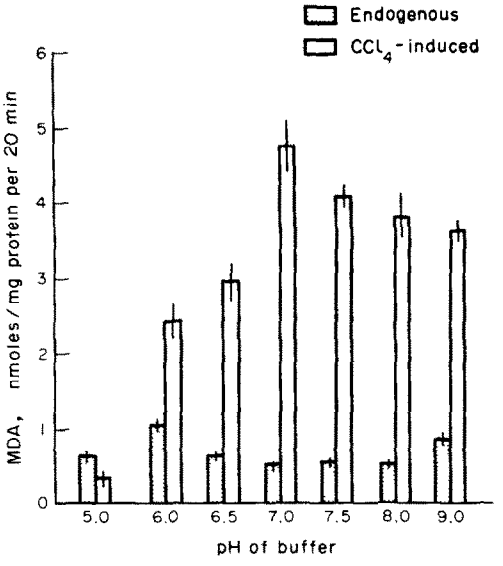

Fig. 2. Effect of pH on endogenous and CCl_4-induced malonic dialdehyde (MDA) production in microsomes. Microsomes were suspended in 0.15 M potassium phosphate buffer of different pH. Other conditions are as described for Fig. 1. Each column represents the mean ± S.E.M. (bar) of 4 experiments.

altered by changing pH. Concentration of the buffer greatly affected both endogenous and CCl_4-induced MDA production: both increased in low buffer concentrations and then decreased in higher concentrations, in which the former was much suppressed, and optimum concentration for the former (50 mM) was always lower than that for the latter (75 mM). These different sensitivities to the changes of pH and concentration of phosphate buffer indicate that mechanisms involved in both peroxidation are different.

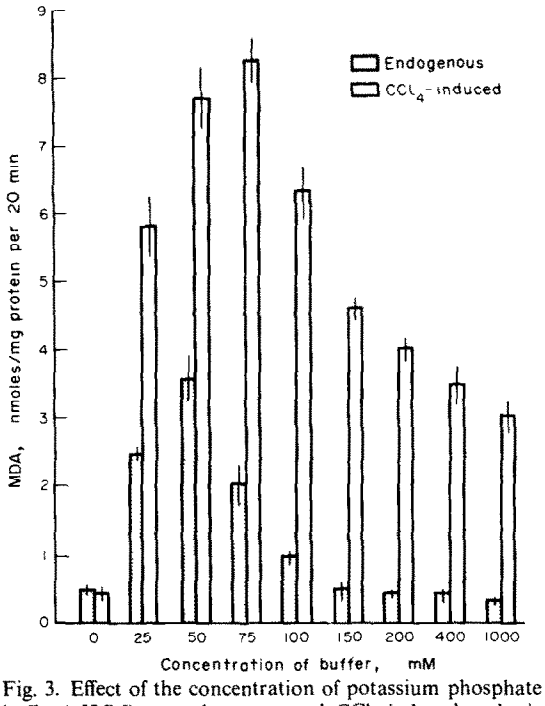

Fig. 3. Effect of the concentration of potassium phosphate buffer (pH 7.5) on endogenous and CCl_4-induced malonic dialdehyde (MDA) production in microsomes. Experimental conditions are as described for Fig. 1. Each column represents the mean ± S.E.M. (bar) of 4 experiments.

