

Mass spectrometric detection of cross-linked fatty acids formed during radical-induced lesion of lipid membranes*

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A mass spectrometric method is described for the quantitative determination of dimers of polyunsaturated fatty acids (PUFA) formed in the hepatic endoplasmic reticulum of rats upon inhalation of tetrachloromethane. The results show that dimers account for a considerable fraction of microsomal PUFA which disappear during CCl_4 metabolism. Cross-linking of the membrane lipids of the endoplasmic reticulum seems to be a significant process with respect to cell toxicity.

INTRODUCTION

Acute hepatotoxicity caused by tetrachloromethane and other xenobiotics, derangements associated with various diseases, and formation of fluorescent lipids upon ageing are all believed to result from radical-induced degradation of polyunsaturated fatty acids (PUFA) (Yagi, 1982). In the case of acute CCl_4 toxicity, loss of arachidonic and docosahexenoic acid from the phospholipids of the hepatic endoplasmic reticulum is one of the characteristic pathobiochemical effects (Miller & Cornatzer, 1969; Ilyas *et al.*, 1978; James *et al.*, 1982; Frank *et al.*, 1987a). Various low-molecular-mass products such as malonaldehyde (Wilbur *et al.*, 1949), hydroxyalkenals (Esterbauer *et al.*, 1982), hydroxy- and hydroperoxy-fatty acids (Hughes *et al.*, 1983; Frank *et al.*, 1984, 1987b), and the volatile scission products pentane and ethane (Riley *et al.*, 1974) have been considered as indicators of radical-induced degradation of PUFA. However, in stoichiometric terms, these products account for only a small fraction of the degraded PUFA (Frank *et al.*, 1989).

We have recently shown that upon CCl_4 metabolism, the fatty acids of the hepatic endoplasmic reticulum are partially trichloromethylated (Link *et al.*, 1984). In addition, chemical entities were found which have similar chromatographic properties to dimers and oligomers. There is evidence that phospholipids containing fatty acid dimers and/or oligomers are resistant to catabolism by phospholipases (Yoshida & Alexander, 1983; Frank & Link, 1984), while oxygen-modified fatty acids seem to be preferentially excised by these enzymes (Barker & Brin, 1975; Sevanian *et al.*, 1983).

Fatty acid dimers and oligomers are difficult to detect and to quantify by biochemical or histochemical techniques; their staining properties are not different from normal fatty acids, and no specific reactions are known which would serve to monitor such species. They have relatively high molecular masses and probably include many isomers and homologues, with different PUFA

cross-linked at various positions along the hydrocarbon tail. Due to the absence of a typical chromophore, they are not detectable by photometric means.

We have now developed a mass spectrometric method for the quantitative determination of fatty acid dimers in membrane lipids. The method has been applied to analysis of lipids from microsomes incubated with CCl_4 and from microsomes of rats previously exposed to CCl_4 by inhalation.

EXPERIMENTAL

Chemicals

Triaccontanoic acid was purchased from Sigma Chemicals. The fatty acid dimer (C_{36}) Pripol 1009 was a gift from Unichema International (Emmerich, Germany). All other chemicals were of the highest purity available and were from E. Merck (Darmstadt, Germany); biochemicals were from Boehringer–Mannheim (Mannheim, Germany).

Experiments *in vivo*

Male Sprague–Dawley rats (200 g body weight) were exposed to 80 mg of CCl_4 /kg for 6 h as described previously (Frank *et al.*, 1987a). Microsomes were prepared as described by Remmer *et al.* (1967). Protein was determined as described previously (Frank *et al.*, 1987a).

