*Biochem. J.* (1984) **223**, 577–586
*Printed in Great Britain*



# Binding of trichloromethyl radicals to lipids of the hepatic endoplasmic reticulum during tetrachloromethane metabolism

Bernhard LINK, Heinz DÜRK, Dietmar THIEL and Hartmut FRANK*
*Institut für Toxikologie, Wilhelmstrasse 56, D-7400 Tübingen 1, Federal Republic of Germany*

(*Received 20 February 1984/Accepted 10 July 1984*)

Metabolism of tetrachloromethane (carbon tetrachloride) by liver microsomal fraction under anaerobic conditions and *in vivo* leads to covalent binding of trichloromethyl radicals to lipids. The resulting covalently modified lipids contain two different types of fatty acids: a group of monomeric trichloromethyl fatty acid residues, usually with one double bond less than the precursor fatty acids, and a group of fatty acids that are not sufficiently volatile for gas chromatography. The liquid-chromatographic properties of the latter indicate high molecular mass, presumably due to cross-linking. The chemical structures of the monomeric fatty acids were elucidated, and these support the view that the most significant reactive metabolite of tetrachloromethane is the trichloromethyl radical. The isomer patterns of the monomeric trichloromethyl fatty acids *in vitro* and *in vivo* are almost identical, which shows that anaerobic incubation of tetrachloromethane with microsomal fraction very well reflects the processes involved in hepatotoxicity of tetrachloromethane *in vivo*.

The obligatory primary step towards the hepatotoxic manifestation elicited by tetrachloromethane (carbon tetrachloride) is the metabolic formation of trichloromethyl radicals (Butler, 1961; Recknagel & Ghoshal, 1966; Slater, 1966). The mechanism has been elucidated (Ahr *et al.*, 1980; Kubic & Anders, 1981), and the involvement of radicals has been proven directly by spin-trapping experiments (Poyer *et al.*, 1980). However, the molecular causes for the ensuing morphological and pathological degenerations are still obscure; *in vivo* metabolism of tetrachloromethane ceases within a few hours after application of the compound (Dürk & Frank, 1984), whereas the degeneration towards necrosis develops in the course of about 1 day. One or several deleterious processes are initiated that develop spontaneously and that escape the regulating or restoring activities of the cells.

A widely accepted hypothesis is that trichloromethyl radicals trigger a chain reaction, which is propagated by oxygen and involves transient formation of lipid hydroperoxides, leading to massive oxidative destruction of polyunsaturated fatty acids of the endoplasmic reticulum (Recknagel & Ghoshal, 1966; Recknagel *et al.*, 1977). The mechanism, usually denoted as 'lipid peroxidation', is founded on experiments performed *in vitro* with hepatic microsomal fraction under normobaric oxygen. The oxygen partial pressure in the liver cells is certainly considerably lower, especially in the pericentral region of the liver lobule, where the damaging effect is most pronounced; the peroxidative degradation of lipids is strongly diminished at low oxygen pressure (Kieczka & Kappus, 1980). Moreover, exhalation of alkanes, considered as undisputable proof of peroxidative degradation of polyunsaturated fatty acids *in vivo*, comes to an end almost immediately after termination of the metabolic formation of trichloromethyl radicals (Dürk & Frank, 1984). The reaction of 'lipid peroxidation' may be significant during metabolism, but not beyond this stage.

A consequence of tetrachloromethane metabolism is strong covalent binding of reactive intermediates to biomolecules, and most typically to lipids (Reynolds, 1967; Gordis, 1969; Diaz Gomez *et al.*, 1973). A fraction of the modified lipids is resistant towards cleavage by phospholipase $A_2$ (Frank & Link, 1984); these are likely to play a critical role in the development towards liver cell necrosis. We have now isolated and further

Abbreviations used: h.p.l.c., high-performance liquid chromatography; g.c.–m.s., gas chromatography–mass spectrometry; e.c.d., electron-capture detection.

* To whom correspondence should be addressed.

characterized a major part of the covalently modified lipids. Their chemical structures corroborate that the trichloromethyl radical is an important reactive metabolite; further conclusions on the pathway leading to cell death are drawn.

## Experimental

### Animals

Male Sprague–Dawley rats (Ivanovas, Kisslegg, Germany) had been fed on a standard diet (Altromin, Lage, Germany) and tap water *ad libitum*. For experiments performed *in vivo*, animals with body weights of 160–200g were used. Microsomes for experiments performed *in vitro* were prepared from animals with body weights of 300–500g that had been pretreated with an intraperitoneal injection of 80mg of phenobarbital (sodium salt)/kg and with 0.1% phenobarbital in the drinking water for the following 5 days.

### Chemicals and biochemicals

$BF_3$ [14% (w/v) in methanol] and DL-isocitric acid (sodium salt) (98%, w/w) were from Serva (Heidelberg, Germany); $NADP^+$ (disodium salt) was from Boehringer (Mannheim, Germany); platinum dioxide was from Ventron (Karlsruhe, Germany); all other chemicals were of analytical grade from E. Merck (Darmstadt, Germany) and Riedel de Haen (Seelze, Germany). Tetrachloro-[$^{14}C$]methane was from Amersham Buchler (Braunschweig, Germany), with a specific radioactivity of 26.9Ci/mol and a radiochemical purity of 99%. The material was transferred by isothermal distillation into a conical test tube containing 200μl of ethanol cooled with liquid $N_2$. The test tube was sealed with a spring-tightened ground-glass stopper; starch/glycerol grease was used for the stopper. This solution was diluted with non-labelled tetrachloromethane in ethanol (10%, v/v) to yield 552nmol of tetrachloromethane (1.0μCi)/μl, specific radioactivity 1.81 Ci/mol.