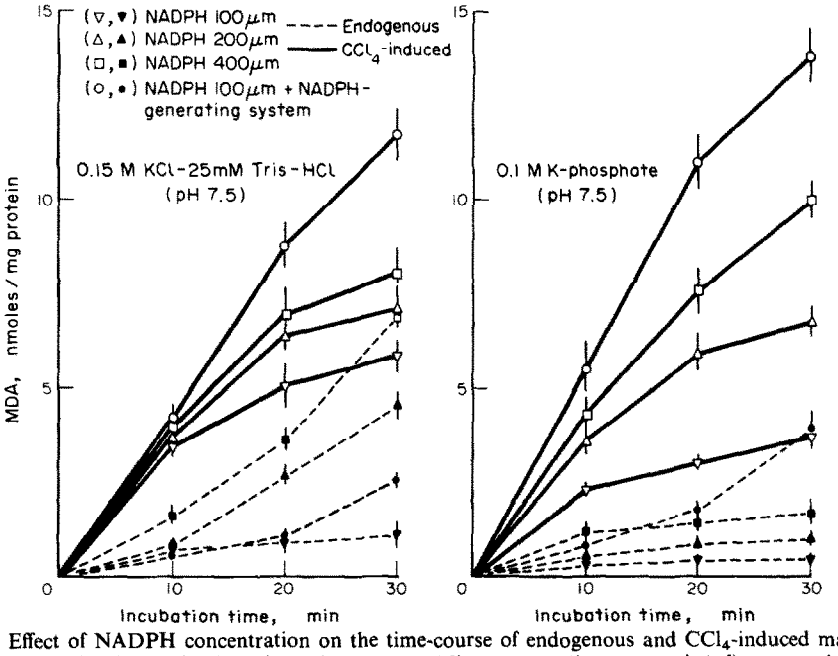

Fig. 4. Effect of NADPH concentration on the time-course of endogenous and CCl_4-induced malonic dialdehyde (MDA) production in microsomes. Microsomes (1 mg protein/ml) suspended in 0.15 M KCl–25 mM Tris–HCl (pH 7.5) or 0.1 M potassium phosphate buffer (pH 7.5) were incubated with or without CCl_4 (0.5 μl/ml) in the presence of a different amount of NADPH. CCl_4-induced MDA production does not include the endogenous. NADPH-generating system contained 2.5 mM nicotinamide, 5 mM $MgCl_2$, 10 mM isocitrate and 0.1 mg/ml of isocitrate dehydrogenase. Each point represents the mean ± S.E.M. (bar) of 3 experiments.

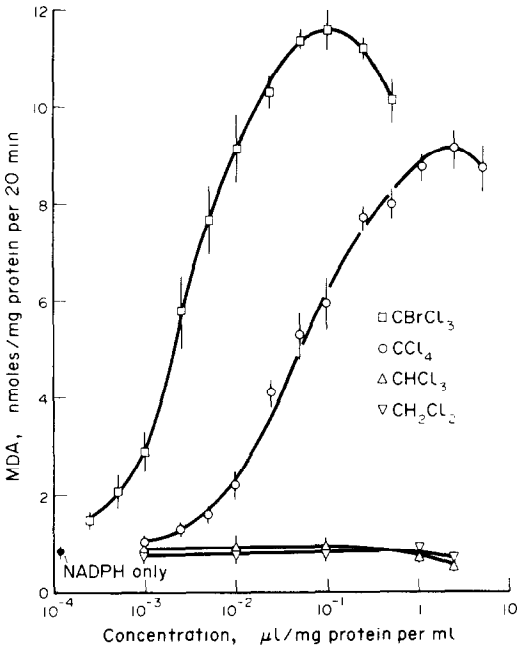

Fig. 5. Comparison of the effects of some halogenated methanes on malonic dialdehyde (MDA) production in microsomes. Experimental details are given in the Methods section. 0.1 M potassium phosphate buffer was used. Endogenous values are not subtracted. Each point represents the mean ± S.E.M. (bar) of 3 to 4 experiments.

NADPH requirement for the stimulation of lipid peroxidation by CCl_4. Figure 4 shows a time-course of endogenous and CCl_4-induced MDA production in different media with various concentrations of NADPH. Consistent with the above data, endogenous MDA production increased remarkably in 0.15 M KCl-25 mM Tris–HCl buffer (pH 7.5) with increasing NADPH concentration and prolonged incubation time, whereas it remained quite low in 0.1 M potassium phosphate buffer (pH 7.5). The stimulation by CCl_4 was dependent on the concentration of NADPH: the dependency was more distinct with 0.1 M potassium phosphate buffer. A decreased rate of MDA production with time may be due to consumption of NADPH as (1) MDA production increased almost linearly in the presence of NADPH-generating system, (2) additional NADPH restored the initial rate, and (3) the oxidation of NADPH, measured spectrophotometrically by recording the optical density change at 340 mμ, was nearly completed at 20 min with 200 μM NADPH (hardly affected by the presence or absence of CCl_4) (not shown).

The stimulation by CCl_4 required NADPH as reported by other workers (7,8); neither NADH nor ascorbic acid could be replaced. In the following experiments, 0.1 or 0.2 M potassium phosphate buffer (pH 7.5) was used in order to keep the endogenous as low as possible.

