

Separation and characterization of the aldehydic products of lipid peroxidation stimulated by ADP-Fe²⁺ in rat liver microsomes

H. ESTERBAUER,* K. H. CHEESEMAN,† M. U. DIANZANI,‡ G. POLI‡ and T. F. SLÄTER†
*Institute of Biochemistry, University of Graz, Schubertstrasse 1, A-8010 Graz, Austria, †Department of Biochemistry, School of Biological Sciences, Brunel University, Uxbridge, Middx., U.K., and ‡Istituto di Patologia Generale, Università di Torino, Corso Raffaello 30, Torino 10124, Italy

(Received 1 June 1982/Accepted 5 July 1982)

1. Methods using t.l.c. and high-pressure liquid chromatography (h.p.l.c.) have been used to separate the complex variety of substances possessing a carbonyl function that are produced during lipid peroxidation. 2. The major type of lipid peroxidation studied was the ADP-Fe²⁺-stimulated peroxidation of rat liver microsomal phospholipids. Preliminary separation of the polar and non-polar products was achieved by t.l.c.; further separation and identification of individual components was performed by h.p.l.c. Estimations were performed on microsomal pellets and the supernatant mixture after incubation of microsomes for 30 min at 37°C. 3. The polar fraction was larger than the non-polar fraction when expressed as nmol of carbonyl groups/g of liver. In the non-polar supernatant fraction the major contributors were n-alkanals (31% of the total), α -dicarbonyl compounds (22%) and 4-hydroxyalkenals (37%) with the extraction method used. 4. Major individual contributors to the non-polar fraction were found to be propanal, 4-hydroxynonenal, hexanal and oct-2-enal. Other components identified include butanal, pent-2-enal, hex-2-enal, hept-2-enal, 4-hydroxyoctenal and 4-hydroxyundecenal. The polar carbonyl fraction was less complex than the non-polar fraction, although the identities of the individual components have not yet been established. 5. Since these carbonyl compounds do not react significantly in the thiobarbituric acid reaction, which largely demonstrates the presence of malonaldehyde, it is concluded that considerable amounts of biologically reactive carbonyl derivatives are released in lipid peroxidation and yet may not be picked up by the thiobarbituric acid reaction.

Lipid peroxidation in tissues and in tissue fractions is a degradative free-radical process that primarily involves polyunsaturated fatty acids, especially arachidonic acid, in biomembranes (Slater, 1972; Mead, 1976); it has been implicated as a major contribution to many types of tissue damage (Dianzani & Ugazio, 1978; Plaa & Witschi, 1976; Halliwell, 1981). Lipid peroxidation can be stimulated in the membranes of the endoplasmic reticulum by ADP-Fe²⁺ (Hochstein & Ernster, 1963), cumene hydroperoxide (O'Brien & Rahimtula, 1975), ascorbate (Thiele & Huff, 1964) and also by the metabolic activation of a variety of toxic agents such as carbon tetrachloride (Comporti *et al.*, 1965; for background discussion, see Slater, 1966, 1972; Recknagel & Glende, 1973). Microsomal lipid peroxidation has been extensively studied in relation to such types of metabolic activation, and the

peroxidation reactions are accompanied by disturbances to membrane structure and function (Orrenius *et al.*, 1964; Ghoshal & Recknagel, 1965; Glende *et al.*, 1976; Reynolds & Moslen, 1980; Benedetti *et al.*, 1980).

The non-enzymic autoxidation of polyunsaturated fatty acids is known to be accompanied by the formation of a complex mixture of products including aldehydes such as alkanals, alk-2-enals, 4-hydroxyalkenals and malonaldehyde (Schauenstein, 1967; Esterbauer & Schauenstein, 1967; Loury, 1973; Schauenstein *et al.*, 1977; Esterbauer, 1982). The 4-hydroxyalkenals have especially interesting biological reactivities: 4-hydroxyoctenal, for example, can exert strongly inhibitory effects on various enzyme reactions (Schauenstein, 1967). The related 4-hydroxypentenal exerts strongly inhibitory effects on DNA and protein synthesis (Seeber *et al.*, 1969; Dostal *et al.*, 1974; Dianzani, 1979), reacts rapidly with thiols such as cysteine (Esterbauer *et al.*, 1976) and glutathione (Esterbauer *et al.*,

Abbreviations used: h.p.l.c., high-pressure liquid chromatography; AU, absorbance unit; AUFS, absorbance units full scale.

1975) and has inhibitory actions on certain tumour cell populations both *in vitro* and *in vivo* (for detailed references, see Schauenstein *et al.*, 1977; Schauenstein & Esterbauer, 1979; Conroy *et al.*, 1975).

Recently, 4-hydroxynonenal was isolated from peroxidizing liver microsomes (Benedetti *et al.*, 1980) and shown to inhibit several microsomal and plasma-membrane enzymes in the 1–100 μM concentration range (Ferrali *et al.*, 1980; Dianzani, 1982). It is of interest and importance, therefore, to study the patterns of products produced by lipid peroxidation in biological situations where many different ways of stimulating lipid peroxidation are known to exist. In this study we have concentrated on the stimulation of lipid peroxidation produced by ADP–Fe²⁺, a peroxidation system that has been well characterized in previous studies (Hochstein & Ernster, 1963; Orrenius *et al.*, 1964; Slater, 1968; for review, see Slater 1972).

A major objective of this study was to develop satisfactory h.p.l.c. procedures for separating the main aldehydic components of peroxidizing liver microsomes, in a manner suitable for their chemical identification, using ADP–Fe²⁺ as the stimulus for lipid peroxidation (the effects of other stimulators of lipid peroxidation are the subject of subsequent studies). A second objective was to identify major non-polar aldehydic products of the ADP–Fe²⁺-stimulated lipid peroxidation; in particular to determine the amounts of 4-hydroxyalkenals produced. A third objective was to evaluate the previously reported biological consequences of ADP–Fe²⁺-stimulated peroxidation in relation to the aldehydic products identified in this study.

Experimental

Materials

Chemicals were obtained from Boehringer Mannheim G.m.b.H. or The Boehringer Corporation (London) Ltd., Lewes, Sussex, U.K. (NADP, ADP and glucose 6-phosphate dehydrogenase), Sigma London Chemical Co., Poole, Dorset, U.K. (nicotinamide and glucose 6-phosphate), T. F. Baker Chemicals, Deventer, Holland (MgO), Serva, Heidelberg, Germany (Celite 545) and Merck, Darmstadt, Germany (all other chemicals, including t.l.c. plates). Desferrioxamine (Desferal) was kindly provided by Ciba-Geigy, Horsham, Sussex, U.K.

Quartz-distilled water was used for the preparation of all aqueous solutions. The rat liver microsomes were prepared as previously described (Slater & Sawyer, 1971) from male rats (Wistar; 180–220 g body wt.) fed with a standard diet (TACO T779; Tagger & Co., Graz, Austria) and starved overnight before killing.