### Experiments performed in vitro

*Isolation of microsomal fraction.* Microsomal fraction (referred to below simply as 'microsomes') was prepared by the procedure of Remmer *et al.* (1967). The microsomal pellet was suspended in 50mM-Tris/HCl buffer, pH7.4, containing 120mM-KCl, centrifuged at 100000*g* for 50min and resuspended in the same buffer. Microsomal protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Cytochrome *P*-450 was measured by the method of Omura & Sato (1964) and found to be 1.8nmol/mg of protein.

*Incubation of microsomes with tetrachloro[$^{14}C$]-methane.* Microsomes were incubated with tetrachloro[$^{14}C$]methane in a 15ml-volume flask with two stop-cock connections for $N_2$-purging and a Quickfit connection with polytetrafluoroethylene-laminated septum for addition of tetrachloromethane and $NADP^+$. An ice-cold solution (10.25ml), pH7.4, containing 50mM-Tris/HCl, 120mM-KCl, 5mM-$MgCl_2$, 8mM-sodium isocitrate, isocitrate dehydrogenase (50μg/ml) and liver microsomes (3mg of protein/ml) was purged with $N_2$ for 30min. $NADP^+$ (10μmol in 100μl of $N_2$-purged incubation buffer) was added, the stopcocks were closed and 11μmol of tetrachloro[$^{14}C$]-methane (radioactivity 1.82Ci/mol, total 20μCi) dissolved in 20μl of ethanol was injected. The flask was then incubated at 37°C for 60min.

The reaction was stopped by cooling to 0°C; volatile metabolites were removed by purging with $N_2$ and collected in a trap cooled with liquid $N_2$.

*Extraction of microsomal lipids.* 2,6-Di-t-butyl-4-methylphenol (10mg) was added to the incubation mixture, the lipids were extracted with 150ml of methanol plus 320ml of trichloromethane (chloroform) and the solution was filtered through a glass-fibre filter. Polar substances were removed by addition of 1g of Sephadex G-25 (coarse grade) and subsequent filtration. The solvent was evaporated and lipids were dissolved in 9ml of trichloromethane.

*Preparation of fatty acid methyl esters.* Half of the extracted lipid material was subjected to trans-esterification, as follows. After evaporation of the solvent under $N_2$, the residue was dissolved in 2ml of 14% (w/v) $BF_3$ in methanol and kept for 1h at 100°C. After the mixture had cooled, 1ml of water was added and the fatty acid methyl esters were extracted with three 2ml portions of n-hexane.

*Catalytic hydrogenation and acidolysis.* The fatty acid methyl esters were dissolved in 2ml of ethanol, a small quantity of $PtO_2$ was added and $H_2$ was introduced for 2h. After this period the catalyst was sedimented by centrifugation and the solution was transferred with a pipette to another vial. For acidolysis of trichloromethyl groups to carboxy groups, the fatty acids are heated with 100μl of 93% (w/w) $H_2SO_4$ at 80°C for 20min. The acid mixture was poured on to 2g of ice, and the aqueous solution was extracted with dichloromethane. After removal of solvent, carboxy groups were esterified with diazomethane in methanol/diethyl ether (1:2, v/v) for 20min at room temperature.

*T.l.c.* Samples of the hexane extract were applied to thin-layer plates that had been activated at 120°C for 1h before use (precoated plates Silica Gel 60, 5cm × 20cm; Merck). The plates were developed with n-hexane/diethyl ether/acetic acid (80:20:1.5, by vol.). Labelled compounds were

detected with a t.l.c. radioactivity scanner (Berthold, Wildbad, Germany). After charring with copper acetate reagent (Fewster *et al.*, 1969), spots were detected by scanning the plate with a t.l.c. densitometer (Zeiss, Oberkochen, Germany) at 600nm. In analogous experiments, the spots detected by radioactivity scanning were scraped from the plate and eluted with methanol/water (6:1, v/v) for scintillation counting. Quantitative determination of radioactivity was done as described previously (Frank & Link, 1984).

*H.p.l.c.* Samples of the hexane extract were separated by h.p.l.c. on a reversed-phase column (LiChrosorb RP-18, 10μm; 25cm × 0.4cm) guarded with a precolumn (Perisorb RP-18, 30–40μm; 3cm × 0.4cm). Samples were injected with an autosampler (WISP; Waters Associates), the mobile phase was methanol/water (23:2, v/v), and the flow rate was 1ml/min. After 50min n-hexane/propan-2-ol (2:1, v/v) was used as eluent. The absorbance of the eluate at 206nm was monitored; fractions of volume 1 or 3ml were collected.

*G.l.c.* H.p.l.c. fractions were analysed by g.l.c. on a glass capillary (50m × 0.25mm) coated with OV-101. $H_2$ was used as carrier gas at an inlet pressure of 100kPa. Injector and detector temperatures were 300°C. Samples were injected in the split-less mode; the split was opened after 30s. The oven temperature programme was as follows: 1min isothermal at 60°C, to 180°C at a rate of 40°C/min, 2min isothermal, to 280°C at a rate of 4°C/min. For selective detection of the adducts of tetrachloromethane metabolites to unsaturated fatty acids, an electron-capture detector with $^{63}Ni$ as radioactive β-emitting source (model HT-25; Carlo Erba) was used. Conditions for e.c.d. analysis were as follows: temperature of the electron-capture detector, 300°C; make-up gas argon/methane (9:1) with a flow rate of 30ml/min; excitation, constant current mode; voltage amplitude 40mV; pre-set pulse duration 0.5μs; under these conditions the standing current was 2.6nA.