Incubation of microsomes with CCl_4

Liver microsomes from male Sprague–Dawley rats (body weight 200 g) were suspended in buffer, pH 7.4, containing 50 mM-Tris/HCl, 120 mM-KCl, 5 mM-MgCl₂, 8 mM-sodium isocitrate, and isocitrate dehydrogenase (50 µg/ml), to yield a concentration of 3 mg of protein/ml. Incubations of 8 ml of the microsomal suspension with CCl_4 were performed in 15 ml round-bottom flasks with stopcock connections for N_2 purging, and a Quickfit adapter with polytetrafluoroethylene-laminated septum for addition of CCl_4 and NADP^+ . The

Abbreviations used: PUFA, polyunsaturated fatty acid(s); PFB, pentafluorobenzyl; n.i.c.i.m.s., negative-ion chemical-ionization mass spectrometry.

* Dedicated to H. Remmer on the occasion of his 70th birthday.

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vessel was placed in an ice bath and purged at 0 °C with N₂ for 30 min. NADP⁺ (10 μmol in 100 μl of incubation buffer) was added through the septum, the N₂ flow was stopped and 1 μl of CCl₄ was injected. The flask was then placed in a water bath at 37 °C and gently shaken for 60 min. The reaction was stopped by adding 3 ml of 3 M-KOH in methanol.

Extraction of fatty acids

The lipids were saponified by heating to 80 °C for 60 min under a slight flow of N₂. Triacontanoic acid is added as standard, at a concentration of 20 μg/mg protein to samples from experiments *in vitro*, and at 2 μg/mg of protein to samples from exposures *in vivo*. After cooling, the solution was adjusted to pH 1 with 12 M-HCl. The fatty acids were extracted three times, with 5 ml of ethyl acetate on each occasion. The extract was washed with 3 ml of distilled water and was brought to complete dryness on a rotary evaporator. The residue was dissolved in 1 ml of ethyl acetate.

Hydrogenation and derivatization

A small quantity of PtO₂ was added to the ethyl acetate solution, the mixture was sonicated for 5 min and H₂ was introduced for 4 h. After centrifugation, the solution was transferred to a 10 ml round-bottom flask with a glass pipette, the solvent was removed on a rotary evaporator, and the residue was dissolved in 220 μl of acetonitrile; 20 μl of pentafluorobenzyl (PFB) bromide and 60 μl of ethyl di-isopropylamine were added and the solution was kept at 45 °C for 45 min (Blair *et al.*, 1983). Excess reagent was removed by evaporation under a stream of N₂. The resultant PFB esters were dissolved in 1 ml of ethyl acetate.

M.s. analysis

Negative-ion chemical-ionization mass spectrometry (n.i.c.i.m.s.) was performed on a Finnigan 4021 with Incos data system. The ion source conditions were: electron energy, 100 eV; filament current, 0.2 mA; methane gas pressure, 30 Pa; ion source temperature, 280 °C. Scans from *m/z* 250 to 1000 were taken every second. A 1 μl portion of the sample in ethyl acetate was injected into the glass tube (2 mm × 10 mm) of the direct probe. Data acquisition was started when the probe was introduced into the ion source. For 1 min 40 s the probe was not heated, slowly warming up to about 130 °C. Under these conditions the monomeric PFB fatty esters evaporated; when their ions had disappeared, the probe was then ballistically heated to 320 °C in about 1.5 min. The dimeric PFB fatty esters started to vaporize at about 260 °C.

For quantitative determination, the ion currents of the respective molecular species including all isotopic ions were integrated over the evaporation period of 20–30 s (see Fig. 3). The ratio of the area under the evaporation curve of the dimer to the area under the curve of the triacontanoate anion (*m/z* 451) was determined. The quantity of each dimer (C₃₆, C₃₈, etc.) was calculated using a calibration with the commercial C₃₆-dimer Pripol 1009 which had previously been fully hydrogenated; absence of olefinic protons was ascertained by ¹H-n.m.r. Calibrations were run every day both before and after a series of determinations, since absolute and relative ion yields depend upon the actual ion source conditions. In general, the molar response of the C₃₆-dimer acid was

about four times larger than that of the triacontanoic acid PFB-ester. The detection limit is about 1–5 pmol of C₃₆-dimer acid, depending upon the nominal resolution of the mass spectrometer and ion source conditions.