2,4-Dinitrophenylhydrazine reagent

2,4-Dinitrophenylhydrazine (50 mg recrystallized from n-butanol) was added to 100 ml of 1 M-HCl. The suspension was kept for 1 h at 50°C, cooled down to ambient temperature and extracted twice with 50 ml of n-hexane. This extraction removed the greater part of the carbonyl impurities (mostly acetone, formaldehyde and acetaldehyde) present in trace amounts in quartz-distilled water and in the HCl. The extracted reagent was flushed with N₂ and stored at ambient temperature in the dark for not more than 24 h before use.

Incubation procedure

The incubation mixture (48 ml) contained 4.8 ml of microsomal suspension (equivalent to 4.8 g wet wt. of liver), 1.6 ml of ADP–Fe²⁺ (48 mM-ADP/0.54 mM-FeSO₄), 6.4 ml of water and 35.2 ml of an NADPH-generating stock solution (Slater & Sawyer, 1971). Experimental samples, controls (minus ADP–Fe²⁺) and reagent blanks (minus microsomes) were incubated at 37°C for 30 min.

Preparation of the 2,4-dinitrophenylhydrazones

At the end of the incubation period, the microsomal suspension was centrifuged for 40 min at 100 000 g and both the supernatant solution and the sediment so obtained were allowed to react with 2,4-dinitrophenylhydrazine as follows.

Carbonyl compounds of the supernatant samples. The supernatant was mixed with an equal volume of dinitrophenylhydrazine at room temperature for 12 h in the dark and then extracted with chloroform (2 × 3 ml); phase separation was improved by centrifugation. Traces of water were removed by freezing (–20°C, 2 h) and filtering. Excess dinitrophenylhydrazine reagent, and also the hydrazones of acetone, formaldehyde and acetaldehyde that were always present as contaminants even when highly purified water and solvents were used, were removed from the extracts by t.l.c. (silica-gel 60; pre-coated; plate dimensions 20 cm × 20 cm) using dichloromethane as first developer (5 cm) and benzene as second developer (15 cm). All hydrazones with R_f values greater than or equal to 0.12 (the R_f of 4-hydroxynonenal), except for the contaminants just mentioned, were scraped off and extracted with methanol (2 × 5 ml), and brought to volume with methanol (40 ml for u.v. spectra; 0.5 ml for h.p.l.c.). This extract contains all the carbonyl compounds of medium and low polarity and these are referred to hereinafter as ‘non-polar carbonyl compounds’ of the supernatant. The hydrazones from the origin R_f values less than 0.12 were similarly extracted and are referred to hereinafter as ‘polar carbonyl compounds’ of the supernatant. The polar carbonyl compounds were subjected to an

additional t.l.c. pre-separation (silica gel; ethyl acetate as developer) to remove some hydrazones that were also present in the reagent-blank sample. All hydrazones absent from the reagent blank were scraped off, eluted with methanol (2 × 5 ml) and concentrated to 0.5 ml.

Carbonyl compounds of the microsomal sediment. The sediment was resuspended with 8 ml of dinitrophenylhydrazine reagent and allowed to react as previously described for the supernatant sample. The extraction and pre-separation by t.l.c. were essentially the same as described above for the carbonyl compounds contained in the supernatant sample. The descriptions 'polar' and 'non-polar' for the carbonyl compounds are used as defined above.

Class separation of the dinitrophenylhydrazine derivatives on MgO

The mixture of the dinitrophenylhydrazine derivatives of the non-polar carbonyl compounds was separated into individual classes (alkanones, alkanals, alk-2-enals, alka-2,4-dienals) by t.l.c. on MgO-Celite. The method used was essentially as described by Schwartz & Parks (1963) as modified by Rachelsperger (1980). The carbonyl compounds belonging to each class were extracted into chloroform/methanol (9:1, v/v), dried and dissolved in methanol for analysis by h.p.l.c.

Quantitative estimation of total carbonyl compounds and individual carbonyl classes

The dinitrophenylhydrazine derivatives of the total non-polar and polar carbonyl compounds isolated from the microsomal supernatant samples and sediments as described above were dissolved in methanol (8.3 ml per g of original liver) and the concentration was calculated from the u.v. absorbance (at 365 nm) using an average ϵ value of 25 000 litre·mol⁻¹·cm⁻¹. The dinitrophenylhydrazine derivatives of 4-hydroxyalkenals (mostly due to 4-hydroxynonenal) and the osazones were isolated from the fraction of the dinitrophenylhydrazine derivatives of the non-polar carbonyl compounds by t.l.c. on silica-gel plates (20 cm × 20 cm) with benzene as developing solvent. The zone of the 4-hydroxyalkenals was identified using a reference sample (Esterbauer & Weger, 1967); the osazone-bands were localized (on a part of the plate) through the characteristic change of their colour (orange-red to blue), which occurred after spraying the plate with a methanolic solution of KOH. The classes of alkanones, n-alkanals, alk-2-enals and alka-2,4-dienals were recovered by separation of the dinitrophenylhydrazine derivatives of the non-polar carbonyl compounds (free of 4-hydroxyalkenals and osazones) on MgO-Celite plates as described above. The dinitrophenylhydrazine derivatives of the various carbonyl classes so obtained were dissolved

in an appropriate volume of methanol and their concentrations were calculated from the u.v. spectra. The ϵ values used for calculations are: 4-hydroxyalkenal dinitrophenylhydrazine, ϵ 28 000, 370 nm; osazones, ϵ 44 000, 430 nm; alkanones, ϵ 22 500, 362 nm; n-alkanals, ϵ 21 600, 360 nm; alk-2-enals, ϵ 28 000, 378 nm; alka-2,4-dienals, ϵ 37 000 litre·mol⁻¹·cm⁻¹, 392 nm. [A collection of many different dinitrophenylhydrazine derivatives was available due to the previous work of one of us (Esterbauer & Weger, 1967). The ϵ values given are averages of carbonyl compounds with different chain length.]

Analysis of the dinitrophenylhydrazine mixture by h.p.l.c.

Analysis by h.p.l.c. was performed with a DuPont Liquid Chromatograph (model 830) and a variable-wavelength detector (DuPont model 837 or Perkin-Elmer LC 65T). The column mostly used in the experiments described in this paper was a Zorbax ODS model of dimensions 4.6 mm × 25 cm. Other columns tested were Lichrosorb 5 RP 18 and Spherisorb 5 μ ODS. The mobile phase was a methanol/water mixture; the concentration is given in the legends to the Figures.

Thiobarbituric acid reaction with microsomal suspensions and individual aldehydes

The thiobarbituric acid reaction was performed essentially as described by Slater & Sawyer (1971). Microsomal suspension (1 ml) was mixed with 2 ml of 10% (w/v) trichloroacetic acid; 2 ml of the clear supernatant obtained after centrifuging was then mixed with 2 ml of aqueous thiobarbituric acid solution (0.67%, w/v) and heated for 10 min in a boiling-water bath. The solutions were then cooled with tap water for 5 min and the absorbance measured at 535 nm.