*G.c.–m.s.* The instrument employed was a Finnigan 4021 with Incos data system. The g.c. conditions were as follows. Helium was used as carrier gas, inlet pressure 100 kPa; the samples were injected in the split-less mode (injector temperature 280°C) on to a glass capillary (20m × 0.25mm) coated with OV-101; after 0.5min the split-valve was opened; oven temperature was programmed as follows: isothermal for 0.5min at 60°C, to 170°C at a rate of 40°C/min, then at a rate of 3°C/min to 200°C, and at a rate of 15°C/min to 300°C. The interface was a silane-treated fused-silica capillary (40cm × 0.1mm) maintained at 290°C.

The m.s. conditions were as follows. The ion-source temperature was 290°C, and the secondary electron-multiplier voltage was 1.5kV. Chlorine-containing fatty acid methyl esters were selectively monitored by negative chemical ionization with methane (40Pa) as reagent gas. Scans from $m/z$ 32 to 40 were taken every second; electron energy was 100eV; mass fragmentograms of $m/z$ 35 and $m/z$ 37 ($Cl^-$) were reconstructed.

The chlorinated fatty acid methyl esters were identified by electron-impact ionization (electron energy 35eV) and chemical ionization (electron energy 100eV) with isobutane and ammonia as reagent gases (32Pa) respectively. Scans over the mass ranges from 50 to 550 (electron-impact ionization) or 200 to 550 (chemical ionization) were taken at rates of one scan/s.

*Experiments performed in vivo*

The animals were placed in a desiccator as described by Bolt *et al.* (1976), modified and equipped with a sampling system for automatic g.c. determination of tetrachloromethane and alkanes (Frank & Dürk, 1983; Dürk & Frank, 1984). The exposure system had a volume of about 1 litre and was occupied by a rat with a body weight of 200g. At the beginning of the experiment, 10μl of tetrachloromethane was injected, corresponding to an initial concentration of about 130μmol/l.

During equilibration for 3h the atmospheric concentration decreased to 20μmol/l, corresponding to an uptake of about 80mg/kg. At this time the $O_2$ partial pressure was decreased from 200mbar to 65 ± 7mbar (Dürk & Frank, 1984). After an additional 3h the animals were killed and the livers were removed. Preparation of microsomes, extraction of lipids and analysis of fatty acid methyl esters were done as described above.

## Results and discussion

Anaerobic incubation of microsomes from phenobarbital-treated rats with NADPH and tetrachloro[$^{14}C$]methane led to covalent binding of 16% of the total radioactivity to lipids. Calculations of the specific binding of reactive metabolites to lipids yielded an average value of 5mol% (Frank & Link, 1984).

Separation of the fatty acid methyl esters by capillary g.l.c. in combination with e.c.d. yielded chromatograms with several peaks (Fig. 1*a*). Since the detector exhibits high and selective sensitivity towards halogen-containing compounds, most of these peaks represent fatty acids with covalently bonded tetrachloromethane metabolites. There is some resemblance to the g.l.c. chromatograms as published by Bendetti *et al.* (1977), namely that

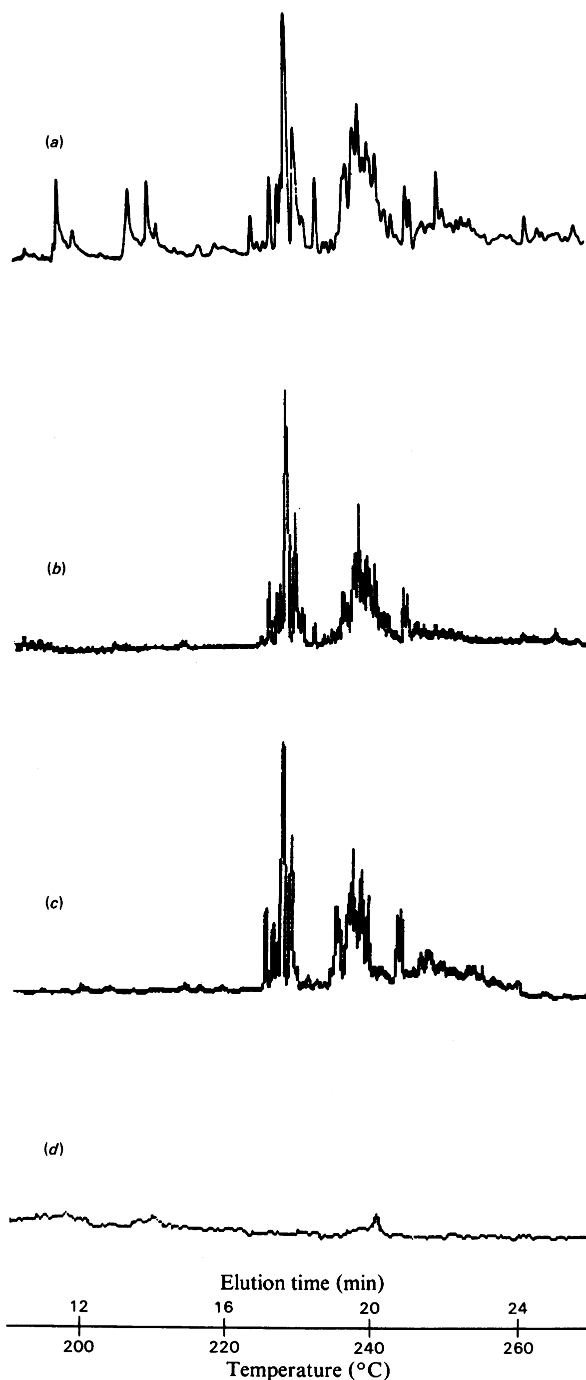



Fig. 1. *G.l.c. of trichloromethyl fatty acid methyl esters*
Fatty acid methyl esters were prepared by transesterification of lipids from anaerobic microsomal incubations (*a* and *b*) or rat liver microsomes from an '*in-vivo*' experiment (*c*) with tetrachloromethane; chlorine-containing fatty acids were monitored by e.c.d. (*a*) or selected ion detection of *m/z* 35 (*b*, *c* and *d*); (*d*) represents an '*in-vivo*' control experiment without tetrachloromethane. For full experimental details see the text.

two clusters are found; as we have employed more-efficient capillaries instead of a packed column, distinct peaks are registered.