RESULTS AND DISCUSSION

Procedures for the analysis of dimeric fatty acids have been developed mainly for determination of thermally-polymerized unsaturated fatty acids, which are used widely for industrial purposes. Commonly, their analysis is based on chromatographic methods including g.l.c. (Nelson & Milun, 1975), h.p.l.c. (Veazey, 1986; Zeman *et al.*, 1986) and gel-permeation chromatography (Inoue *et al.*, 1970; Harris *et al.*, 1973; Jensen & Moller, 1986). Sen Gupta (1969) found that heating of methyl linolenate to 280 °C under high vacuum gives rise to dimers with more than one carbon-carbon bridge, while peroxide-induced dimerization at 140 °C entails mainly acyclic dimers. Dimers with a single carbon-carbon bridge are formed during the anaerobic lipoxygenase-catalysed reaction between linoleic acid and its peroxide (Garssen *et al.*, 1972). Their structures have been derived from m.s. analysis of their fully hydrogenated methyl esters.

We have previously found evidence for the formation of dimeric and oligomeric fatty acids in lipids from microsomes incubated with CCl₄ in the absence of oxygen (Link *et al.*, 1984). In order to assess the toxicological significance of lipid cross-linking, a method had to be developed to estimate the amounts present in rat liver microsomes.

None of the previously published procedures was found to be suitable, for a variety of reasons. The main requirement of the adopted method was sufficient sensitivity. Cross-linking of the three PUFA present in microsomal membranes, i.e. linoleic, arachidonic and docosahexenoic acids, can yield homo- and heterodimers with bridges between all carbon atoms of double bonds and bis-allylic methylene groups in the hydrocarbon tail. Therefore, each possible isomer or homologue is present only in small amounts. A sufficiently sensitive technique for detection and quantitative determination is n.i.c.i.m.s. of the pentafluorobenzyl (PFB) esters.

PUFA have the tendency to thermally dimerize; during direct-probe n.i.c.i.m.s., varying amounts of dimers can form from monomeric PUFA upon heating the probe; however, this can be minimized by cautious evaporation of the fatty acids at low temperature (100–130 °C) before the temperature is raised to vaporize the dimers. Thermal cross-linking may also occur during high-temperature gas chromatography. When quantitative determination of fatty acid dimers is attempted without previous hydrogenation, the integrated ion currents are relatively small and the reproducibility for replicate analyses is low. This is due to the fact that polyunsaturated dimers polymerize further during heating of the probe, similar to the PUFA.

Therefore, the microsomal lipids are saponified and the fatty acids are hydrogenated in the presence of PtO₂, reducing all double bonds and eliminating the possibility of adventitious dimer formation. Polyunsaturated dimeric acids are also fully reduced under these conditions.

An n.i.c.i.m.s. spectrum of hydrogenated dimers from an experiment *in vivo* is shown in Fig. 1. The dimers

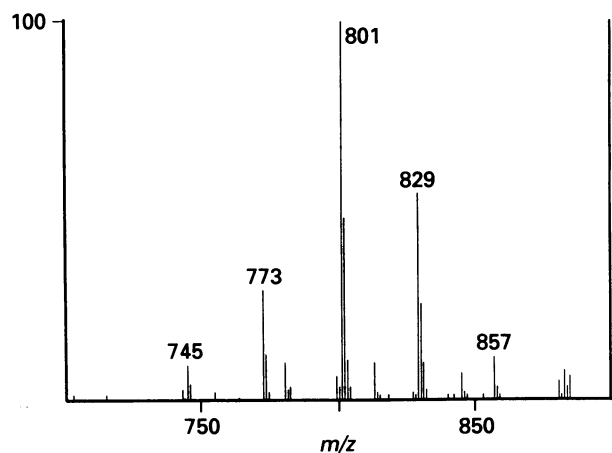


Fig. 1. N.i.c.i.-mass spectrum (m/z 700 to m/z 900) of the PFB esters of hydrogenated fatty acid dimers in hepatic microsomal lipids from a rat previously exposed to CCl_4