Stock solutions (0.1 M) of individual aldehydes in methanol were diluted in buffer solution (60 ml of 0.1 M-Tris, pH 7.4, mixed with 90 ml of 0.15 M-KCl and 71 ml of water) or buffer solution mixed with FeSO₄ solution (29 ml of buffer solution and 1 ml of 0.539 mM-FeSO₄) to give final aldehyde concentrations of 10 mM. Samples (1 ml) of these solutions were then treated as described above for the microsomal suspensions, except that heating was performed in tubes sealed with Teflon-lined screw-caps to avoid loss of volatile aldehydes. Turbid solutions were centrifuged before spectral analysis. The absorbance spectra of the coloured solutions were then recorded over the wavelength range of 360–600 nm against blank solutions from which the aldehyde had been omitted. After these measurements (see Table 4, trichloroacetic acid solutions), the 'complete' thiobarbituric acid reaction mixtures were extracted with 6 ml of n-butanol; the butanol

phases were cleared by centrifugation and spectra were recorded against the butanol extract of a blank sample (see Table 4, butanol extracts).

Fatty acid analysis in microsomal fractions

Samples (20 ml) of microsomal suspensions (1.5–1.8 mg of protein/ml) were extracted with 20 vol. of chloroform/methanol (2:1, v/v) containing 10 mg of butylated hydroxyanisole, and 5 mg of heptadecanoic acid as an internal standard. The lipid extract was trans-esterified with 2 ml of benzene and 4 ml of BF₃/methanol (14%, w/v) for 1 h at 100°C as described by Morrison & Smith (1964). After addition of 10 ml of water, the methyl esters were extracted with 3 × 5 ml of benzene. G.l.c. separation was performed on a Perkin–Elmer F 22 instrument with a 2 m glass column containing 10% SP 2330 on 100/120 Chromosorb WAW and with a temperature programme of: 170–210°C, 2°/min; 210–250°C, 4°/min. The chromatograms were evaluated with the LDC 308 computing integrator. The detector response factors for the individual fatty acids were estimated in separate experiments. The mean values of three determinations were: C_{16:0} 1.02 ± 0.02; C_{17:0} 1.00 (internal standard); C_{18:0} 1.01 ± 0.04; C_{18:1} 1.03 ± 0.01; C_{18:2} 1.15 ± 0.01; C_{20:4} 1.17 ± 0.05; C_{22:6}, not estimated (factor used, 1.17). The procedures described above were used in the University of Graz and similar experiments were done in Brunel University; in the latter case the experimental procedure followed was that described by McDonald-Gibson & Young (1974).

Determination of malonaldehyde by h.p.l.c.

Analysis of free malonaldehyde concentrations on microsomal suspensions (with and without ADP–Fe²⁺ stimulation of lipid peroxidation) was performed as described by Esterbauer & Slater (1981).

Results

The microsomal fractions used in most of the experiments reported here were prepared in Graz; the major points were checked using microsomes prepared from liver samples obtained from rat colonies in Brunel University and in the University of Turin; no significant differences were found in the experiments using the different microsomal preparations. Table 1 gives data for the polyunsaturated fatty acid content of microsomal fractions used, and includes some values previously reported in the literature. Table 1 also includes the changes observed in the polyunsaturated fatty acid composition of microsomal suspensions after a 30 min peroxidation with ADP–Fe²⁺, and the malonaldehyde production after various times of incubation at 37°C. It can be seen that ADP–Fe²⁺ stimulated the production of malonaldehyde to a large extent in the microsomal suspensions used here; this is consistent with previous reports that ADP–Fe²⁺ stimulates lipid peroxidation as measured by the uptake of O₂ and the production of malonaldehyde (Hochstein & Ernster, 1963), or by the loss of polyunsaturated fatty acids (Tam & McCay, 1970). Table 1 shows that the microsomal suspensions used here have

Table 1. Effect of ADP–Fe²⁺ on the fatty acid content of total lipids in rat liver microsomes, and on the production of malonaldehyde

Microsomes (equivalent to 4.8 g of liver) were incubated in the presence of ADP–Fe²⁺ and an NADPH-generating system in a final volume of 48 ml at 37°C for 30 min. The lipids contained in 20 ml of the incubation mixture were extracted with a 20-fold volume of chloroform/methanol (2:1, v/v) and transesterified with BF₃/methanol as described in the Experimental section. For controls all conditions were identical except that ADP–Fe²⁺ was omitted. The control values are means ± S.E.M. of four experiments; individual results for the incubations with ADP–Fe²⁺ are shown. Some values for microsomal fatty acid composition previously reported are included for comparison. Also included are values for malonaldehyde production during microsomal incubation with ADP–Fe²⁺. For further details see the Experimental section.

| Fatty acid | Fatty acid (nmol/mg of microsomal protein) | | | | | | Malonaldehyde production | |
|-------------------|--|-----------------------|--------------------|-----------------------------|--------------------|--|--------------------------|-------------------|
| | The present study | | May & McCay (1968) | Weddle <i>et al.</i> (1976) | Tam & McCay (1970) | K. Cheeseman & T. F. Slater (unpublished work) | Time (min) | (nmol/g of liver) |
| | Control | +ADP–Fe ²⁺ | | | | | | |
| C _{16:0} | 250 ± 4 | 259, 278 | 302 | 226 | — | 243 | 0 | 16 |
| C _{18:0} | 229 ± 10 | 247, 223 | 348 | 323 | 667 | 211 | 2 | 33 |
| | | | | | | | 5 | 320 |
| | | | | | | | 10 | 513 |
| C _{18:1} | 79 ± 7 | 72, 54 | 112 | 101 | 21 | 63 | 15 | 554 |
| C _{18:2} | 173 ± 9 | 147, 129 | 190 | 184 | 451 | 158 | 30 | 563 |
| C _{20:4} | 225 ± 21 | 137, 126 | 276 | 314 | 549 | 190 | 60 | 573 |
| C _{22:6} | 83 ± 15 | 28, 82 | 96 | 51 | 205 | 62 | | |

similar polyunsaturated fatty acid content to samples previously reported in the literature, and confirms that the major changes produced by ADP-Fe²⁺-stimulated peroxidation are in the highly unsaturated fatty acids.

In preliminary experiments microsomes were peroxidized for 30 min at 37°C with ADP-Fe²⁺, and then 2,4-dinitrophenylhydrazones derivatives were formed. The resultant hydrazones were separated by t.l.c. The results showed clearly that the peroxidized samples contained complex varieties of aldehydic materials other than malonaldehyde (free malonaldehyde does not form a dinitrophenylhydrazone derivative; Kwon & Watts, 1964). To simplify the subsequent separation and identification stages, it was found advantageous to centrifuge the peroxidized microsomal samples before derivative formation, and to analyse the carbonyl compounds in the microsomal pellet and supernatant mixture separately. Moreover, to obtain a clear h.p.l.c. separation profile of the many dinitrophenylhydrazone derivatives it was necessary to subdivide the complex mixtures of dinitrophenylhydrazone derivatives by t.l.c. into two fractions differing in their polarity. The non-polar carbonyl fraction included all dinitrophenylhydrazone derivatives giving R_F values between 0.12 (the R_F of 4-hydroxynonenal dinitrophenylhydrazone) and 1.0, and the polar carbonyl fraction included the material that remained at or near the point of application. The u.v.-visible absorption spectrum was recorded from each of the four dinitrophenylhydrazone fractions so obtained. The amounts of carbonyl compounds in each fraction were then calculated from the maxi-

mum absorption (between 365 and 368 nm) and the average molar absorption coefficients previously reported by Esterbauer (1982).