A detection mode that is more selective for chlorine-containing compounds is negative chemical-ionization mass spectrometry with monitoring of the masses *m/z* 35 or *m/z* 37 (Fig. 1*b*). The chromatograms were considerably cleaner; a number of peaks in the g.l.c.–e.c.d. chromatograms apparently do not contain chlorine.

The lipids extracted from microsomes of animals exposed to non-labelled tetrachloromethane were transesterified and analysed in the same manner, yielding a very similar peak pattern to that observed for the experiments performed *in vitro* (Fig. 1*c*). As expected, no chlorine-containing fatty acids could be detected in the microsomal lipids from control animals (Fig. 1*d*).

Since the chromatograms were relatively complex, the fatty acid methyl esters from the experiment performed *in vitro* with tetrachloro[$^{14}$C]-methane were fractionated by reversed-phase h.p.l.c. The effluent was collected and the radioactivity in each fraction was determined by liquid-scintillation counting (Fig. 2). Most of the radioactivity was found at positions different from those of normal fatty acids. Equal-sized portions of each fraction were taken and analysed by g.l.c. and e.c.d. A typical example is the gas chromatogram obtained from fraction 24 (Fig. 3).

The h.p.l.c. fractions were used for identification of individual compounds by g.c.–m.s. The mass spectra of the first major g.l.c. peak in fraction 24 are shown in Fig. 4. Although the electron-impact-ionization spectrum (not shown) was not informative, the chemical-ionization mass spectrum with isobutane (Fig. 4*a*) displayed a conspicuous quasi-molecular ion $[M\mathrm{H}]^+$ (*m/z* 413/415) and a fragmentation pattern resulting from consecutive loss of three HCl fragments with and without re-addition of hydrogen. The mass of the quasi-molecular ion and the fragmentation sequence indicated that this compound is an isomer of trichloromethyloctadecenoic acid methyl ester. This was confirmed by the chemical-ionization mass spectrum with ammonia as reagent gas (Fig. 4*b*): the quasi-molecular ion $[M+\mathrm{NH}_4]^+$ (*m/z* 430/432) was even more abundant and an analogous fragmentation (430/432 → 394/396/398 → 358/360/362 → 322/324/326) was observed.

Hydrogenation of double bonds and acidolysis of trichloromethyl groups to carboxy groups yielded the corresponding alkanedioic acids. Fig. 4(*c*) shows the electron-impact-ionization mass spectrum of a mixture of nonadecanedioic acid methyl ester isomers. No molecular ion (*m/z* 356) was found, but fragments arising from sequential loss of a methyl radical (*m/z* 341), $CO_2$ (*m/z* 297) and methanol (*m/z* 265) were observed. The conspicuous even-electron ions from McLafferty re-arrangement (Scheme 1) indicated the branching position ($C^9$: *m/z* 200, 230; $C^{10}$: *m/z* 186, 244); since the g.l.c. separation of different alkanedioic

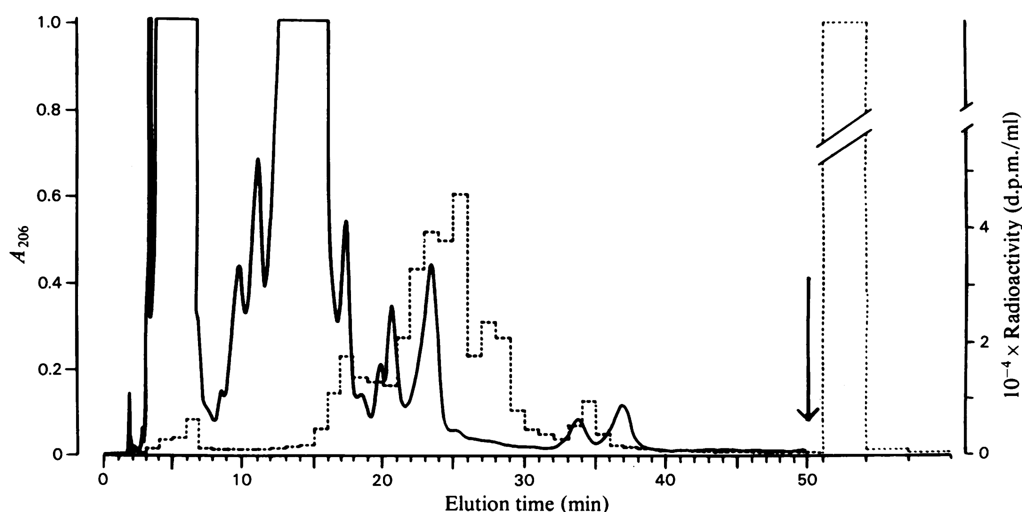



Fig. 2. *H.p.l.c. of fatty acid methyl esters*

Fatty acid methyl esters were prepared by transesterification of lipids extracted from anaerobic microsomal incubation with tetrachloro[$^{14}$C]methane and NADPH. ——, $A_{260}$; ·····, radioactivity of collected fractions. The arrow indicates the change to the less-polar eluent n-hexane/propan-2-ol. For full experimental details see the text.