Comparison of the stimulatory effects of some halogenated methanes. CCl_4, $CHCl_3$ and $CBrCl_3$, all of which produce hepatic dysfunction *in vivo* [1, 11, 28], were compared for their ability to stimulate microsomal lipid peroxidation *in vitro* (Fig. 5). The stimulation of MDA production by CCl_4 was obvious at a concentration as low as 0.01 μl CCl_4/mg microsomal protein, increased linearly with increasing logarithmic concentrations, and reached a maximum at 1.0 μl CCl_4/mg protein, approximately 10 times the endogenous MDA production. Thus, the stimulation by CCl_4 was quite apparent as compared with that reported by Slater and Sawyer [8]. $CBrCl_3$, whose hepatotoxic action is stronger than that of CCl_4 (28), had a more powerful stimulatory effect than CCl_4 which is in accordance with their report [8]. However, no stimulatory effect was observed with $CHCl_3$. The stimulation may not be simply due to the solvent action, as CH_2Cl_2 and $CHCl_3$ as well as ethanol and acetone (not shown) were ineffective.

Inhibition of CCl_4-induced lipid peroxidation by some antioxidants and radioprotectors. Compounds that have an antioxidative, radioprotective or chelating action have been reported to ameliorate or protect against the liver damage produced by CCl_4 [1, 29–31] and also to inhibit lipid peroxidation

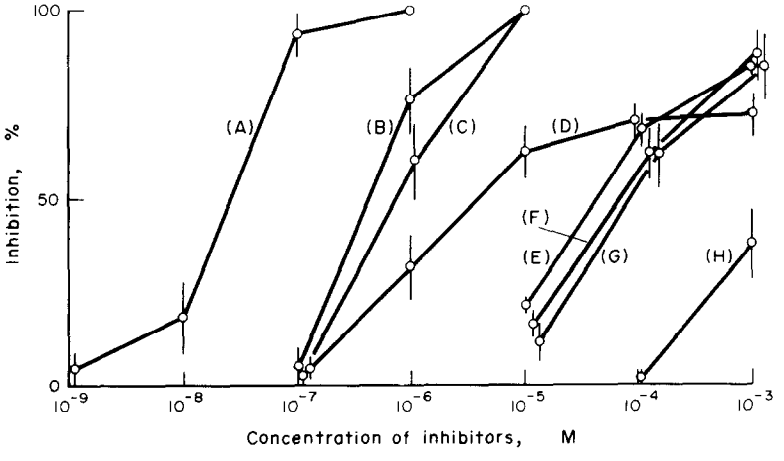

Fig. 6. Comparison of the effects of various inhibitors on CCl_4-induced malonic dialdehyde (MDA) production in microsomes. Experimental details are given in the Methods section. 0.2 M potassium phosphate buffer was used. Inhibitors: (A) *N,N*-diphenyl-*p*-phenylenediamine, (B) diethyldithiocarbamate, (C) disulfiram, (D) EDTA, (E) glutathione, (F) dithiothreitol, (G) cysteamine, (H) penicillamine. Each point represents the mean of 2 experiments with the range (bar).

Table 1. Effect of various substances interacting with NADPH-dependent electron transport system on CCl_4-induced malonic dialdehyde (MDA) production in microsomes*

Substances	Conc (M)	Inhibition of CCl_4-induced MDA production (%)	Substances	Conc (M)	Inhibition of CCl_4-induced MDA production (%)
p-Chloromercuric benzoate	10^{-5}	0	Aminopyrine	10^{-4}	43– 47
	10^{-4}	99–100		10^{-3}	74– 76
Ferricyanide	1.7×10^{-4}	19– 22		5×10^{-3}	100
	3.3×10^{-4}	100	SKF-525A	10^{-4}	61– 70
Cytochrome *c*	2.5×10^{-5}	24– 55		10^{-3}	88–100
	5×10^{-5}	67– 80		2×10^{-3}	96–100
	10^{-4}	94–100	CO		−20–0
Vitamin K_3	1.2×10^{-7}	67– 88	CO + aminopyrine	5×10^{-3}	96–100
	1.2×10^{-6}	95–100			

* Experimental procedures are given in the Methods section. Potassium phosphate buffer (0.2 M) was used. Carbon monoxide was directly bubbled into the microsomal suspension for 30 sec through a fine capillary. Results are given as the ranges of 2 to 3 experiments.

in vitro [19, 22, 32, 33]. Some were tested to determine if they inhibit CCl_4-induced lipid peroxidation (Fig. 6). *N,N*-Diphenyl-*p*-phenylenediamine, a powerful free radical scavenger, inhibited it at extremely low concentrations. Diethyldithiocarbamate, a radioprotector, and interestingly its dimer disulfiram, which have a strong protective effect *in vivo* [31], were also strong inhibitors *in vitro*. The former had no effect on microsomal aminopyrine demethylase activity at the concentrations that inhibited the lipid peroxidation. Their strong iron-chelating as well as radioprotective action may contribute to the inhibition. Inhibitions by thiol compounds such as gluthathione, dithiothreitol, cysteamine and penicillamine were rather weak. Inhibition by EDTA was apparent at low concentrations but not complete even at 10^{-2} M.