After 30 min incubation at 37°C with ADP-Fe²⁺ the microsomal suspension contained approx. 950 nmol of carbonyl compounds/g liver (Table 2), of which about 40% were non-polar carbonyl compounds. A proportion (approx. 40%) of the polar and non-polar carbonyl compounds is released from the microsomes into the incubation medium, whereas the remainder remains associated with the microsomal structure.

The relatively large variations in the amounts of carbonyl compounds recovered in the various fractions may be caused, at least in part, by differences in the extent to which the carbonyl compounds are released from the microsomes into the surrounding medium. The main reason, however, appears to be that polar aldehydes, in particular, were not completely recovered by the chloroform-extraction method employed in this study. That the chloroform-extracted residue still contained carbonyl hydrazones was clearly evident from its intense yellow colour. When this residue was re-extracted with chloroform/methanol (2:1, v/v) an additional amount of approx. 300–400 nmol of carbonyl compounds/g of original liver was recovered. In other experiments, where the complete microsomal suspension was subjected to derivative formation with dinitrophenylhydrazine reagent and extracted with chloroform/methanol (2:1, v/v), reproducible results were obtained. For example, in three experiments using chloroform/methanol extraction the total amount of alkanones + n-

Table 2. *Effect of ADP-Fe²⁺ on the formation of carbonyl compounds by rat liver microsomes*

For the experimental sample, microsomes (equivalent to 4.8 g of liver) were incubated in the presence of ADP-Fe²⁺ (48 mM/0.54 mM) and an NADPH-generating system in a final volume of 48 ml (Tris/KCl, pH 7.4, at 37°C for 30 min). The suspension was then centrifuged (100 000 g), and then dinitrophenylhydrazone-derivatives were prepared from both the supernatant samples and the pellets, and separated into non-polar and polar carbonyl compounds by t.l.c. (see the Experimental section). Each of the dinitrophenylhydrazone-derivative fractions so obtained was dissolved in 40 ml of methanol and the u.v.-visible spectra were recorded in 1 cm cuvettes (see Esterbauer, 1982). The carbonyl concentrations were calculated from the maximum absorbance (in the range of 365–370 nm) and an average ϵ value of 25 000 litre·mol⁻¹·cm⁻¹. Mean values \pm S.E.M. are shown (numbers of experiments are shown in parentheses). For the control sample, all conditions were identical with those for the experimental sample, except that ADP-Fe²⁺ was omitted.

| | Carbonyl compounds (nmol/g of original liver) | | |
|------------------------------------|---|-----------------|------------|
| | Experimental | Control | Difference |
| Microsomal supernatant | | | |
| Non-polar | 178 \pm 30 (6) | 21 \pm 9 (3) | 157 |
| Polar | 192 \pm 61 (8) | 50 \pm 20 (4) | 142 |
| Microsomal pellet | | | |
| Non-polar | 159 \pm 46 (5) | 27, 20 | 136 |
| Polar | 425 \pm 111 (6) | 88, 20 | 371 |
| Total non-polar carbonyl compounds | 407 \pm 52 (7) | 37, 60 | 359 |
| Total polar carbonyl compounds | 554 \pm 92 (7) | 152, 40 | 458 |
| Total carbonyl compounds | 945 \pm 113 (6) | 189, 100 | 801 |

alkanals + alk-2-enals was found to be 246 ± 6 nmol/g of liver. The chloroform/methanol extract, however, contained a large amount of lipid material, which severely disturbed all further attempts to perform clear t.l.c. or h.p.l.c. separations necessary to identify the individual compounds. It should be stressed, therefore, that the work-up procedure used in the present study (using chloroform extraction) has to be considered as a preliminary approach for a semi-quantitative analysis since it was mainly designed to obtain the complex mixture of carbonyl dinitrophenylhydrazones in a form suitable for their further identification. The knowledge of the structure of the carbonyl compounds together with their approximate concentrations will allow further developments of a method for the quantitative estimation of total carbonyl groups and the various carbonyl classes, including individual carbonyl compounds of biological interest.

Separation and characterization of carbonyl compounds by h.p.l.c.

(a) *Non-polar carbonyl compounds in the supernatant solution from microsomes peroxidized with ADP-Fe²⁺*. The separation by h.p.l.c. of the mixture of non-polar carbonyl dinitrophenylhydrazone derivatives prepared from microsomal supernatant solutions revealed a complex chromatogram with at least 31 peaks of different intensities (Fig. 1). Some of the peaks overlapped and were not completely resolved. Attempts to obtain clearcut separations of all peaks by numerous variations of the separation conditions have not been successful; the conditions used in the experiment shown in Fig. 1 are the best so far available.

The major peaks identified are the dinitrophenylhydrazone derivatives of propanal, hexanal, 4-hydroxynonenal and three osazones (peaks 19, 23 and 25). In addition to these major compounds, minor amounts of butanal, pentanal, pent-2-enal, hex-2-enal, hept-2-enal, oct-2-enal, 4-hydroxyoctenal and 4-hydroxyundecenal were also present. The remaining other small peaks of the chromatogram have not yet been identified.

That the production of the carbonyl compounds is in fact due to the stimulated lipid peroxidation was proved by the analysis of the control and reagent blanks. The control (Fig. 1) contained only trace amounts of the carbonyl compounds. From the retention times of the dinitrophenylhydrazone peaks it seems likely that these traces of carbonyl compounds are propanal, butanal, hexanal and two osazones; the sum of all was less than 10% of the amount present in the ADP-Fe²⁺-stimulated sample.

The reagent blank was nearly free of any carbonyl compounds (Fig. 1). The only noteworthy peak (no. 5) was also present in the experimental and control

samples and most probably results from incompletely removed 2,4-dinitrophenylhydrazine reagent. The other very small peaks in the reagent-blank chromatogram had retention times agreeing with peaks 13, 17, 19 and 25. Because of the very low

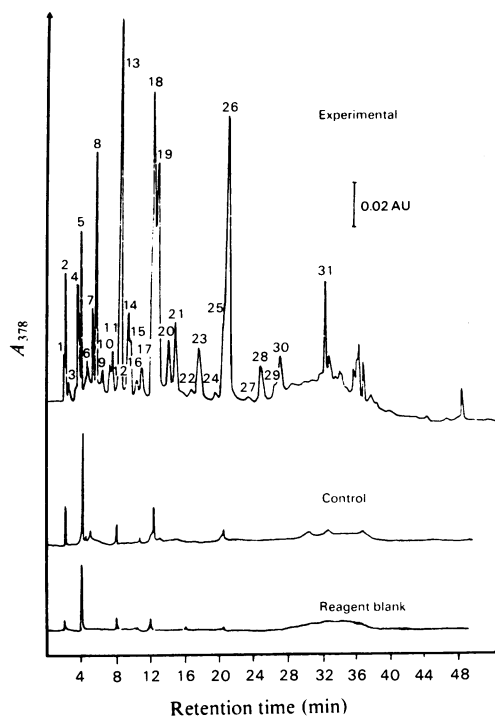


Fig. 1. Separation by h.p.l.c. of the dinitrophenylhydrazone derivatives of the non-polar carbonyl compounds obtained from the supernatant samples of microsomes incubated in the presence (experimental) and absence (control) of ADP-Fe²⁺