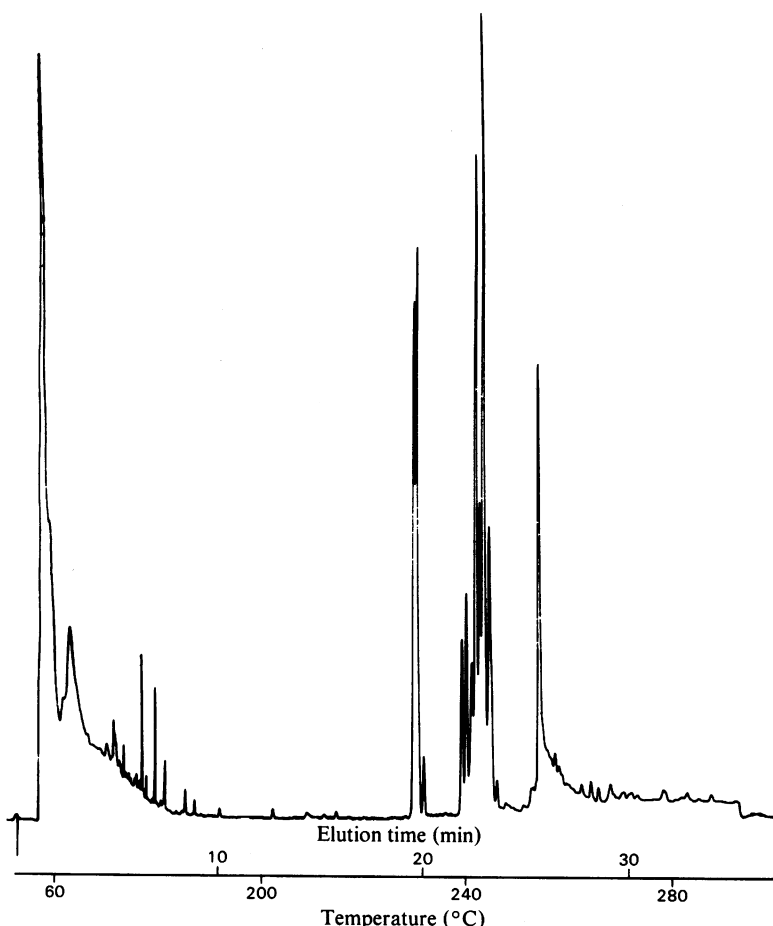



Fig. 3. *G.l.c.–e.c.d. of h.p.l.c. fraction 24*
H.p.l.c.-purified fatty acid methyl esters were from a microsomal incubation with tetrachloromethane. The peaks eluted after 25 min are plasticizers. For full experimental details see the text.

acid methyl esters is only slight, it was difficult to obtain mass spectra of pure isomers. The formation of the derivatives requires relatively large amounts of sample, and, as the exact location of the trichloromethyl group in each isomer from the biological point of view may be irrelevant, we did not investigate this aspect further.

The chemical-ionization mass spectra of the other chlorine-containing fatty acids showed that the first peak cluster in Fig. 1(*b*) or 1(*c*) represented mainly the isomers of trichloromethyloctadecenoic acid methyl ester, and the second cluster the isomers of trichloromethyleicosatrienoic acid methyl ester. Obviously, a considerable fraction of trichloromethyl fatty acid radicals formed on addition of trichloromethyl radicals to unsaturated fatty acids was stabilized by the addition of a hydrogen atom (Scheme 2). A small amount of trichloromethyloctadecadienoic acid was found, presumably arising from elimination of a hydrogen atom.

The direct abstraction of hydrogen from bisallylic methylene groups to form resonance-stabilized pentadienyl radicals, as also shown in Scheme 2, is often considered as the most important primary reaction between trichloromethyl radicals and membrane lipids; mechanistically this is reasonable, but so far there is only indirect evidence for it. The first metabolite of tetrachloromethane, trichloromethane, is probably formed via such a reaction (Slater, 1972).

Combination of h.p.l.c. and g.c.–m.s. analysis afforded identification of most of the monomeric trichloromethyl fatty acids (Fig. 5). Some minor peaks are unidentified as yet, but more than 90% of the monomeric trichloromethyl fatty acids were

