Biochemical Pharmacology, Vol. 25, pp. 2163–2170. Pergamon Press, 1976. Printed in Great Britain.

CRITICAL ROLE OF LIPID PEROXIDATION IN CARBON TETRACHLORIDE-INDUCED LOSS OF AMINOPYRINE DEMETHYLASE, CYTOCHROME P-450 AND GLUCOSE 6-PHOSPHATASE*

ERIC A. GLENDE, JR., ANDREW M. HRUSZKEWYCZ† and RICHARD O. RECKNAGEL
Department of Physiology, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, U.S.A.

(*Received* 7 *July* 1975; *accepted* 13 *February* 1976)

Abstract—Liver microsomal glucose 6-phosphatase, cytochrome P-450 and aminopyrine demethylase all decrease rapidly in the CCl_4-poisoned rat. It has been suggested that such enzyme loss may be due to direct attack on enzyme catalytic sites by free radical cleavage products of CCl_4 metabolism. An alternate view has favored the suggestion that peroxidative decomposition of lipids is an intermediate link between initial homolytic cleavage of the CCl_3–Cl bond and eventual loss of these enzymes. We have subjected these two ideas to a critical test. In an anaerobic system *in vitro* containing liver microsomes supplemented with an NADPH-generating system, and in the presence of EDTA, all added CCl_4 is metabolized in 90 min. About one-third appears as $CHCl_3$ amd most of the remainder is covalently bound to microsomal lipids and proteins. In this anaerobic system *in vitro* there is no evolution of malonic dialdehyde. During the period of conversion of CCl_4 to $CHCl_3$, when extensive binding of ^{14}C from $^{14}CCl_4$ is also taking place, there was no detectable loss of either glucose 6-phosphatase or cytochrome P-450, and aminopyrine demethylase activity decreased minimally. In the same system, under aerobic conditions and without EDTA, there is vigorous lipid peroxidation and all three of these enzyme systems decrease markedly. These experiments demonstrate conclusively that CCl_3—Cl bond cleavage and covalent binding of products of CCl_4 metabolism do not constitute a mechanism for loss of microsomal glucose 6-phosphatase, cytochrome P-450 or aminopyrine demethylase for the particular anaerobic conditions employed *in vitro*. By extension they suggest, but do not prove, that covalent binding of CCl_4 carbon probably does not constitute a mechanism for CCl_4-induced loss of these enzymes *in vivo*. Rather, these experiments support the view that lipid peroxidation is an obligatory link between initial CCl_3—Cl bond cleavage and loss of these enzymes.

It is widely known that a variety of hepatic enzymic functions, especially those associated with the hepatocellular endoplasmic reticulum, are lost shortly after carbon tetrachloride administration to rats [1]. During the past 14 yr, two dominant viewpoints have emerged regarding possible chemical mechanisms by means of which CCl_4 effects such loss of enzymic function. Both of these points of view accept that the initial event is cleavage of the CCl_3—Cl bond, and many workers (including ourselves) incline to the view that the cleavage is homolytic (2–6). Where there have been differences of opinion, and where there have been differences of emphasis, relate to events believed to take place immediately after the initial CCl_3—Cl bond cleavage. One view, favored by our laboratory [1–3, 7], has suggested that peroxidative decomposition of structural lipids in the endoplasmic reticulum of liver cells is probably the key intermediate process linking initial CCl_4 metabolism and the subsequent display of structural and functional abnormalities. An alternate view, although in some instances recognizing a possible role for lipid peroxidation, has tended to stress covalent binding of presumed toxic metabolites of CCl_4 as an equally if not more important part of the chemical pathological mechanism of CCl_4-induced cellular injury [8–16]. The idea [17] that covalent binding of a neutral radical such as $\cdot CCl_3$ to protein—SH groups may play a significant role was recently subjected to close scrutiny, and in a critical review [3] no evidence could be found for its support.

In our opinion, it is not yet possible to resolve these differences of view in a general sense. However, recent developments have permitted an exceptionally definitive answer with respect to a limited aspect of the general problem. Certain liver enzymes, notably glucose 6-phosphatase [3, 18] and cytochrome P-450 [19–23], decrease rapidly in the CCl_4-poisoned rat. The conclusion that CCl_4-induced loss of cytochrome P-450 is due to lipid peroxidation and not to direct attack by "toxic metabolites" of CCl_4 metabolism was reached recently [24–26] on the basis of the following argument. Cytochrome P-450 is destroyed rapidly *in vitro* when liver microsomes are incubated in the presence of NADPH‡ and oxygen [25]. Addition of CCl_4 enhances both the rate of production of MDA and the destruction of cytochrome P-450. However, it turns out that addition of EDTA to these systems

*This work was supported by Grant AM-01489 from the National Institute of Arthritis, Metabolism, and Digestive Diseases, United States Public Health Service.

† Submitted for partial fulfillment for requirements for the Ph.D. degree in Physiology.