The reagent blank was as for the experimental mixtures but without the addition of microsomes. Incubation and work-up were as described in the legend to Table 2. The hydrazones obtained were dissolved in 0.5 ml of methanol and 50 μ l were separated on a Zorbax ODS column (4.6 mm \times 25 cm) by isocratic elution for 20 min with methanol/water (4:1, v/v) followed by a linear gradient of 80–100% methanol for 20 min; flow rate was 0.9 ml/min, ambient temperature, detector wavelength 378 nm, attenuation 0.2 AUFS. Peak identification was performed as described in the text. 1–12, Unknown; 13, propanal; 14, 4-hydroxyoctenal; 15, 16, unknown; 17, butanal; 18, 4-hydroxynonenal; 19, osazone; 20, pent-2-enal; 21, pentanal; 22, unknown; 23, osazone; 24, hex-2-enal; 25, osazone; 26, hexanal; 27, unknown; 28, 4-hydroxyundecenal; 29, unknown; 30, hept-2-enal; 31, oct-2-enal.

amounts, however, it was impossible to identify them.

(b) *Non-polar carbonyl compounds in microsomal pellets.* Separations similar to that shown in Fig. 1 were performed with the non-polar carbonyl compounds recovered from the microsomal sediment. These results (not shown) indicated that the pattern of the peaks is qualitatively very similar to that found for the supernatant solution (Fig. 1).

(c) *Proportions of alkanals and 4-hydroxyalkenals in the non-polar fractions obtained from microsomes peroxidized with ADP-Fe²⁺.* The n-alkanals, 4-hydroxyalkenals and the α -dicarbonyl compounds represent the major classes of the non-polar carbonyl compounds (Table 3). The proportions of n-alkanals contained in the microsomal supernatant samples and in the microsomal sediments were approx. 30% and 50% respectively of the total carbonyl compounds present; the corresponding values for the 4-hydroxyalkenals were 37% and 20% respectively.

(d) *Identification of the carbonyl products of microsomal peroxidation stimulated by ADP-Fe²⁺.* The identifications of the individual 4-hydroxyalkenals were made by chromatographic methods; the identification of 4-hydroxynonenal (a major peak in the h.p.l.c. separation of the dinitrophenylhydrazone derivatives of 4-hydroxyalkenals) as a microsomal lipid peroxidation product has already been conclusively proved (Benedetti *et al.*, 1980) by a

combination of unequivocal procedures. The chromatographic identifications performed in this study consisted of the isolation of the dinitrophenylhydrazone derivatives of the 4-hydroxyalkenals by t.l.c. followed by a separation into the homologous members by h.p.l.c. as described previously (Benedetti *et al.*, 1980).

The dinitrophenylhydrazone derivatives of the n-alkanals were first recovered by t.l.c. on silica gel and on MgO-Celite (see the Experimental section), and then separated into the individual aldehydes by h.p.l.c. on a reversed-phase column. The peak identity was ascertained by using a standard reference mixture (Fig. 2).

The class of the alk-2-enals was similarly analysed and gave a chromatogram indicating the presence of pent-2-enal, hex-2-enal, hept-2-enal, oct-2-enal and non-2-enal (Fig. 3).

The carbonyl compounds yielding osazones during the formation of derivatives (α -dicarbonyl compounds and/or α -hydroxyaldehydes) were detected easily and specifically, without any additional pre-separation, by monitoring the h.p.l.c. effluent at 460 nm, where osazones exhibit a high absorbance, whereas hydrazones do not. If separations, as shown

Table 3. Distributions of the various classes of carbonyl compounds in the non-polar carbonyl fractions recovered from the microsomal pellets and microsomal supernatant samples

Incubation conditions and preparation of the dinitrophenylhydrazone derivatives were as described in the legend to Table 2; the dinitrophenylhydrazone derivatives of the non-polar carbonyl compounds were then separated into individual classes by t.l.c. on MgO and silica gel as described in the Experimental section. Each dinitrophenylhydrazone fraction so obtained was dissolved in an appropriate volume of methanol, the u.v.-visible spectra were recorded and the carbonyl concentration was calculated by using the ϵ values given in the Experimental section. The values are expressed as percentages of the total carbonyl compounds recovered in classes 1-6. Values are means \pm s.d. of three determinations, or single determinations.

| Carbonyl class | Microsomal pellet (%) | Microsomal supernatant sample (%) |
|-----------------------------------|-----------------------|-----------------------------------|
| 1. Alkanones | 3 | 3 |
| 2. n-Alkanals | 50 | 31 \pm 5 |
| 3. Alk-2-enals | 7 | 9 \pm 0.4 |
| 4. Alka-2,4-dienals | 7 | 4 |
| 5. α -Dicarbonyl compounds | 14 | 22 \pm 8 |
| 6. 4-Hydroxyalkenals | 20 | 37 \pm 6 |

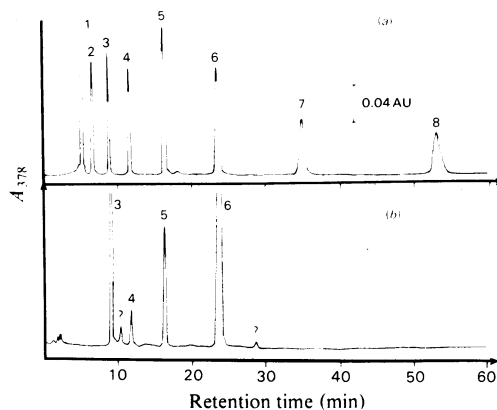


Fig. 2. Identification by h.p.l.c. of the n-alkanals formed by ADP-Fe²⁺-stimulated peroxidation of microsomes. Incubation and general work-up were as described in the legend to Table 2. (a) H.p.l.c. of a reference mixture of the dinitrophenylhydrazone derivatives of n-alkanals with chain length of one (formaldehyde), two, three, four, five, six, seven and eight (octanal) carbon atoms. (b) H.p.l.c. of the dinitrophenylhydrazone derivatives of the n-alkanals obtained by t.l.c. on MgO from the non-polar carbonyl compounds of the supernatant (see Fig. 1). 3, Propanal; 4, butanal; 5, pentanal; 6, hexanal. Operating conditions: Zorbax ODS column (4.6 mm \times 25 cm); isocratic elution with methanol/water (4:1, v/v); flow rate, 0.78 ml/min; 378 nm. 0.4 AUFS; injected volume, 50 μ l.

in Fig. 1, were monitored at 460nm, only three major peaks were visible, which agreed in retention times with peaks 19, 23 and the unresolved peak 25. A small amount of the main peak 19 was isolated in pure form by repeated h.p.l.c. This material gave a u.v.-visible spectrum with two maxima at 378 and 430nm typical for osazones. An additional structure analysis, however, was not possible with the limited amounts of material available.