The most abundant ions (m/z 745, 773, 801, 829, 857) correspond to the loss of one PFB moiety from the molecular ion which is not observed as such; to each base peak the corresponding ion 180 mass units lower (m/z 565, 593, etc.) is found at a relative abundance of 15% (results not shown).

are derived from linoleic, arachidonic and docosahexaenoic acids, the most abundant PUFA in rat liver microsomes. The main fragments represent the carboxylate anions formed upon loss of one PFB residue ($\text{C}_{18}\text{-C}_{20}$, m/z 773; $\text{C}_{20}\text{-C}_{20}$ and $\text{C}_{18}\text{-C}_{22}$, m/z 801; $\text{C}_{20}\text{-C}_{22}$, m/z 829; $\text{C}_{22}\text{-C}_{22}$, m/z 857). A few ions with lower mass and a relative abundance of about 15% (results not shown) are present, probably corresponding to elimination of the second PFB residue as carbene moiety: m/z 593 (773-180); m/z 621 (801-180), m/z 649 (829-180) and m/z 677 (857-180). All possible isomeric homo- and heterodimers with the same number of carbon atoms but with linkages at different points are collectively counted as a single peak. Some ions of unknown origin are also present, but there is no evidence for multiple cross-links or for fatty acids linked by ether bridges, which are to be expected when hydroperoxides are involved (Miyashita *et al.*, 1985).

For quantitative determination, the ion currents of the respective dimers are integrated as they evaporate from the probe. A typical example of dimer fatty acids from hepatic microsomes of a rat exposed to CCl_4 is shown in Fig. 2. The collective ion currents of all isotopic ions are monitored, e.g. m/z 798-805 for the C_{40} dimer (see Fig. 1). Integration of the area under the time/ion-current curve, calculation of the ratio relative to the area under the curve for tricontanoic acid (m/z 451) and comparison with the calibration line constructed on the same day with the commercial, hydrogenated C_{38} diacid enables determination of the amounts present in the sample. This assumes that ionization yields for the different dimer species (C_{36} , C_{38} , etc.) are similar.

The dimer contents in microsomes from experiments *in vitro* and *in vivo* are shown in Table 1. In control microsomes, dimers are not usually present, only occasionally are amounts close to the detection limit found. Fatty acid dimers are formed to a considerable extent

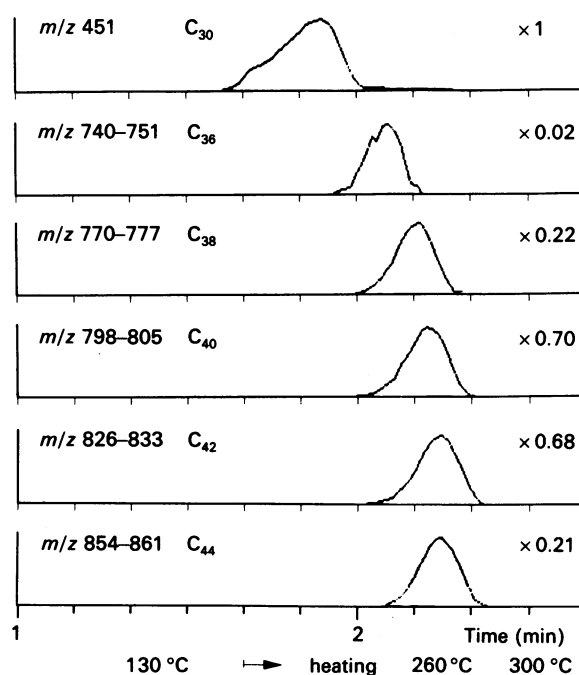


Fig. 2. Ion current profiles from n.i.c.i.m.s.