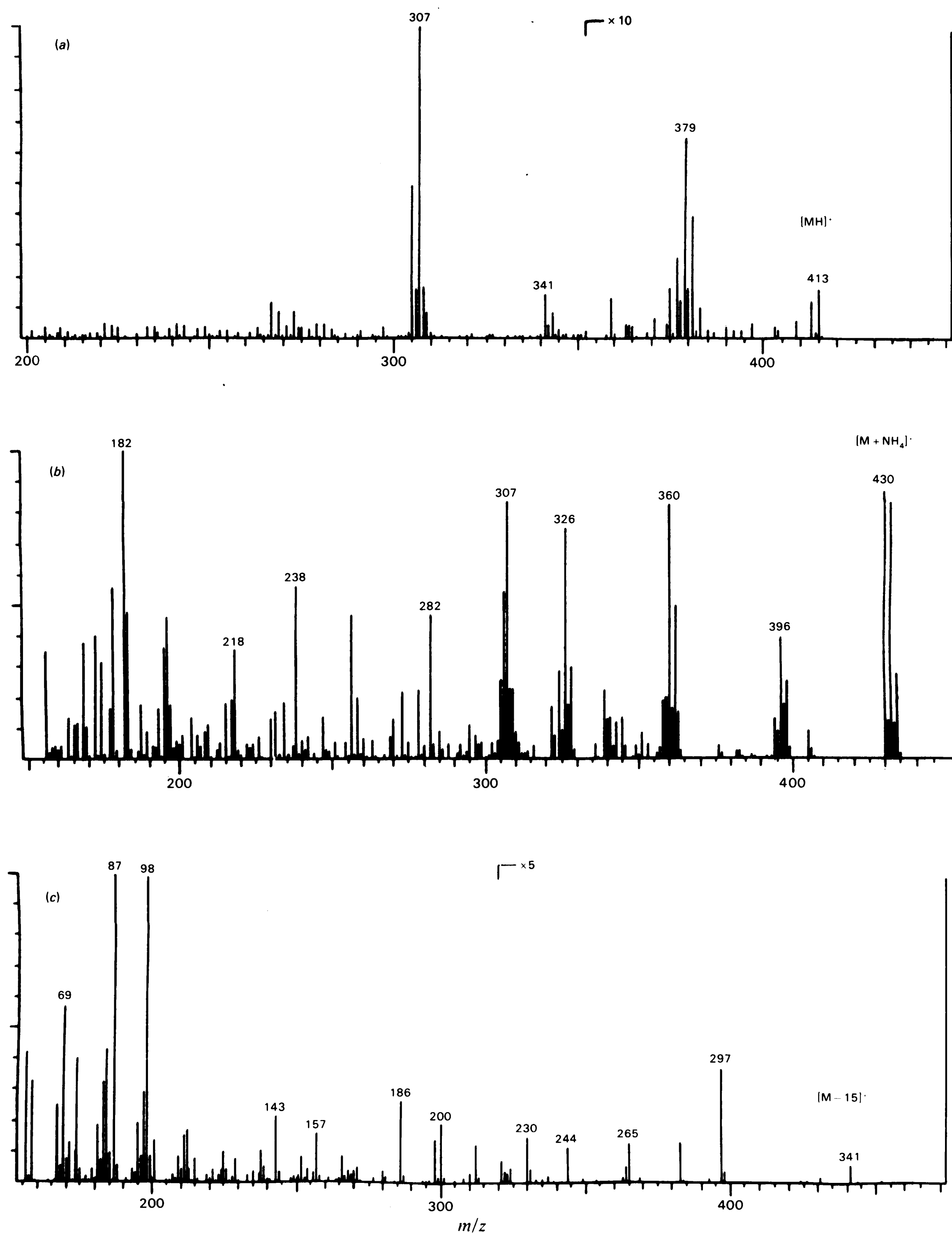
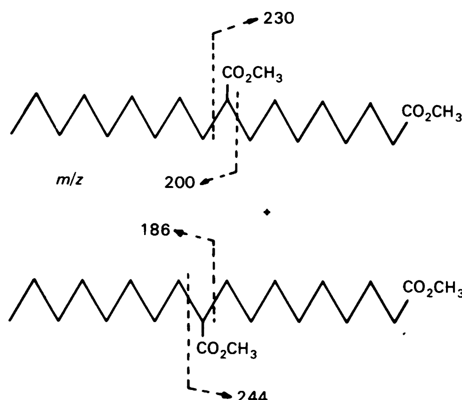



Fig. 4. *Mass spectra of trichloromethyloctadecenoic acid methyl ester*

The microsomal fatty acids are from an anaerobic incubation with tetrachloromethane. (*a*) Chemical-ionization spectrum, reagent gas isobutane; (*b*) chemical-ionization spectrum, reagent gas ammonia; (*c*) electron-impact-ionization spectrum after hydrogenation, acid hydrolysis and re-esterification. For full experimental details see the text.

products arising from addition of the radical to linoleic acid, which yielded trichloromethyl-octadecenoic acid, and to arachidonic acid to yield trichloromethyleicosatrienoic acid.

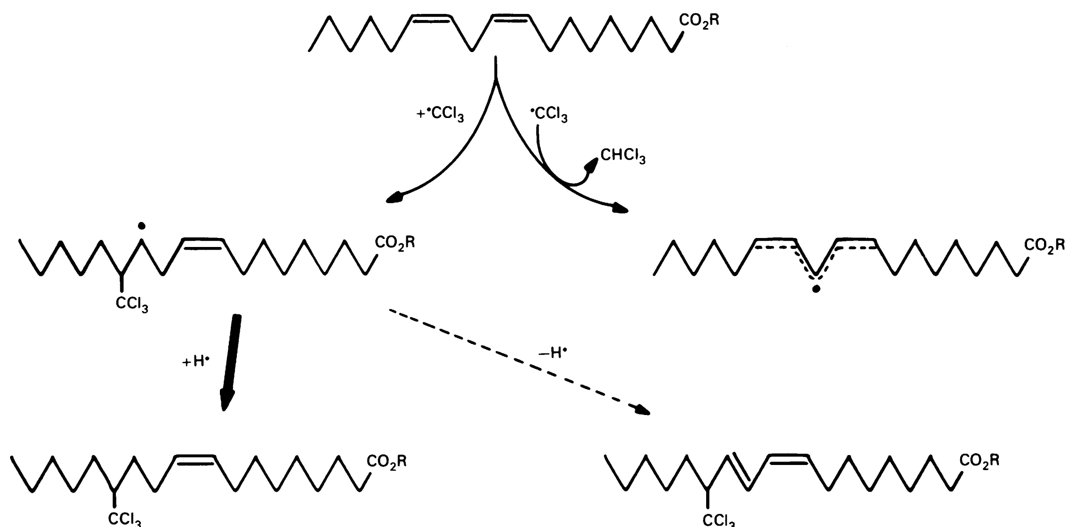
The relative elution volumes in reversed-phase h.p.l.c. supported the identification based upon g.c.–m.s.; retention volumes increased with the number of carbon atoms and decreased with the number of double bonds. Interestingly, relative to the losses of the corresponding polyunsaturated fatty acids after tetrachloromethane metabolism, the yields of trichloromethyl fatty acids were smaller with increasing number of double bonds; it is reasonable to assume that the greater unsaturation facilitated secondary reactions leading to other end products.