‡ Abbreviations and definitions: NADPH, reduced nicotinamide adenine dinucleotide phosphate; MDA, malonic dialdehyde; G-6-Pase, glucose 6-phosphatase; EDTA, ethylene diaminetetraacetate; VLDL, very low density lipoproteins. One mg equivalent of liver microsomes is the entire yield of microsomes derived from 1 mg wet weight of fresh liver.

in vitro does not influence either the aerobic conversion of CCl_4 to CO_2 [27] or the anaerobic conversion of CCl_4 to $CHCl_3$ [26]. In other words, the metabolism of CCl_4 *in vitro* by the microsomal mixed-function oxidase system does not require ionic metal cofactors chelatable by EDTA. However, EDTA is known to protect cytochrome P-450. The implication of this set of circumstances was recognized by Uehleke *et al.* [26], who drew the conclusion that when cytochrome P-450 is destroyed by CCl_4 either *in vitro* or *in vivo*, the destruction is due indirectly to lipid peroxidation and not to direct attack by split products of CCl_4 metabolism on the hemoprotein. These developments set the stage for carrying out of definitive experiments in which cytochrome P-450 content and other enzymic activities of a microsomal preparation could be assayed both before and after an anaerobic incubation *in vitro* in which conversion of CCl_4 to its metabolic products has occurred in the presence of EDTA. It was recently pointed out [3] that until such data became available, judgment would have to be witheld on the question as to whether rapid loss of cytochrome P-450 in CCl_4 poisoning is due to direct attack by metabolites of CCl_4, or whether lipid peroxidation is more significant. The data presented in this paper strongly support the view that, for CCl_4-induced loss of G-6-Pase, cytochrome P-450 and aminopyrine demethylase, lipid peroxidation is the decisive event.

METHODS

Animals. Animals used in these studies were male rats of the Sprague–Dawley strain obtained from Zivic–Miller Laboratories, Inc. or Flow Laboratories, Inc. The animals were fed Purina rat chow and had water available *ad lib.* Fed rats were killed by decapitation and allowed to exsanguinate. Most of the rats used were within a weight range of 200–300 g body weight, although occasionally lighter or heavier rats were used. No effect of rat body weight was noted with respect to any of the various parameters which were monitored.

Preparation of liver microsome fraction. After decapitation and exsanguination, the liver was removed and chilled in ice-cold homogenization medium, after which a 10% whole liver homogenate was prepared. The homogenization medium was 0.25 M sucrose, which contained 0.001 M EDTA, except for experiments in which lipid peroxidation would be promoted. For the latter, EDTA was omitted. Homogenization was carried out with a glass Potter–Elvehjem homogenizer equipped with a Teflon pestle. The 10% whole homogenate was centrifuged at 5900 *g* for 10 min in the SS-34 rotor of the RC2-B Sorvall centrifuge, at 2–4°. The supernatant fraction was removed and set aside at 0–1°. The sediment of mixed red cells, nuclei, mitochondria and so-called "fluffy layer" [28] was resuspended in the appropriate homogenization medium and centrifuged again for 10 min at 5900 *g* in the SS-34 rotor. The fluffy layer was removed from the centrifuged pellet and along with the supernatant medium was added to the original supernatant fraction from the first centrifugation step. This procedure yields the so-called S_2, or post-mitochondrial supernatant, fraction. The S_2 fraction was then centrifuged at 80,000 *g* for 30 min in the type 40 or type 42.1 rotor of a Spinco preparative ultracentrifuge.

Incubation procedures: overall experimental design. The essential experimental design for the experiments reported in this paper involved an initial incubation of the isolated microsomes under a number of precisely defined conditions. These conditions were designed either on the one hand to maximize microsomal metabolism and binding of CCl_4 in the absence of lipid peroxidation, or, on the other hand, to allow lipid peroxidation to take place. Details pertinent to particular experiments are given in full in legends to the appropriate figures. Our experiments have confirmed the report [26] that conversion of CCl_4 to $CHCl_3$ and binding of CCl_4 carbon to microsomal lipids and proteins are greatly enhanced under anaerobic conditions. Anaerobiosis was chosen, therefore, in order to achieve maximal CCl_4 metabolism and binding. After the appropriate initial incubation, the microsomes were recovered and assayed for enzymic activity. For assay of cytochrome P-450 the microsomes were not sedimented (see below). Any residual enzymic activity was compared with the corresponding enzymic activity of intact control microsomes which had not supported either CCl_4 metabolism or undergone lipid peroxidation. Thus, one point of these experiments was to determine whether and to what degree rat liver microsomes would be affected by incubation conditions which supported CCl_4 metabolism and binding in the absence of lipid peroxidation. The other point of these experiments was to observe any effects of lipid peroxidation on the same microsomal enzymes.

Enzyme assays, analysis for CCl_4 and $CHCl_3$, and other analytical procedures. Assay for cytochrome P-450 content was carried out on complete incubation systems without prior sedimentation and resuspension of the microsomes, according to Omuro and Sato [29]. The fact that CCl_4 was present in the experimental flasks had to be taken into account: we found that, at input levels of CCl_4 as used in these experiments, the 450 nm absorbance of the CO–cytochrome P-450 complex of intact microsomes was decreased 15–20 per cent. Therefore, measurement of cytochrome P-450 content in control flasks was carried out by adding CCl_4 at the end of the incubation period at the same concentration as was present in experimental flasks. Details for assay of G-6-Pase and aminopyrine demethylase are given in Figs. 5 and 8 respectively. Conditions for assay of CCl_4 and $CHCl_3$ are given in Fig. 1. This figure illustrates capacity of the gas chromatography (g.c.) procedures to effect separation of CCl_4 and $CHCl_3$.