Integration of ion current profiles (direct-probe n.i.c.i.m.s.) over time of the standard compound tricontanoic acid (m/z 451) and the respective dimer ion clusters (C_{36} , m/z 740-751; C_{38} , m/z 770-777, etc.) in a lipid sample isolated from hepatic microsomes of a rat exposed to CCl_4 . The area correction factors relative to tricontanoic acid (m/z 451, relative area = 1) are shown on the right.

following CCl_4 metabolism *in vivo*, although the amounts are smaller than upon anaerobic incubation of microsomes with CCl_4 *in vitro*. The difference may be due to the fact that microsomes from liver tissue of CCl_4 -treated animals stem mainly from non-affected periportal hepatocytes.

From the determined amounts, the molar contribution of the three main PUFA to dimer formation can be deduced (lower half of Table 1). The calculation may be slightly incorrect for the $\text{C}_{20}\text{-C}_{20}$ species, as the latter does not only represent dimerized arachidonic acid, but probably includes also heterodimers of linoleic acid and docosahexaenoic acid. For comparison, the differences in microsomal PUFA between controls and the animals exposed to CCl_4 are shown on the bottom line of Table 1 (Frank *et al.*, 1987a); arachidonic and docosahexaenoic acid are greatly reduced, whereas linoleic acid is increased. The dimer fraction represents about 5% of the losses of arachidonic and docosahexaenoic acid. Although linoleic acid is increased upon CCl_4 exposure, it is also incorporated into the dimer fraction. This peculiar fact has been addressed before (Frank *et al.*, 1987a); most probably it results from unbalanced linoleic acid import and desaturation (Carreau *et al.*, 1980). The latter pathway is blocked upon damage to the endoplasmic reticulum, and hence there is a resulting accumulation of linoleic acid.

Some mechanistic conclusions can be drawn from the occurrence of dimeric fatty acids. There is little doubt that fatty acid dimers are generated by a free radical

Table 1. Fatty acid dimer content in microsomes

The fatty acid dimer content (nmol/mg of protein, n.d., not detected) in control microsomes, microsomes incubated with CCl_4 and microsomes isolated from male Sprague-Dawley rats exposed to 75 mg of CCl_4/kg of body weight for 6 h. In the lower half of the table the amounts of polyunsaturated fatty acids (Σ) incorporated into dimers *in vivo* are compared to the losses of polyunsaturated fatty acids (Δ) determined under the same exposure conditions (Frank *et al.*, 1987a).

Treatment of microsomes	Fatty acid dimer content (nmol/mg of protein)			
	$\text{C}_{18}\text{-C}_{20}$	$\text{C}_{20}\text{-C}_{20}$	$\text{C}_{20}\text{-C}_{22}$	$\text{C}_{22}\text{-C}_{22}$
Control	n.d.	n.d.	n.d.	n.d.
Exposure to CCl_4 <i>in vitro</i>	6.3 ± 1.5	13.4 ± 2.7	10.6 ± 1.5	4.4 ± 0.6
Exposure to CCl_4 <i>in vivo</i>	0.3 ± 0.05	1.6 ± 0.2	1.4 ± 0.4	0.2 ± 0.1
	(18:2)	(20:4)	(20:4)	(22:6)
Σ	0.3 ± 0.05	4.9 ± 0.8		1.8 ± 0.6
Δ	+30	-100		-30

process initiated by $\cdot\text{CCl}_3$ radicals. From the n.i.c.i.m.s. spectra of non-hydrogenated dimers (Fig. 3) the numbers of double bonds may be determined, giving insight into the cross-linking mechanism. In each cluster of dimers with identical numbers of carbon atoms dehydrodimers containing all double bonds of the original polyunsaturated acids ($\text{C}_{18:2}\text{-C}_{20:4}$, m/z 761; $\text{C}_{20:4}\text{-C}_{20:4}$, m/z 785; $\text{C}_{20:4}\text{-C}_{22:6}$, m/z 809; $\text{C}_{22:6}\text{-C}_{22:6}$, m/z 833) predominate over those formed with saturation of one double bond ($\text{C}_{18:2}\text{-C}_{20:3}$, m/z 763; $\text{C}_{20:4}\text{-C}_{20:3}$, m/z 787; $\text{C}_{20:4}\text{-C}_{22:5}$, m/z 811; $\text{C}_{22:6}\text{-C}_{22:5}$, m/z 835).