Scheme 1. *McLafferty fragments of the electron-impact mass spectra of nonadecanedioic acid methyl esters formed from 9- and 10-trichloromethyloctadecanoic acid by acid hydrolysis and esterification*

The involvement of covalently modified phospholipids in the pathogenicity of tetrachloromethane has been recognized before (Reynolds, 1967; Gordis, 1969; Villarruel *et al.*, 1975; Benedetti *et al.*, 1977). Their experiments indicated indirectly that the group attached to the lipids is a trichloromethyl radical. This was further supported by the findings obtained by Ansari *et al.* (1982), who tentatively identified by selected ion monitoring a product of trichloromethyl-radical addition to cholesterol. Binding products of trichloromethyl radicals have also been found in a reconstituted system containing cytochrome *P*-450, dioleoyl phosphatidylcholine, NADPH–cytochrome *P*-450 reductase, cytochrome $b_5$ and tetrachloromethane by Trudell *et al.* (1982). They identified the binding product to oleic acid as trichloromethyl-octadecanoic acid.

It must be expected that both types of fatty acid radicals, formed on reaction with the trichloromethyl radical via addition or hydrogen abstraction (Scheme 2), initiate secondary reactions that propagate the radical chain, e.g. addition of oxygen to form peroxy radicals or addition to a double bond of a neighbouring polyunsaturated fatty acid leading to cross-linkage (Scheme 3); this could proceed for several cycles. Also, terminating reactions can be envisaged such as transfer of a hydrogen atom from an endogenous donor, e.g. vitamin E, or dimerization with a relatively stable long-lived radical, possibly a pentadienyl or peroxy




Scheme 2. *Main reactions of trichloromethyl radicals with polyunsaturated fatty acids in vitro and in vivo*

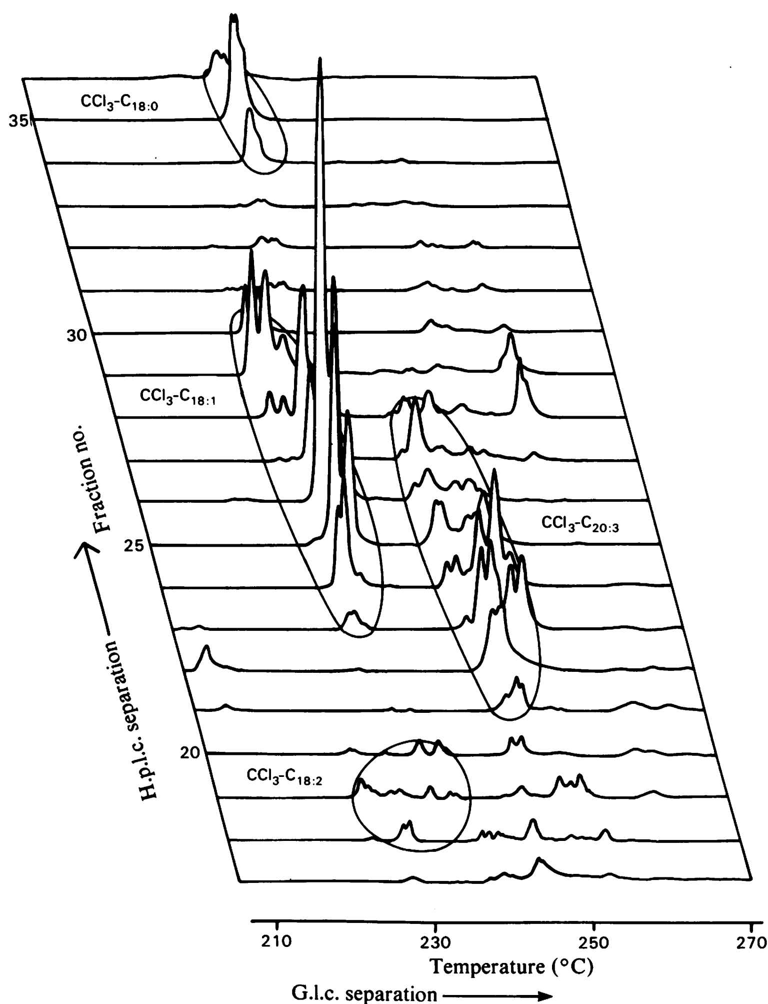
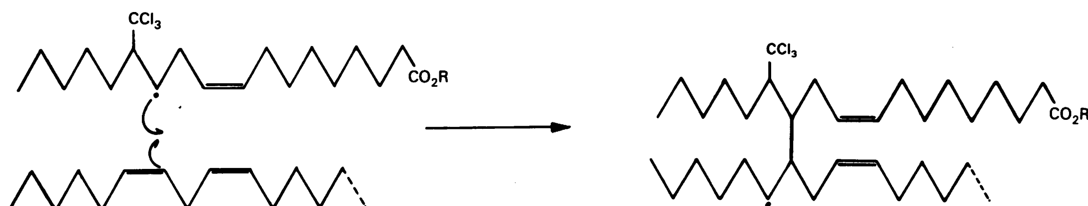



Fig. 5. *H.p.l.c. and g.l.c. of trichloromethyl fatty acid methyl esters*
Chlorine-containing fatty acid methyl esters from the h.p.l.c. fractions were detected by negative chemical-ionization mass spectrometry ($m/z$ 35) and are depicted in their relative quantities and their chromatographic properties in a three-dimensional array. For full experimental details see the text.




Scheme 3. *Hypothetical reaction of a secondary trichloromethyl fatty acid radical with a neighbouring unsaturated fatty acid, leading to cross-linkage*

radical; however, it must be pointed out here that the relative significance of all these and other possibilities depends on many unknown parameters, such as absolute and relative reactivities and concentrations of all potential reactants; it would be highly speculative to make suggestions in

this general direction before the main end products of the reactions causing the loss of polyunsaturated fatty acids are identified and quantitatively determined.