Malonic dialdehyde was measured according to Ghoshal and Recknagel [30], formaldehyde according to Nash [31], inorganic phosphorus according to Fiske and Subbarow [32], and protein according to Lowry *et al.* [33].

Covalent binding of ^{14}C from $^{14}CCl_4$. In order to determine covalent binding of CCl_4 carbon, $^{14}CCl_4$ was present when incubation was initiated. After the incubation, the microsomal suspension was centrifuged at 80,000 *g* for 30 min. Appropriate control experiments demonstrated that all radioactivity present in the sedimented pellet was covalently bound. The

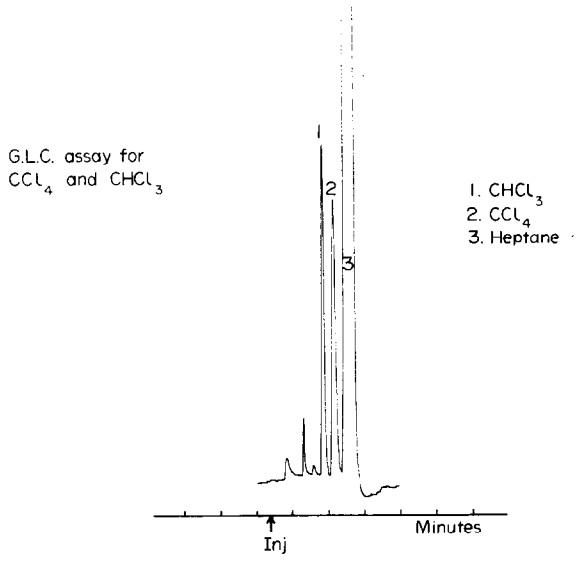

Fig. 1. Assay of CCl_4 and $CHCl_3$ by gas chromatography. CCl_4 and $CHCl_3$ were extracted from 8.0-ml incubation mixtures with 8.0 ml *n*-heptane. A suitably diluted heptane extract (1 μl) was injected into an F & M model 402 gas chromatograph equipped with a ^{63}Ni electron capture detector. Column: 6ft × 4 mm i.d., glass, packed with 3% SE-30 on 80/100 Supelcoport. Temperatures: flash heater, 53–58°; column, 45–49°; detector, 145–152°. Carrier gas: argon with 5% methane. The separation of CCl_4 from $CHCl_3$ by this method is illustrated after injection of known standards.

sedimented microsomes were resuspended in the buffer medium appropriate for subsequent enzymic assay. To measure binding, an aliquot of resuspended microsomes was added to Unisol (Isolab, Inc.) and allowed to stand at room temperature for 12–24 hr, after which were added 0.5 ml methanol and 13 ml Unisol-Complement (Isolab, Inc.). Radioactivity was monitored in a liquid scintillation spectrometer. The degree of quenching, which was minor, was determined by use of an internal standard of [^{14}C]toluene.

Distribution of ^{14}C covalently bound as a result of metabolism *in vitro* of $^{14}CCl_4$ was determined with respect to lipid and non-lipid moieties of the microsomal preparation. Total lipids of recovered microsomes were extracted in chloroform–methanol according to Rao and Recknagel [34]. An aliquot of extracted lipid in $CHCl_3$ was added to a scintillation vial and dried down under a stream of nitrogen. The recovered lipid was redissolved in fresh $CHCl_3$ and again dried down. The final lipid fraction was then dissolved in Scintisol-Complete (Isolab, Inc.), after which radioactivity was determined. In order to determine ^{14}C covalently bound to the non-lipoidal moiety of the microsomes, the sedimented microsomes recovered after metabolism *in vitro* of $^{14}CCl_4$ were processed according to Rao and Recknagel [35], except that the step involving hot trichloroacetic acid (TCA) extraction was omitted.

Controls. As pointed out above, the essential feature of these experiments was to determine the effects on various microsomal enzymes of two distinct incubation conditions, viz. a system supporting CCl_4 metabolism in the absence of lipid peroxidation and a system in which lipid peroxidation was allowed to occur. In both cases residual enzymic activity was compared with enzymic activity of a control system. In all cases the control system consisted of one or more flasks containing the complete incubation medium, including microsomes, CCl_4, NADPH-generating system, etc., identical with the incubated experimental flasks except that the controls were maintained at 0–1° during the initial incubation period. Enzymic activity of microsomes recovered from such control flasks represented baseline activity. It may be pointed out that such baseline activity did not differ significantly from corresponding activity of the original microsomal preparation, which was simply stored at 0–1° without additives. Ethanol, the vehicle used for introducing small quantities of CCl_4 into the flasks, had no effect on the parameters measured. Control experiments were also carried out with reference to experiments involving binding of ^{14}C from $^{14}CCl_4$. For these controls, microsomes were incubated with $^{14}CCl_4$ for up to 120 min, but without a source of NADPH. In such control systems, with no electron transport occurring over the mixed-function oxidase system, radioactivity of recovered microsomes was negligible.