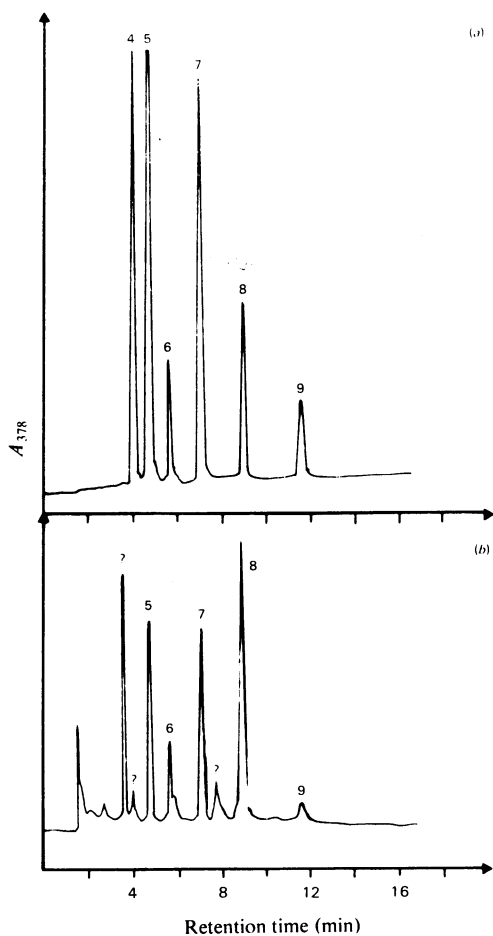


Fig. 3. Identification by h.p.l.c. of the alk-2-enals formed by ADP- Fe^{2+} -stimulated peroxidation of microsomes. Incubation and general work-up were as described in the legend to Table 2. (a) H.p.l.c. of a reference mixture of dinitrophenylhydrazone derivatives of alk-2-enals with chain length of four (but-2-enal), five, six, seven, eight and nine (non-2-enal) carbon atoms. (b) H.p.l.c. of alk-2-enals obtained by t.l.c. on MgO from the non-polar carbonyl compounds of the supernatant samples (see Fig. 1). Operating conditions were all identical with those in Fig. 2, except that elution was performed with methanol/water (9:1, v/v).

(e) Time course of carbonyl production during microsomal peroxidation by ADP- Fe^{2+} . Similar separations to that shown in Fig. 1 were performed after various incubation times (0, 5, 10, 15, 30 and 60 min) at 37°C. The results showed that, in general, the production of most aldehydes, as measured by the peak height, closely follows the time curve of malonaldehyde formation over the first 20 min of incubation at 37°C; after this time, the concentrations of most aldehydes remained constant, whereas the concentration of malonaldehyde continued to increase slowly in extent up to 30–60 min.

(f) Polar carbonyl compounds in microsomal fractions after ADP- Fe^{2+} -stimulated lipid peroxidation. Fig. 4 shows chromatograms of the dinitrophenylhydrazone derivatives of the polar carbonyl compounds recovered from the microsomal supernatant samples and sediments respectively. Compared with the non-polar carbonyl compounds, the peak patterns were much less complex and showed only 12 (supernatant) and seven (sediment) clearly resolved peaks. If a gradient elution was performed, a number of additional minor peaks were visible. Up to now, however, we have been unable to isolate sufficient amounts of any peak material in pure form for further structure analysis; therefore the identity of these carbonyl compounds has not been established. Nonetheless, from the chromatographic behaviour during t.l.c. separation (see the Experimental section) it is obvious that these carbonyl compounds must possess additional polar groups such as, for example, a carboxylic acid or a phospholipid residue.

Reaction of aldehydes with thiobarbituric acid

A variety of aldehydes detected by h.p.l.c. as products of lipid peroxidation were allowed to react with thiobarbituric acid to determine their molar absorption coefficients. The results obtained are given in Table 4. It can be seen that major carbonyl products of peroxidation (n-alkanals and 4-hydroxy-alkanals) gave much smaller molar absorption coefficients than found with malonaldehyde.

Direct measurement of malonaldehyde by h.p.l.c.

Table 5 gives results obtained by the direct measurement of malonaldehyde by h.p.l.c. compared with values obtained by the thiobarbituric acid reaction. It can be seen that there is a close correspondence; this is not surprising in view of the results given in Tables 3 and 4.

Discussion

Several previously reported studies have shown that peroxidation of fatty acids results in a variety of products. For example, Fränz & Cole (1962) reported that four peaks, which reacted positively

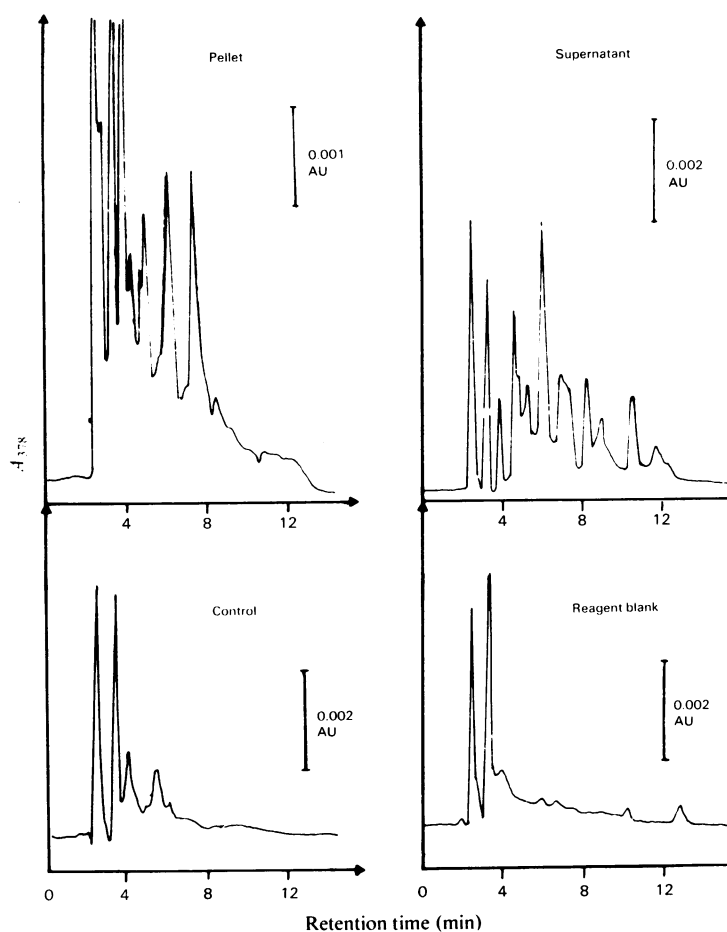


Fig. 4. H.p.l.c. separation of the dinitrophenylhydrazone derivatives of the polar carbonyl compounds obtained from the pellet and the supernatant of microsomes incubated in the presence (pellet and supernatant) and absence (control) of ADP-Fe²⁺

The reagent blank was prepared as for the supernatant sample except that microsomes were omitted. Incubation and work-up were as described in the legend to Table 2. Operating conditions: Zorbax ODS column (4.6 mm × 25 cm); isocratic elution with methanol/water (4:1, v/v); flow rate, 0.9 ml/min; 378 nm (Perkin-Elmer LC 65T), 0.02 and 0.01 AUFs; injected volume, 50 μ l.