The presence of these two main species suggests the following pathways (Scheme 1). Hydrogen abstraction by a $\cdot\text{CCl}_3$ radical from an allylically activated methylene group of a PUFA (1 in Scheme 1) yields a pentadienyl radical (2). This can then react with a neighbouring polyunsaturated acid in one of two ways, leading to intermolecular cross-linking of lipid moieties. Predominantly it combines with another fatty acid radical (3); to a smaller extent, the pentadienyl radical adds to the double bond of a neighbouring PUFA. The resultant

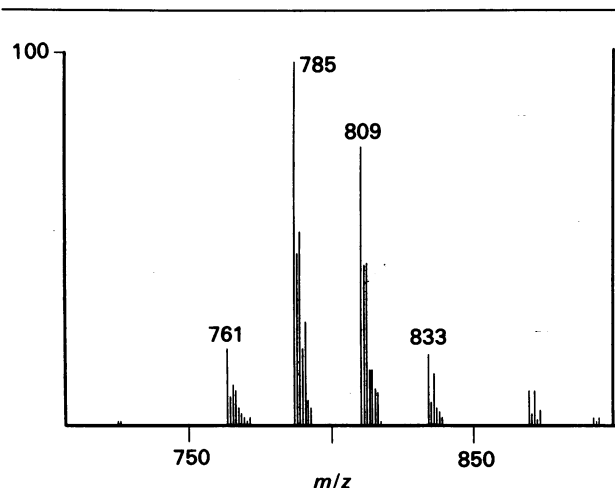
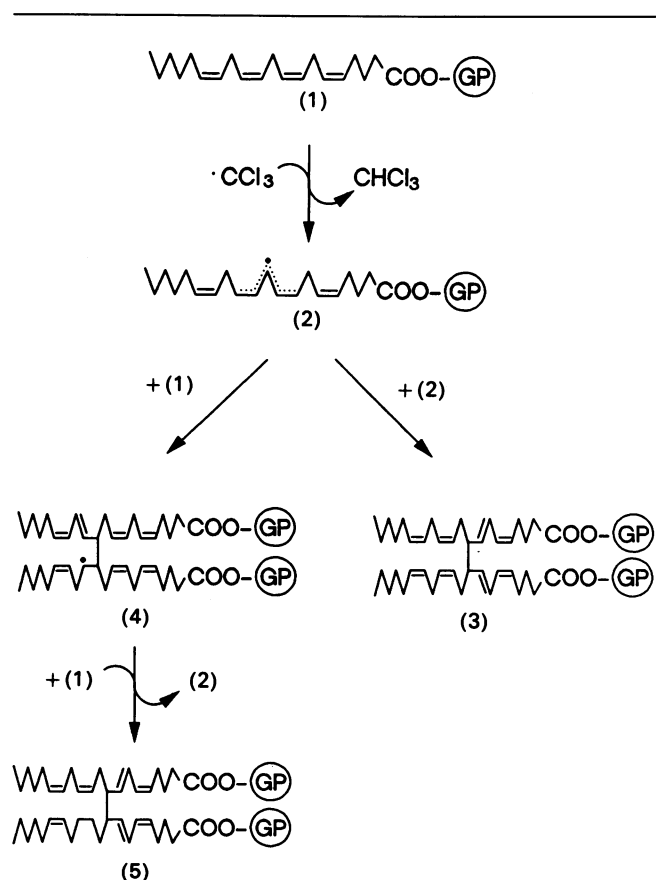


Fig. 3. N.i.c.i.-mass spectrum of the PFB esters of non-hydrogenated fatty acid dimers in hepatic microsomal lipids from rats after CCl_4 exposure *in vivo*

dimer radical (4) probably stabilizes by abstraction of a hydrogen atom (5) from an adjacent PUFA, generating another bisallylic fatty acid radical (2) which may again participate in either pathway.