RESULTS

When rat liver microsomes, supplemented with an NADPH-generating system, are incubated *in vitro* under anaerobic conditions, added CCl_4 disappears rapidly (Fig. 2). About one-third of added CCl_4 appears as $CHCl_3$ and about one-half is covalently bound (Table 1). The time course for the covalent binding is shown in Fig. 3. Of the CCl_4 covalently bound, 80–92 per cent was found in the lipids and about 18 per cent was found in the non-lipoidal microsomal residue (Table 2). Note that metabolism of CCl_4 in this system takes place in the presence of added EDTA. In this system *in vitro*, the magnitude of CCl_4 metabolism/mg of microsomal protein is considerably in excess of the rate of CCl_4 metabolism *in vivo* (see Discussion).

During the anaerobic conversion *in vitro* of CCl_4 to $CHCl_3$, if lipid peroxidation is prevented, there is no detectable diminution in microsomal cytochrome P-450 content (Fig. 4, left panel; note absence of MDA production). In an exactly equivalent incubation system which is, however, aerobic and without added EDTA, there is vigorous production of MDA, indicating lipid peroxidation, and concomitantly cytochrome P-450 content is decreased about 60 per cent (Fig. 4, right panel). An identical finding occurred with respect to G-6-Pase. During 90 min of incubation under anaerobic conditions in the presence of EDTA, although there was substantial conversion of CCl_4 to $CHCl_3$, G-6-Pase activity was essentially unaffected (Fig. 5). For the experiment reported in Fig. 5, there was no lipid peroxidation. When the same system *in vitro* is aerobic and with no added EDTA, there is vigorous lipid peroxidation and almost complete destruction of G-6-Pase (Fig. 6). Hogberg *et al.* [37] also noted an initial decline in rat liver microsomal G-6-Pase activity at a low level of lipid peroxidation (low MDA), then a return to control levels, and finally a large irreversible loss of enzyme activity at higher MDA levels.

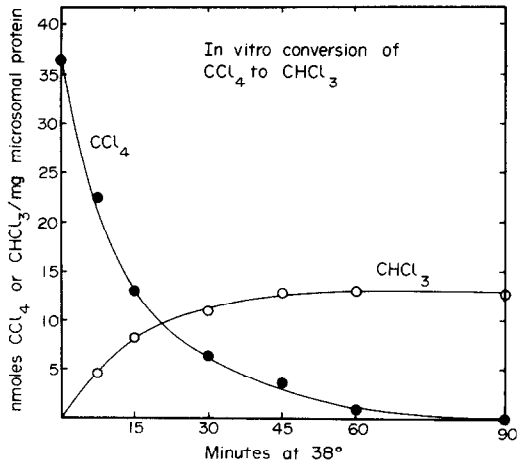

Fig. 2. Disappearance of CCl_4 and appearance of $CHCl_3$ catalyzed by rat liver microsomes *in vitro* under anaerobic conditions in the presence of EDTA. Liver microsomes were incubated at 38° in 0.15 M KCl buffered at pH 7.4 with 0.05 M Tris–maleate [36]. The final volume of 8.0 ml contained 100 mg equivalents of microsomes/ml and an NADPH-generating system [final concentrations: 0.10 mM NADP, 20 mM DL-isocitrate, 5.0 mM $MgCl_2$, 2.5 mM nicotinamide. Isocitric dehydrogenase was present at 0.02 μM units (Sigma)/ml]. Lipid peroxidation was prevented by addition of 3.0 mM EDTA, final concentration. Anaerobiosis was achieved by prior flushing of the microsome suspension with nitrogen and by flushing the flask contents with 500 ml nitrogen (reaction vessel: 25 ml Reacti-flask, Pierce Chemical Co.). After nitrogen flush, 0.08 μl CCl_4 was injected into the flask as 27 μl of 0.3% solution of CCl_4 in ethanol, v/v. Assay of CCl_4 and $CHCl_3$ was carried out by gas chromatography as indicated in Fig. 1.

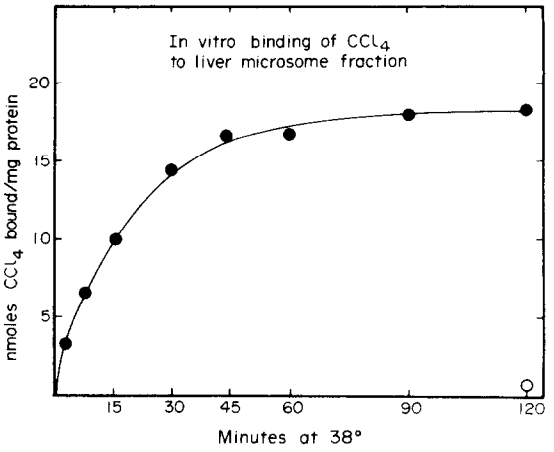

Fig. 3. Time course of covalent binding of ^{14}C during anaerobic metabolism *in vitro* of $^{14}CCl_4$ in the presence of EDTA. Incubation conditions were identical to those given in Fig. 2. The added $^{14}CCl_4$ contained 64,000 dis./min. After incubation the microsomal suspension was centrifuged at 80,000 *g* for 30 min. Details for radioactivity assay of the microsomal pellet are given in Methods.