Effects of some substances that interact with NADPH-dependent microsomal electron transfer chain on CCl_4-induced lipid peroxidation. CCl_4 is considered to be converted to more toxic free radicals on the NADPH-dependent electron transport system [7, 8, 18] in which flavoprotein and cytochrome P-450 are involved. The exact site of activation, however, is still not clear. Slater and Sawyer [18] carried out extensive work on this point by using microsomal suspensions although in their experiment the stimulation of MDA production by CCl_4 is quite low as compared with the endogenous. We attempted a similar type of experiment. Table 1 (left) shows the inhibition at a flavoprotein step. *p*-Chloromercuric benzoate—a SH-inhibitor of the flavoprotein enzyme—, cytochrome *c* and ferricyanide—artificial electron acceptors at the flavoprotein level—, and Vitamin K_3—a stimulator of microsomal NADPH oxidation by the flavoprotein [34]—were all inhibitory.

Inhibitions at cytochrome P-450 level are also shown in Table 1 (right). Aminopyrine—its oxidative *N*-demethylation is coupled with this heme-protein—and SKF-525A—a competitive inhibitor for the binding of drug to heme-protein—were inhibitory at rather high concentrations. On the contrary, carbon monoxide treatment, which inhibited aminopyrine demethylase activity by 91 per cent (our own data), was not at all inhibitory. Furthermore, the inhibition by aminopyrine was observed even after the treatment of microsomes with carbon monoxide, suggesting that it may not be coupled with the demethylation process or rather may be a non-specific inhibition of lipid peroxidation.

Effects of CCl_4-induced lipid peroxidation on microsomal enzyme activities and heme-protein contents. Some liver microsomal enzymes have been reported to be most susceptible to the administration of CCl_4 in rats and the enzymic changes of microsomes are considered to be due to a lipid peroxidative breakdown of microsomal membranes produced by CCl_4 [1–3, 11–15]. Therefore, this point was checked

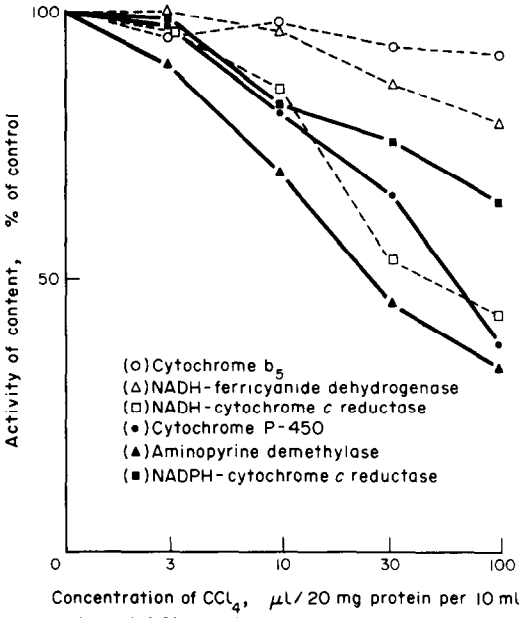

Fig. 7. Effect of CCl_4 on the enzyme activities of electron transport systems and heme-protein contents in liver microsomes. Microsomes (20 mg protein) were incubated with various amounts of CCl_4 in 10 ml of 0.2 M potassium phosphate buffer (pH 7.5) for 20 min at 37°. One ml of the suspension was then assayed for malonic dialdehyde (MDA). No appreciable amount of MDA was formed under these conditions. The remainder was cooled and centrifuged at 127,000 *g* for 1 hr and the sediment suspended in 0.1 M potassium phosphate buffer (pH 7.5) for the enzyme assays. Neither enzyme activities nor heme-proteins were detectable in the supernatant.