with thiobarbituric acid, could be detected by g.l.c. after u.v. irradiation of methyl linolenate. Saslaw *et al.* (1966) recognized two products other than malonaldehyde, which were positive in the thiobarbituric acid reaction, after t.l.c. of peroxidized linolenic acid or arachidonic acid. Gutteridge *et al.* (1974) used t.l.c. to separate products of autoxidizing linoleic acid and linolenic acid: 15 separate bands stained with Schiff's reagent, and many of the components of those bands reacted positively with thiobarbituric acid. Schauenstein and colleagues have studied in great detail the aldehydic products formed by autoxidation of methyl linoleate in aqueous suspensions (see Schauenstein, 1967); several of the

products were identified and found to have considerable biological reactivity (e.g. 4-hydroxy-octenal). Tam & McCay (1970) were able to isolate as 2,4-dinitrophenylhydrazone derivatives from peroxidized microsomes by t.l.c. 13 different fatty acids having a carbonyl function; the identity of those products, however, was not established. It is evident, therefore, that peroxidation of fatty acids and of microsomes gives a mixture of products, many having carbonyl functions, and several of which react positively with thiobarbituric acid.

The application of modern h.p.l.c. techniques together with some other chromatographic methods, for example, class separation on layers of MgO, has

Table 4. *Molar absorption coefficients at 535 nm of the reaction products of different aldehydes with thiobarbituric acid*
The thiobarbituric acid test was performed in the presence (3 μM) and absence of FeSO_4 as described in the Experimental section. The TCA-solution refers to measurements made on the trichloroacetic acid-supernatant solution; the butanol extract refers to measurements made on butanol extracts of whole reaction mixtures (see the Experimental section).

| | ϵ (litre \cdot mol $^{-1}$ \cdot cm $^{-1}$) | | | |
|-----------------------------|--|--------------------|--------------------|--------------------|
| | TCA-solution | | Butanol extract | |
| | + Fe^{2+} | - Fe^{2+} | + Fe^{2+} | - Fe^{2+} |
| Alkanals | | | | |
| Propanal | 0 | 0 | 0 | 0 |
| Butanal | 0 | 0 | 0 | 0 |
| Pentanal | 0 | 0 | 0 | 0 |
| Hexanal | 0 | 0 | 0 | 0 |
| Alk-2-enals | | | | |
| Nonenal | 90 | 66 | 68 | 56 |
| Undecenal | 30 | 14 | 34 | 10 |
| Alka-2,4-dienals | | | | |
| Heptadienal | 280 | 160 | 248 | 168 |
| Nonadienal | 184 | 48 | 108 | 52 |
| 4-Hydroxyalk-2-enals | | | | |
| Hydroxyoctenal | 124 | 119 | 90 | 63 |
| Hydroxynonenal | 80 | 47 | 63 | 38 |
| Hydroxyundecenal | 38 | 12 | 57 | 17 |
| Malonaldehyde | | | | |
| Sinnhuber & Yu (1958) | | 156 000 | | |
| Placer <i>et al.</i> (1966) | | 152 000* | | |
| Slater & Sawyer (1971) | | 149 000 | | |
| Jordan & Schenkman (1982) | | 156 000 | | |

* Measured at 531 nm.

Table 5. *Analysis of free malonaldehyde by h.p.l.c. in a microsomal suspension in the presence of ADP- Fe^{2+}*

Values obtained by the thiobarbituric acid method are given for comparison. Analysis by h.p.l.c. was performed with both the complete incubation mixture (20 μl of microsomal suspension injected directly into the h.p.l.c. column) and the supernatant solution obtained after addition of 50 μM -Desferal at the indicated times followed by centrifuging at 100 000 g. The thiobarbituric acid reaction was performed with the complete incubation mixtures after addition of 50 μM -Desferal. (The values are from one representative incubation experiment. In other experiments a similarly good agreement of the malonaldehyde values obtained by the different methods was found).

| | Incubation time (min) | Malonaldehyde (nmol/ml of incubation mixture) | | | | | |
|---------------------------------------|-----------------------|---|------|------|------|------|------|
| | | 0 | 5 | 15 | 30 | 35 | 60 |
| H.p.l.c. complete mixture | ... | 2.3 | 17.0 | 38.0 | | 62.7 | 68.5 |
| H.p.l.c. supernatant | | 2.2 | 13.4 | 32.8 | 57.9 | — | 69.0 |
| Thiobarbituric acid, complete mixture | | 2.8 | 18.2 | 38.0 | 56.1 | — | 67.9 |

now enabled us to separate and identify many of the dinitrophenylhydrazones of the water-soluble short-chain carbonyl compounds that are formed during the ADP- Fe^{2+} -stimulated peroxidation of microsomal lipids. The significance of the present study lies in the fact that (a) for the first time the true complexity of the group of carbonyl compounds is shown, (b) the identity of many of these carbonyl compounds has been established and (c) chromatographic methods have been developed that permit the separation and identification of trace amounts of

lipid-derived carbonyl compounds, which will be useful in analysing the carbonyl patterns produced by different peroxidation systems.

The results obtained here for microsomal peroxidation stimulated by ADP- Fe^{2+} show some interesting differences to results obtained by similar h.p.l.c. methods on peroxidizing non-esterified fatty acids (Esterbauer, 1982). For example, the molar ratios of malonaldehyde to other carbonyl compounds are 1:1.7 for microsomes stimulated by ADP- Fe^{2+} , 1:38.4 for non-esterified arachidonic acid and

1:26.4 for non-esterified linoleic acid. All the aldehydes positively identified in this study (non-polar carbonyl compounds; see Fig. 1) are fragmentation products derived from the methyl end of the polyunsaturated fatty acid. Our efforts in separation and structure analysis were focused towards this group of aldehydes, since they contain substances such as 4-hydroxynonenal with high biological reactivities of interest to our other studies. The two major aldehydic fragmentation products found are hexanal and 4-hydroxynon-2-enal; other aldehydes identified include propanal, butanal, pentanal, pent-2-enal, hex-2-enal, hept-2-enal, oct-2-enal, 4-hydroxyoctenal, 4-hydroxyundecenal, alk-2,4-dienals and three carbonyl compounds (α -dicarbonyl or α -hydroxyaldehyde compounds) giving osazones with dinitrophenylhydrazine.

The counterparts of the aldehydic products derived from the methyl end of the polyunsaturated fatty acids (e.g. phospholipids containing aldehydic functions in their alkyl residues) have not been investigated in detail. Tam & McCay (1970) and Poyer & McCay (1971) have shown that aldehydes containing lipid phosphorus are formed during peroxidation of liver microsomes in the presence of ADP-Fe²⁺. It seems likely that the polar carbonyl fraction investigated by us (Fig. 4) contained 'phospholipid aldehydes' of the type described by them. The major proportion of the hydrazones of the phospholipid aldehydes, however, was not recovered by the extraction method we employed (using only a small volume of chloroform), and which was designed to allow good h.p.l.c. separations principally of the hydrazones of the non-polar carbonyl compounds.