The results given in Figs. 4 and 5 clearly show that during long incubation of rat liver microsomes at 38°, during which substantial conversion of CCl_4 to $CHCl_3$ occurs, there is no loss of either cytochrome P-450 or G-6-Pase, if lipid peroxidation is prevented. Clearly, the metabolism of CCl_4, in the sense of CCl_3—Cl bond cleavage, does not, in and of itself, affect these enzymes. However, lipid peroxidation is one of the consequences of CCl_3—Cl bond cleavage, and if this particular consequence is allowed to occur, there are large losses of cytochrome P-450 content and G-6-Pase activity (Figs. 4 and 6).

Binding of CCl_4 carbon is another consequence of CCl_4 metabolism which occurs *in vivo* in parallel with lipid peroxidation. When covalent binding of ^{14}C from $^{14}CCl_4$ metabolism occurs anaerobically *in vitro* in the presence of EDTA, during 120 min of incubation there is virtually no effect on microsomal cytochrome P-450 content (Fig. 7). As for cytochrome P-450, the entire mixed-function oxidase system (assayed as aminopyrine demethylase) appears to be indifferent toward substantial covalent binding of ^{14}C from $^{14}CCl_4$, provided lipid peroxidation is prevented (Fig. 8). However, in the same incubation system, if lipid peroxidation is allowed to occur, within the first 7–8 min there is an abrupt decline in aminopyrine demethylase activity (Fig. 9) followed by a further more gradual loss such that at 90 min residual activity is only about 25 per cent of control levels.

As for cytochrome P-450 (Fig. 7) and aminopyrine demethylase (Fig. 8), rat liver G-6-Pase is also not affected even though substantial covalent binding of ^{14}C from $^{14}CCl_4$ has occurred (Table 3).

DISCUSSION

The significance of the work presented in this study would be greatly lessened if the degree of CCl_4 metabolism and covalent binding in the system *in vitro* employed was small in comparison with CCl_4 metabolism and binding *in vivo* in the CCl_4-poisoned rat. However, rather than small, the degree of metabolism and binding *in vitro* was considerably greater than corresponding rates *in vivo*. For example, Rao and Recknagel [35] reported for an experiment *in vivo* that ^{14}C corresponding to 40 μg CCl_4 was bound into the microsomal lipids of the liver for each 100 g of

Table 1. Distribution of carbon tetrachloride metabolized *in vitro* under anaerobic conditions*

	Fraction of added CCl_4 (%)
CCl_4 metabolized	99.5
CCl_4 converted to $CHCl_3$	34.3
CCl_4 bound to whole microsomes	50.5
CCl_4 unaccounted for (by difference)	14.7

* Rat liver microsomes were incubated for 90 min at 38° under conditions described in Figs. 2 and 3.

Table 2. Distribution of bound CCl_4*

	Fraction of covalently bound CCl_4 (%)	
	Expt. 1	Expt. 2
Bound CCl_4 recovered in microsomal lipids	80.2	91.8
Bound CCl_4 recovered in non-lipoidal microsomal residue	17.5	18.1

* Experimental conditions as for Table 1. Values given are per cent of total CCl_4 bound to the unfractionated microsomal pellet.

rat body weight. This value corresponds to 2 nmoles CCl_4 bound to the lipids associated with each mg of microsomal protein. Reynolds and Ree [38] reported a somewhat lower value. As shown in Fig. 3, for the system *in vitro* used here, a total of 18 nmoles CCl_4 was bound for each mg of microsomal protein, and of that total, 80–90 per cent (see Table 3) is bound to microsomal lipids. Thus, conservatively, the system *in vitro* binds at least 14 nmoles CCl_4 to the lipids associated with each mg of microsomal protein, which is seven times greater than the corresponding rate *in vivo*.

As a further example, we estimated from data published by Garner and McLean [39] and Seawright and McLean [27] that the rate *in vivo* of conversion of CCl_4 to respiratory CO_2 varied from 0.18 to 0.27 μmole CCl_4 metabolized in 15 min for the whole liver/100 g of rat. During metabolism *in vivo* of CCl_4, 0.26 μmole CCl_4 was bound to liver microsomal lipids [35] in 15 min. Thus, it can be estimated that up to 0.53 μmole CCl_4 is metabolized *in vivo* (binding plus conversion to CO_2) in 15 min/100 g of rat. In our system *in vitro*, 0.9 μmole CCl_4 was converted to $CHCl_3$ in 15 min by liver microsomes/100 g of rat. Further, total CCl_4 disappearance is three times greater than

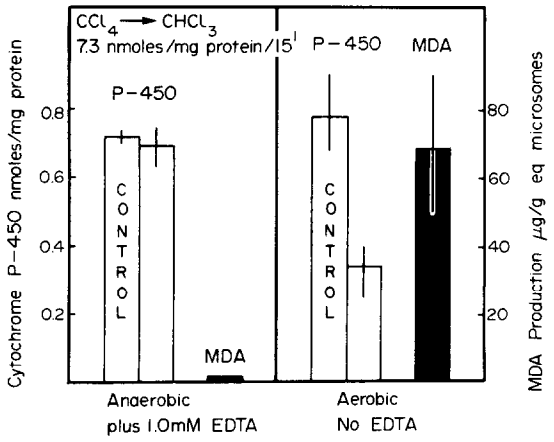

Fig. 4. Preservation of liver microsomal cytochrome P-450 content during conversion of CCl_4 to $CHCl_3$ (left panel) and loss of cytochrome P-450 when microsomal lipids peroxidize (right panel). Microsomes were incubated for 15 min at 38° in saline buffered at pH 7.4 with 0.05 M phosphate in the absence or presence of 1.0 mM EDTA as indicated. Other conditions were as described in Fig. 2 except with 0.003 μM units (Sigma) isocitric dehydrogenase/ml in the NADPH-generating system. Height of bars gives the average of two experiments (left panel) or three experiments (right panel) with the range of values indicated.