Scheme 1. Proposed pathway for the formation of dimeric fatty acids in microsomes

Dimeric fatty acid formation can occur via radical recombination (right branch) or via radical addition and hydrogen abstraction (left branch). (GP), glycerophosphatidyl residue of the phospholipid.

The two mechanisms operating concurrently constitute a typical chain reaction. Reaction with oxygen is also possible, resulting in polar polymers cross-linked by ether bridges (Figge, 1971); however, there was no indication of their presence.

Intramolecular radical attack is unlikely since PUFA occur almost exclusively at the *sn*-2 position of the glyceryl esters. The fact that mainly single carbon-carbon linkages are formed is further evidence of a radical process; thermal, ionic dimerization inevitably leads to cyclic dimers (Sen Gupta, 1969). Dimers containing chlorine atoms are only present in trace amounts; covalent binding of $\cdot\text{CCl}_3$ to PUFA, followed by addition of the non-stabilized radical to an adjacent fatty acid seems to be unfavourable. As shown previously, trichloromethyl fatty acid radicals preferentially abstract hydrogen atoms to form monomeric trichloromethyl fatty acids with one less double bond (Link *et al.*, 1984). It is likely that cross-linking proceeds beyond the dimer stage to yield trimers, tetramers etc.; cross-linking of lipids with membrane-associated proteins is also probable.

Cross-linking of membrane lipids may entail considerable complications in the analysis of radical-induced derangements of endoplasmic membranes: the resinification of lipids may lead to preferential loss of those microsomes which are most strongly affected. Their sedimentation during microsome preparation by ultracentrifugation can be expected to differ from normal microsomal vesicles. This is also suggested by the electron microscopic observation of clumping of the endoplasmic membranes after CCl_4 exposure (Ganote & Rosenthal, 1968).

Obviously, fatty acid dimers constitute a significant 'sink' for PUFA vanishing during radical damage to membranes, more so than other lipid-degradation products identified so far (Frank *et al.*, 1989).

The pathobiochemical and pathophysiological significance of cross-linked fatty acids may reside in their resistance to phospholipases (Yoshida & Alexander, 1983; Frank & Link, 1984), and their effect upon the physicochemical properties of the membrane. The clumping of the endoplasmic reticulum upon CCl_4 administration (Ganote & Rosenthal, 1968) may be one of the consequences of cross-linking and resinification of membrane lipids. Many membrane-bound enzymes involved in ion homeostatic mechanisms and biosynthetic activities of the endoplasmic reticulum are strongly affected by the properties of the membrane (Seelig & Hasselbach, 1971; The & Hasselbach, 1973; Enoch *et al.*, 1976; Pugh & Kates, 1979). As the cell after CCl_4 metabolism is deprived of the vital capabilities of synthesizing proteins and phospholipids, decline seems inevitable. This may be even more important since the catabolic branch, i.e. Ca-dependent phospholipases, are activated by an increased influx of calcium (Moore *et al.*, 1976; Coleman *et al.*, 1988). Interestingly, fatty acid dimers in dietary lipids show pronounced hepatotoxicity when they are fed in sufficiently high doses to ensure systemic uptake (Friedman *et al.*, 1961).

With the availability of a suitable analytical method, the significance and generality of membrane lipid cross-linking can now be assessed; for instance, its role in membrane derangements induced by various xenobiotics, under certain pathological conditions or in the process of cell ageing.

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