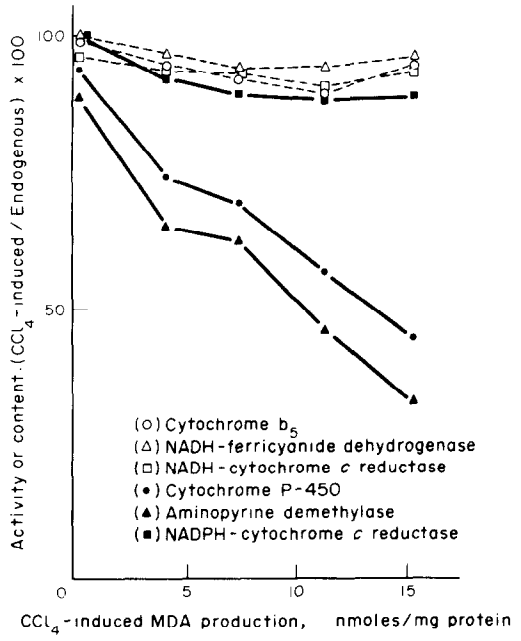

Fig. 8. Effect of CCl_4-induced lipid peroxidation on the enzyme activities of electron transport systems and heme-protein contents in liver microsomes. Microsomes (20 mg protein) were incubated with various concentrations of NADPH in the presence or absence of CCl_4 (3 μl) in 10 ml of 0.2 M potassium phosphate buffer (pH 7.5). After incubation for 20 min at 37°, 1 ml of the suspension was assayed for malonic dialdehyde (MDA). CCl_4-induced MDA production (the endogenous was subtracted) was 0, 4.3, 7.6, 11.1 and 15.5 (nmoles MDA/mg microsomal protein) for 0, 50, 100, 200 and 400 μM NADPH, respectively. The remainder was cooled and centrifuged at 127,000 g for 1 hr. The resulting sediment was suspended in 0.1 M potassium phosphate buffer (pH 7.5) and assayed for enzyme activities and heme-protein contents. Neither enzyme activities nor heme-proteins could be recovered in the supernatant.

in the *in vitro* experimental system in which endogenous lipid peroxidation is so low that its effect on microsomes may be almost neglected. Typical experimental data are presented in Figs. 7, 8 and 9.

Figure 7 shows a direct effect of CCl_4 on microsomal enzymes. Aminopyrine demethylase activity and cytochrome P-450 content decreased markedly as the amount of CCl_4 increased, whereas NADPH-cytochrome c reductase activity was less susceptible. The decrease in cytochrome P-450 content was accompanied by an increase in P-420. NADH-cytochrome c reductase activity also decreased considerably, however, cytochrome b_5 content showed no change when calculated from dithionite-reduced difference spectra. NADH-ferricyanide dehydrogenase was fairly resistant to CCl_4.

Effects of CCl_4-induced lipid peroxidation on microsomal enzymes were examined in the presence of 3 μl of CCl_4/20 mg protein which has a minimum direct effect as shown in Fig. 7. In Fig. 8, the enzyme activities or heme-protein contents were plotted against the different levels of lipid peroxidation produced by changing NADPH concentration. Cytochrome P-450 content together with aminopyrine demethylase activity decreased specifically as a degree of lipid peroxidation progressed. In this case, however, a conversion of cytochrome P-450 to P-420 did not occur. The other enzymes tested were rather resistant to lipid peroxidation. Similar results were obtained when the CCl_4-independent lipid peroxidation system was used (Fig. 9).

These *in vitro* data suggest that a decrease in cytochrome P-450 content and drug metabolizing activity *in vivo* may be partly due to the lipid peroxidative action of CCl_4.

DISCUSSION

Microsomes, isolated from well-perfused rat livers and washed with a medium containing EDTA, exhibited low lipid peroxidative activities when incubated in a phosphate buffer in the presence of NADPH, while CCl_4 markedly stimulated the lipid peroxidation under these conditions. Compared with other investigators' methods in which a supernatant fraction was required [7–9, 17] or CCl_4 was diffused into a microsomal suspension [8, 18, 19], the present method is simple and the stimulation by CCl_4 is quite apparent even at very low concentrations.