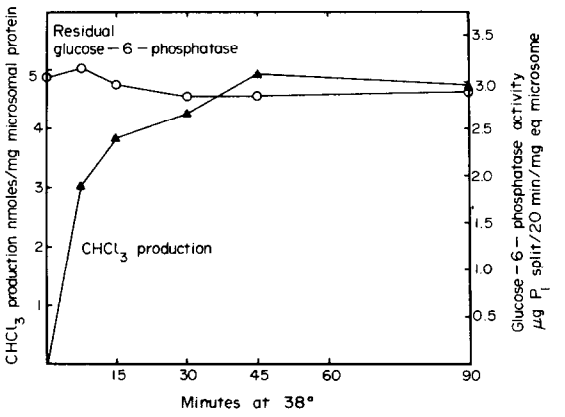

Fig. 5. Preservation of glucose 6-phosphatase activity during conversion of CCl_4 to $CHCl_3$ by rat liver microsomes. Conditions of anaerobic incubation, NADPH generation, and $CHCl_3$ assay were as described in Fig. 2. After the reaction, glucose 6-phosphatase activity of the residual microsomes (resuspended after centrifugation at 80,000 *g* for 30 min) was determined by incubation in 0.15 M KCl and 3.0 mM EDTA buffered at pH 6.6 by 0.05 M Tris–maleate. Included in the final volume of 1.6 ml for assay were 5.0 mg equivalents of microsomes/ml and 20 mM glucose 6-phosphate. After 20 min at 38° the reaction was stopped by addition of 0.5 ml of 1.5 N $HClO_4$. After centrifugation, analysis for inorganic phosphate was made according to Fiske and Subbarow [32].

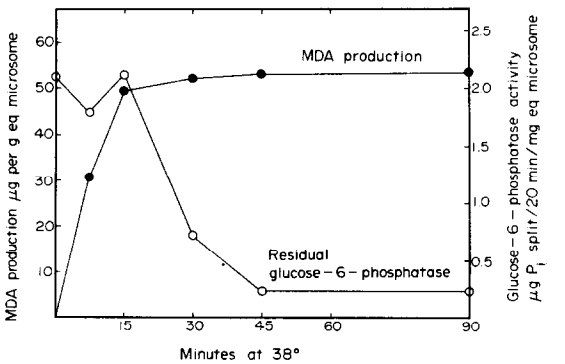

Fig. 6. Loss of glucose 6-phosphatase activity associated with rat liver microsomal lipid peroxidation. Conditions of incubation and glucose 6-phosphatase assay were as described in Fig. 5 except that the system was aerobic with no EDTA added in the initial incubation. Lipid peroxidation was enhanced by the addition of 0.40 mM $FeSO_4$, final concentration. Progress of lipid peroxidation was tracked by measuring malonic dialdehyde (MDA) production.

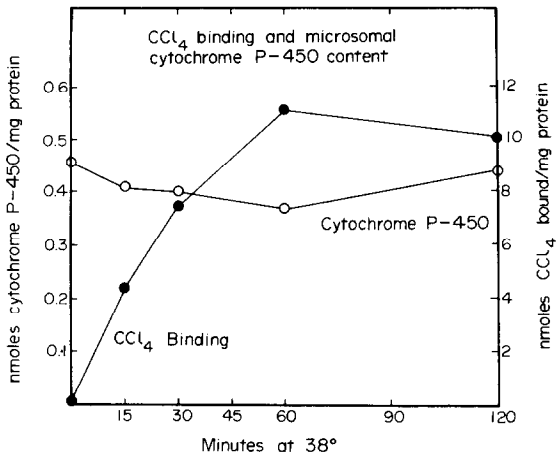

Fig. 7. Stability of rat liver microsomal cytochrome P-450 during covalent binding of ^{14}C from $^{14}CCl_4$. Microsomes were incubated under anaerobic conditions in 0.05 M phosphate buffer at pH 7.4 in the presence of 3.0 mM EDTA. Other conditions were as described in Fig. 2 except with 0.3×10^{-3} μM units (Sigma) isocitric dehydrogenase/ml in the NADPH-generating system.