Nevertheless, the well-known fact of profound losses of polyunsaturated fatty acids and impaired membrane function after tetrachloromethane metabolism suggest a dominant role of cross-linking. We also found evidence for the formation of cross-linked lipids.

The h.p.l.c. fractions collected during elution with methanol/water contained 50% of the applied radioactivity (Fig. 2). The rest was eluted with hexane/propan-2-ol, representing the other half of the applied radioactivity. G.c.–m.s. with this material was unsuccessful, presumably owing to its low volatility or its high molecular mass. T.l.c. of the mixture obtained directly after transesterification of the microsomal lipids indicated the same; besides the spot for the normal fatty acid methyl esters, several labelled spots with smaller $R_F$ values were found, containing 50% of the applied radioactivity. They most probably represent cross-linked fatty acids with an increasing extent of oligomerization.

Cross-linking of membrane lipids should be greatly detrimental to the cell; the strong inhibitory effect of decreased membrane fluidity on certain $Ca^{2+}$-dependent ATPases is well known (Seelig & Hasselbach, 1971). This is noteworthy in view of the findings by Moore *et al.* (1976) that the endoplasmic $Ca^{2+}$-dependent ATPase is inhibited almost immediately after exposure to tetrachloromethane. But other integral membrane enzymes are also already affected at this early stage, for example enzymes involved in protein synthesis (Smuckler & Benditt, 1965) and phospholipid synthesis (Halbreich & Mager, 1969). These observations, and the resistance of cross-linked phospholipids towards phospholipase $A_2$ (Frank & Link, 1984), make cell death seem to be almost inevitable. The cross-linkage of membrane lipids, the aggravating effect of losses of polyunsaturated fatty acids and the ensuing inhibition of enzymes vitally important for the maintenance of cellular ion gradients and/or for the repair of these disturbances of membrane structure and function could explain the seemingly inescapable destiny of the injured cells. However, extensive experimental investigations are still required before such a hypothesis can be firmly established. Especially, the fate of the polyunsaturated fatty acids disappearing during tetrachloromethane intoxications needs to be elucidated in order to follow the sequence of chemical, biochemical and cellular reactions leading to necrosis.

We thank Mrs. E. Steinhilber for secretarial help. Financial support of the Deutsche Forschungsgemeinschaft is acknowledged.

## References

Ahr, H. J., King, L. J., Nastainczyk, W. & Ullrich, V. (1980) *Biochem. Pharmacol.* **29**, 2855–2861

Ansari, G. A. S., Moslen, M. T. & Reynolds, E. S. (1982) *Biochem. Pharmacol.* **31**, 3509–3510

Benedetti, A., Casini, A. F., Ferrali, M. & Comporti, M. (1977) *Chem.-Biol. Interact.* **17**, 151–166

Bolt, H. M., Kappus, H., Buchter, A. & Bolt, W. (1976) *Arch. Toxicol.* **35**, 153–162

Butler, T. C. (1961) *J. Pharmacol. Exp. Ther.* **134**, 311–319

Diaz Gomez, M. I., Castro, J. A., de Ferreyra, E. C., D'Acosta, N. & de Castro, C. R. (1973) *Toxicol. Appl. Pharmacol.* **25**, 534–541

Dürk, H. & Frank, H. (1984) *Toxicology* **30**, 249–257

Fewster, M. E., Burns, B. J. & Mead, J. F. (1969) *J. Chromatogr.* **43**, 120–126

Frank, H. & Dürk, H. (1983) *Arch. Toxicol.* **53**, 213–223

Frank, H. & Link, B. (1984) *Biochem. Pharmacol.* **33**, 1127–1130

Gordis, E. (1969) *J. Clin. Invest.* **48**, 203–208

Halbreich, A. & Mager, J. (1969) *Biochim. Biophys. Acta* **187**, 584–587

Kieczka, H. & Kappus, H. (1980) *Toxicol. Lett.* **5**, 191–196

Kubic, V. L. & Anders, M. W. (1981) *Chem.-Biol. Interact.* **34**, 201–207

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Moore, L., Davenport, G. R. & Landon, E. J. (1976) *J. Biol. Chem.* **251**, 1197–1201

Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2370–2378

Poyer, J. L., McCay, P. B., Lai, E. K., Janzen, E. G. & Davis, E. R. (1980) *Biochem. Biophys. Res. Commun.* **94**, 1154–1160

Recknagel, R. O. & Ghoshal, A. K. (1966) *Lab. Invest.* **15**, 132–146

Recknagel, R. O., Glende, E. A. & Hruszkewycz, A. M. (1977) in *Free Radicals in Biology* (Prior, W. A., ed.), vol. 3, pp. 97–132, Academic Press, New York

Remmer, H., Greim, H., Schenkman, J. B. & Estabrook, R. W. (1967) *Methods Enzymol.* **10**, 703–708

Reynolds, E. S. (1967) *J. Pharmacol. Exp. Ther.* **155**, 117–126

Seelig, J. & Hasselbach, W. (1971) *Eur. J. Biochem.* **21**, 17–21

Slater, T. F. (1966) *Nature (London)* **209**, 36–40

Slater, T. F. (1972) in *Free Radical Mechanisms in Tissue Injury* (Slater, T. F., ed.), pp. 85–170, Pion Press, London

Smuckler, E. A. & Benditt, E. P. (1965) *Biochemistry* **4**, 671–679

Trudell, J. R., Bösterling, B. & Trevor, A. J. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2678–2682

Villarruel, M. C., Diaz Gomez, M. I. & Castro, J. A. (1975) *Toxicol. Appl. Pharmacol.* **33**, 106–114