It is interesting that in the present studies, endogenous lipid peroxidation was enhanced in lower concentrations (25–75 mM) of phosphate buffer and then became suppressed in higher concentrations (more than 0.1 M). Wills [32] also observed increment of peroxidation with phosphate ion and suggested that

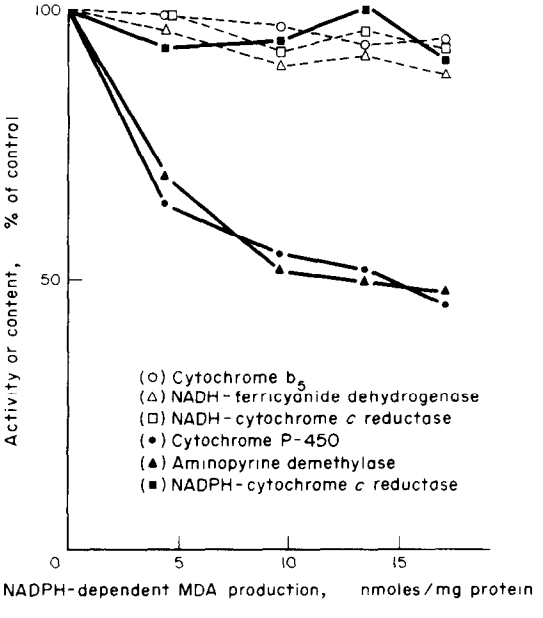

Fig. 9. Effect of NADPH-dependent lipid peroxidation on the enzyme activities of electron transport systems and heme-protein contents in liver microsomes. Microsomes (20 mg protein) were incubated with various concentrations of NADPH in the presence of 20 μM ferric chloride and 0.2 mM pyrophosphate in 10 ml of 0.15 M KCl–25 mM Tris–HCl buffer (pH 7.5). After incubation at 37° for 10 min, the same procedure as that described for Fig. 8 was applied. MDA production due to NADPH was 4.4, 9.8, 13.6 and 17.4 (nmoles MDA/mg microsomal protein) for 4, 10, 20 and 30 μM NADPH, respectively.

active compounds formed from iron compounds and phosphate ion might be responsible for the increased lipid peroxidation [21, 32]. As, in our microsomal preparations, contamination or absorption of iron compounds originated from blood components and other cellular fractions is considered to be negligible, membrane-bound intrinsic iron could be activated in low phosphate ion concentration or even masked with excess phosphate ion, thus resulting in the enhanced or suppressed peroxidation, respectively. CCl_4-induced peroxidation also had a optimum phosphate ion concentration, which was higher than that of the endogenous. The difference in optimum concentration and pH of phosphate buffer between endogenous and CCl_4-induced peroxidation may indicate that each peroxidation process is different.

It is also noteworthy that an increase in endogenous lipid peroxidation did not always accompany a parallel increase in CCl_4-induced peroxidation or rather a ratio of CCl_4-induced to endogenous peroxidation decreased as the endogenous increased when compared in various suspending media. A similar tendency has been reported in different microsomal preparations by Slater and Sawyer [8]. By way of explanation, in normal microsomes, it is considered that lipid peroxidation and drug hydroxylating process compete with each other for NADPH-cytochrome P-450 reductase, a flavoprotein, in the NADPH-dependent electron transport system [35, 36]. Therefore, an increase in the former activity may reduce an electron flow to the latter process involving cytochrome P-450. Accordingly, as will be discussed later, if metabolic conversion of CCl_4 to free radicals is coupled with this heme-protein and free radical products are essentially responsible for the stimulation of lipid peroxidation [1–3], then the reduced electron flow to cytochrome P-450 would consequently decrease CCl_4-induced lipid peroxidation.

The following discussions concern the activation of CCl_4 to free radicals, the site of the activation and the functional alterations of microsomes due to lipid peroxidation.

Firstly, it is generally considered that hepatotoxic action of CCl_4 is initiated by its free radical metabolites [1–3], although there is no direct evidence showing that the free radicals are detectable either *in vivo* or *in vitro*. Indirect evidence supporting the hypothesis includes (1) free radical scavengers or antioxidants suppress or protect against the liver damage *in vivo* [1, 29–31] as well as microsomal lipid peroxidation *in vitro* [19, 22, 32, 33], (2) the potency of halogenated hydrocarbons to produce hepatic lesion or stimulate lipid peroxidation can be correlated to their chemical susceptibility to homolytic cleavage [3, 28, 37], i.e., the lower the bond dissociation energy of carbon–halogen bonds, the greater the toxic action, and (3) free radical products of CCl_4—$\cdot CCl_3$ and $\cdot Cl$—are incorporated into the fatty acid portion of microsomal phospholipids [38–41].