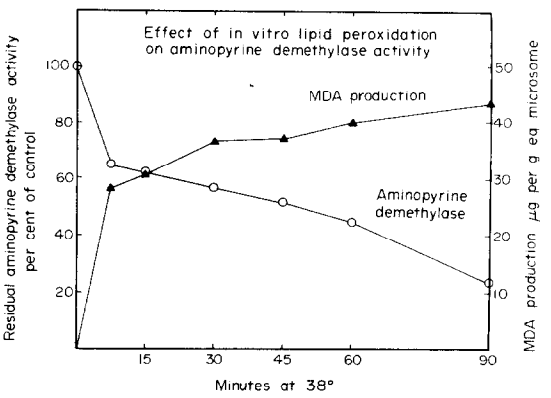

Fig. 9. Loss of rat liver microsomal aminopyrine demethylase activity associated with MDA production. Conditions of incubation and aminopyrine demethylase assay were as described in Fig. 8 except that the system was aerobic with no EDTA added in the initial incubation. Progress of lipid peroxidation was tracked by measuring MDA production. At zero time the average level of demethylase activity for control microsomes was 13.7 nmoles HCHO produced/min/mg of protein; this is shown as 100 per cent.

conversion to $CHCl_3$ (Fig. 2). Thus, the rate *in vitro* of metabolism, based on $CHCl_3$ production and binding, is 2.7 μmoles CCl_4 in 15 min for microsomes derived from the liver/100 g of rat. The rate *in vitro* of total CCl_4 metabolism is thus five times the corresponding rate *in vivo*.

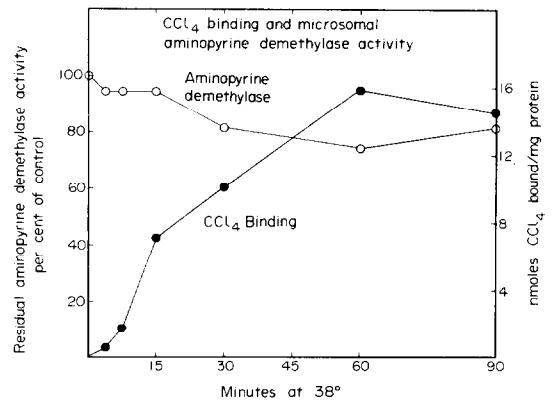

Fig. 8. Stability of rat liver microsomal aminopyrine demethylase during covalent binding of ^{14}C from $^{14}CCl_4$. Microsomes incubated under anaerobic conditions in saline buffered at pH 6.5 with 0.05 M phosphate buffer in the presence of 3.0 mM EDTA. Other conditions were as described in Fig. 2 except with 0.3×10^{-3} μM units (Sigma) isocitric dehydrogenase/ml in the NADPH-generating system. After the reaction, aminopyrine demethylase activity of the residual microsomes (resuspended after centrifugation at 80,000 *g* for 30 min) was determined by incubation in saline buffered at pH 6.5 with 0.05 M phosphate in the presence of 3.0 mM EDTA. Included in the final volume of 6.0 ml were 50 mg equivalents of microsomes/ml, 25 mM aminopyrine, and the NADPH-generating system described in Fig. 2 except with 0.03 μM units (Sigma) isocitric dehydrogenase/ml. After 30 min at 38° HCHO production was measured according to Nash [31]. At zero time the average level of demethylase activity for control microsomes was 11.2 nmoles HCHO produced/min/mg of protein; this is shown as 100 per cent.

The magnitude of CCl_4 metabolism and covalent binding makes the experiments *in vitro* reported here highly relevant with respect to a major issue under debate in the problem of intimate chemical mechanisms involved in CCl_4 liver injury. During 14 yr, from the time of Butler [17], the idea has persisted that binding of toxic metabolites of CCl_4 to cellular macromolecules was probably an important aspect of CCl_4-induced cell injury. In a critical review [3] no evidence could be found to support the original suggestion [17] that binding of toxic metabolites of CCl_4 to protein—SH groups might be involved in CCl_4-induced loss of a number of enzyme systems. It has also been pointed out [3] that the idea of covalent binding of free radical products of CCl_3—Cl bond cleavage as a mechanism of CCl_4 cellular injury suffers from a serious theoretical weakness, the essence of which is as follows. It is now widely accepted that CCl_3—Cl bond cleavage, which releases the toxicological potentiality of CCl_4, occurs in such a way that the trichlormethyl radical ($\cdot CCl_3$) is an initial product. The reaction may be an enzyme-catalyzed homolysis, in which case the monatomic chlorine free radical ($\cdot Cl$) would also be a product, or, more probably, the process occurs by enzyme-catalyzed electron capture, viz. $CCl_4 + e \rightarrow \cdot CCl_3 + Cl^-$. In either case appearance of $\cdot CCl_3$ is probably the key event. The essence of the covalent binding hypothesis is that free radical cleavage products of CCl_4 metabolism bind covalently to various cellular macromolecules, thus inducing pathological alterations in structure and function. However, in complex heterogeneous biological systems free radicals such as $\cdot CCl_3$ and $\cdot Cl$ would be expected to have extremely short half-lives. They could not migrate any significant distances from their point of origin to other loci within the cell. This restriction dictates that any covalent binding of free radical cleavage products of CCl_4 would be confined to regions very close (in molecular terms) to the locus of CCl_3—Cl bond cleavage. However, it is well

Table 3. Failure of CCl_4 covalent binding to affect rat liver microsomal glucose 6-phosphatase*

Additions to rat liver microsomes	Residual G-6-Pase (μg Pi split/20 min/ mg eq microsome)	CCl_4 bound (nmoles/ mg protein)
None	2.44	
NADPH + $^{14}CCl_4$	2.28	18.9

* Rat liver microsomes were incubated under anaerobic conditions for 60 min at 38° as described in Figs. 2 and 3. Residual glucose 6-phosphatase assay was as given in Fig. 5.

known that pathological alteration of a variety of functions of the hepatic endoplasmic reticulum occurs rapidly after CCl_4 administration to the whole animal. These functions, e.g. formation and extrusion of VLDL, protein synthesis, G-6-Pase, and the monooxygenase system involving cytochrome P-450, cannot all be concentrated at single unique molecular loci in the lipoprotein membranes of the endoplasmic reticulum. This is particularly true of protein synthesis, which is associated with polyribosomes. If pathological depression of these spatially disseminated functions is due to covalent binding of CCl_4 free radical cleavage products, then the restriction on movement of such free radical cleavage products demands that sites of CCl_3—Cl bond cleavage must be molecularly contiguous to the enzymic loci known to be susceptible to the action of CCl_4. In other words, any theory of CCl_4 toxicity which has as its central postulate the notion of covalent binding of "toxic metabolites" of CCl_3—Cl bond cleavage onto cellular macromolecules must of necessity postulate as a corollary the existence of manifold sites of CCl_3—Cl bond cleavage which co-exist spatially with an array of structurally dispersed enzymic functions. There is no evidence that such manifold sites exist. On the contrary, on the basis of evidence which has been reviewed [3] it is highly probable that CCl_3—Cl bond cleavage takes place uniquely at the cytochrome P-450 locus. If CCl_3—Cl bond cleavage takes place uniquely at the cytochrome P-450 locus, then any "covalent binding of toxic metabolites to macromolecules" theory suffers from a grave theoretical weakness since any such theory would have to postulate that the free radical products of CCl_3—Cl bond cleavage would have to survive long enough to move from their point of origin to distant sites where they would become covalently bound, and, presumably, there cause cell damage. The lipid peroxidation hypothesis [3] does not suffer from this theoretical weakness. An outstanding feature of the hypothesis is the assumption that peroxidation of microsomal lipids is the indispensable link between the initial highly localized CCl_3—Cl bond cleavage and the progressive fading away of functions far removed from the site of the cleavage. By postulating a spreading peroxidative decomposition of structural lipids within the membrane of the endoplasmic reticulum, and movement of potentially toxic lipid peroxides and hydroperoxides (or their breakdown products) to distant sites, the lipoperoxidation hypothesis can encompass, in a single theoretical framework, unique molecular loci for the CCl_3—Cl bond cleavage, the extremely short half-lives of free radical split products, and destructive action at distances far removed from the locus of the cleavage.

Mahling *et al.* [15] have offered a recent version of what we may call the "covalent binding to macromolecules" hypothesis for CCl_4-induced liver cell injury. These workers comment that free radical products of CCl_4 metabolism "...may bind covalently to the proteins and lipids of the hepatocytes. The binding of free radical derivatives to the double bonds of polyunsaturated fatty acids may initiate processes leading to diene conjugation in liver microsomal phospholipids and lipoperoxidation. Thus, the covalent binding of the free radical derivatives of CCl_4 to liver lipids and proteins presumably initiates the processes responsible for CCl_4 hepatotoxicity." In our opinion this statement places unwarranted emphasis on covalent binding of metabolic products of CCl_4 as the key toxigenic event. The mechanism they suggest would not easily allow for formation of $CHCl_3$ as a product of CCl_4 metabolism. Nor would addition of a free radical onto a double bond of a polyenoic fatty acid lead to formation of a conjugated diene configuration in that particular fatty acid. Some further process, such as formation of an intermediate hydroperoxide, with subsequent cleavage and attack on a neighboring polyenoic fatty acid would have to intervene. Furthermore, as shown in this paper, with respect to G-6-Pase, cytochrome P-450 and aminopyrine demethylase, covalent binding of ^{14}C from $^{14}CCl_4$ to microsomal lipids and proteins *in vitro* can take place to an extent seven times greater than binding *in vivo* with minimal deleterious effects on these enzyme systems. In our opinion the data presented here in Figs. 7 and 8 and in Table 3 show conclusively for these experiments *in vitro* that covalent binding of CCl_4 cleavage products is not the key toxigenic event which follows initial CCl_3—Cl bond cleavage. The hypothesis which we have proposed in a number of places [1–3, 7, 34, 35] has placed emphasis on free radical attack on methylene hydrogens of polyenoic fatty acids (which would explain $CHCl_3$ formation) followed by autocatalytic peroxidative decomposition of the lipids as the key destructive event. The data presented in this paper strongly support the view that lipid peroxidation must intervene between initial CCl_3—Cl bond cleavage and loss of G-6-Pase, cytochrome P-450 and aminopyrine demethylase.

In conclusion, it is clear on the basis of the evidence presented in this paper that losses of liver microsomal G-6-Pase, cytochrome P-450 and aminopyrine demethylase *in vitro* are critically dependent on lipid peroxidation and not on covalent binding of split products of CCl_4 metabolism. However, it is not yet

possible to generalize this conclusion to the entire panorama of pathological consequences of CCl_4 poisoning in the whole animal. Nevertheless, on the basis of the theoretical argument presented here, it seems highly likely that microsomal lipid peroxidation is an obligatory intermediate link which stands between a highly localized initial CCl_3—Cl bond cleavage and unfolding of the well-known pathological consequences of CCl_4 poisoning in the animal as a whole.

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