Our results with inhibitors and some halogenated methanes are in favour of the view above cited. It is strange, however, that $CHCl_3$ did not stimulate lipid peroxidation though it can produce liver damage similar to that induced by CCl_4. Hepatotoxic action of $CHCl_3$ might be different not only quantitatively but also qualitatively from that of CCl_4 as suggested by Klaassen and Plaa [11].

Secondly, microsomal NADPH-dependent electron transport system appears to be responsible for the activation of CCl_4 to free radicals, because hepatotoxic action of CCl_4 *in vivo* is fairly dependent on the drug metabolizing enzyme activity in microsomes [3, 42] and the *in vitro* stimulation of microsomal lipid peroxidation requires the presence of NADPH [7], not replaced by NADH [8]. We also confirmed the latter point. There is no definite evidence, however, which step of the election transfer chain is concerned with a conversion of CCl_4 to free radicals. Slater and Sawyer [8] suggested NADPH-cytochrome *c* reductase, a flavoprotein, as an activation step, Glende and Recknagel [7, 43, 44] suggested it might be after flavoprotein component and involve cytochrome P-450. In our experiments, inhibition of CCl_4-induced lipid peroxidation by electron acceptors at the flavoprotein step such as ferricyanide, cytochrome *c* and vitamin K_3 may not always indicate that an activation step is after flavoprotein because these compounds might competitively inhibit the activation of CCl_4 at the flavoprotein step. Inhibition by aminopyrine and SKF-525A suggests involvement of cytochrome P-450. But, if so, it is incomprehensive that carbon monoxide is not at all inhibitory despite its strong inhibition of aminopyrine demethylation. Further studies with a reconstituted electron transport system are being conducted by our team to clarify the activation step.

The third point concerns the functional alterations of endoplasmic reticulum caused by administration of CCl_4. CCl_4, in an early stage of the liver damage, produces a marked increase in the level of conjugated dienes in microsomal lipids resulting from their peroxidative breakdown [9–11] on the one hand, and a decrease in microsomal enzyme activities such as glucose-6-phosphate and drug metabolizing enzyme together with a loss of cytochrome P-450 [11–15], on the other. There has been no definite evidence, however, indicating that the former event is directly concerned with the latter in the toxigenicity of CCl_4.

Activities of membrane-bound microsomal enzymes are highly dependent on lipids [45], and microsomal lipid peroxidation in the *in vitro* system by NADPH or ascorbic acid in combination with an activator, results in a coincidental loss of these enzymes [5, 6]. These facts are, though indirectly, in favour of the above view.

We attempted more direct experiments concerning the enzymes of microsomal electron transport systems, and confirmed that due to its lipid peroxidative action, a small amount of CCl_4 which would produce a minimal direct effect, could result in a decrease in drug metabolizing enzyme activity in parallel with the loss of cytochrome P-450 content. These findings strongly suggest that the depression of drug metabolizing enzyme activity by CCl_4 administration may be due to a loss of cytochrome P-450 caused by the lipid peroxidative damage of endoplasmic reticulum membranes.

On the other hand, a direct effect of CCl_4 on these enzymes, which was observed *in vitro* with increasing CCl_4 concentrations, may be minimal *in vivo* in consideration of the *in vivo* data on the concentration

of CCl_4 in liver following CCl_4 administration [46]. Another possiblility that this heme-protein is attacked by free radical products of CCl_4 should also be considered [47, 48]. On this point, however, Glende *et al.* [49] have recently reported that the loss of cytochrome P-450, aminopyrine demethylase as well as glucose-6-phosphatase is not caused by direct attack on these enzymes by free radical products of CCl_4, but is mediated by lipid peroxidation.

In view of the finding that among the components of microsomal electron transport systems cytochrome P-450 is particularly sensitive to CCl_4-induced lipid peroxidation and the possibility that this heme-protein may be concerned with the activation of CCl_4 to more toxic free radicals [3, 7, 43, 44], cytochrome P-450 appears to play an important role in 'membrane suicide' due to CCl_4.

Finally, although it is difficult to apply the results obtained from *in vitro* experiments to the explanation of a sequence of events *in vivo*, our experimental system may be useful for the elucidation of the mechanisms involved in the CCl_4-linked lipid peroxidation.

Acknowledgements—Thanks are due to M. Ohara for assistance with preparation of the manuscript. This work was supported in part by the Science and Technology Agency, Tokyo, Japan.

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