The amount of malonaldehyde present in ADP-Fe²⁺-stimulated microsomes after 30 min at 37°C has been reported (Esterbauer & Slater, 1981) to be approx. 700 nmol/g of original liver; the value was obtained by a direct h.p.l.c. method for free malonaldehyde (i.e. not subjected to derivatization, or reaction with thiobarbituric acid). The value corresponds closely to the content estimated from the thiobarbituric acid reaction (see Table 5). Although many other substances react with thiobarbituric acid, the combination of their concentration in the peroxidized microsomal samples used here, and their molar absorption coefficients after reaction with thiobarbituric acid, ensures that a major contribution to the absorption at 535 nm is the product of the malonaldehyde/thiobarbituric acid reaction. Since (Table 2) the amount of non-malonaldehyde carbonyl compounds produced after 30 min stimulation of peroxidation with ADP-Fe²⁺ is approx. 900 nmol/g of original liver, it is clear from the above discussion that a considerable amount of carbonyl-reactive material will not be detected by the standard thiobarbituric acid test.

This conclusion has important biological implications for studies on liver injury associated with lipid peroxidation, since it appears possible that toxic aldehydes such as the 4-hydroxyalkenals may vary considerably in concentration from case to case without significantly affecting the thiobarbituric acid reaction (see Table 4).

It should be stressed here that the free malonaldehyde does not interfere with the estimation of other aldehydes as the dinitrophenylhydrazone derivatives, since it reacts with dinitrophenylhydrazine to give a pyrazole derivative and not a hydrazone (Kwon & Watts, 1964).

The medium-chain-length aldehydic products are partly soluble in water (water solubility of hydroxynonenal, 6.6 g/litre; Esterbauer & Weger, 1967) and less lipophilic than the parent unaltered phospholipids. In consequence, they can escape, at least partially, from the lipid membrane and diffuse into the surrounding medium: after 30 min incubation in the presence of ADP-Fe²⁺ their distribution (Table 2) between the microsomal sediment and the associated supernatant solution was in the ratio of 1:1. In terms of concentrations, it can be estimated that the microsomal supernatant contained approx. 20 μ M aldehydes other than malonaldehyde (Table 2); the concentration of these aldehydes within the membrane lipid layer, although not exactly known, is certainly much higher. On the other hand, malonaldehyde produced during the peroxidative degradation of the microsomal lipids does not remain in the membrane but is released almost completely into the medium as shown previously (Esterbauer & Slater, 1981). From these differences in distribution characteristics it can be concluded that the local membrane concentrations of aldehydes of medium chain length (hydroxyalkenals, hexanal, alk-2-enals) are much greater than that of malonaldehyde, and therefore these aldehydes are more likely than malonaldehyde to produce inhibitions of membrane-bound enzymes. This is supported by other studies (Benedetti *et al.*, 1980) that show that 4-hydroxynonenal (100 μ M) significantly inhibits microsomal glucose 6-phosphatase or cytochrome P-450, whereas malonaldehyde, although otherwise a very reactive molecule, does not, even at much higher concentration.

Previous reports (Schauenstein *et al.*, 1977; Dianzani, 1979, 1982; Benedetti *et al.*, 1980, 1981; Esterbauer, 1982) have emphasized the high biological reactivity of the 4-hydroxyalkenals such as 4-hydroxy-pentenal, -octenal and -nonenal. The latter substance has been found to inhibit enzymes such as adenylate cyclase and S-adenosylmethionine decarboxylase in the 1–100 μ M concentration range; malonaldehyde had no effect even at 5 mM (Dianzani, 1982). Such findings stress again that malonaldehyde estimations alone are not a reliable

index of the likely cytotoxic effects resulting from lipid peroxidation.

We are grateful to the National Foundation for Cancer Research for financial support.

References

- Benedetti, A., Comporti, M. & Esterbauer, H. (1980) *Biochim. Biophys. Acta* **620**, 281–296
- Benedetti, A., Barbieri, L., Ferrali, M., Casini, A. F., Fulceri, R. & Comporti, M. (1981) *Chem.-Biol. Interact.* **35**, 331–340
- Comporti, M., Saccocci, C. & Dianzani, M. U. (1965) *Enzymologia* **29**, 185–204
- Conroy, P. J., Nodes, J. T., Slater, T. F. & White, G. W. (1975) *Eur. J. Cancer* **11**, 231–240
- Dianzani, M. U. (1979) *Submolecular Biology and Cancer; Ciba Found. Symp.* **67**, 245–270
- Dianzani, M. U. (1982) in *Free Radicals, Lipid Peroxidation and Cancer* (McBrien, D. C. H. & Slater, T. F., eds.), pp. 129–151, Academic Press, London, in the press
- Dianzani, M. U. & Ugazio, G. (1978) in *Biochemical Mechanisms of Liver Injury* (Slater, T. F., ed.), pp. 669–707, Academic Press, London
- Dostal, V., Schauenstein, E., Kulnigg, P. & Smeller, E. (1974) *Z. Naturforsch.* **29c**, 76–81
- Esterbauer, H. (1982) in *Free Radicals, Lipid Peroxidation and Cancer* (McBrien, D. C. H. & Slater, T. F., eds.), pp. 101–122, Academic Press, London
- Esterbauer, H. & Schauenstein, E. (1967) *Die Nahrung* **11**, 607–621
- Esterbauer, H. & Slater, T. F. (1981) *IRCS Med. Sci.* **9**, 749–750
- Esterbauer, H. & Weger, W. (1967) *Mh. Chem.* **98**, 1994–2000
- Esterbauer, H., Zollner, H. & Scholz, N. (1975) *Z. Naturforsch.* **30c**, 466–473
- Esterbauer, H., Ertl, A. & Scholz, N. (1976) *Tetrahedron* **32**, 285–289
- Ferrali, M., Fulceri, R., Benedetti, A. & Comporti, M. (1980) *Res. Commun. Chem. Pathol. Pharmacol.* **30**, 99–112
- Fränz, J. & Cole, B. T. (1962) *Arch. Biochem. Biophys.* **96**, 382–385
- Ghoshal, A. K. & Recknagel, R. O. (1965) *Life Sci.* **4**, 2195–2209
- Glende, E. A. Jr., Hruskewycz, A. M. & Recknagel, R. O. (1976) *Biochem. Pharmacol.* **25**, 2163–2170
- Gutteridge, J. M. C., Stocks, J. & Dormandy, T. L. (1974) *Anal. Chim. Acta* **70**, 107–111
- Halliwell, B. (1981) in *Age Pigments* (Sohal, R. S., ed.), pp. 1–62, Elsevier/North-Holland Biomedical Press, Amsterdam
- Hochstein, P. & Ernster, L. (1963) *Biochem. Biophys. Res. Commun.* **12**, 388–394
- Jordan, R. A. & Schenkman, J. B. (1982) *Biochem. Pharmacol.* **31**, 1393–1400
- Kwon, T. W. & Watts, B. M. (1964) *J. Food Sci.* **29**, 294–302
- Loury, M. (1973) *Lipids* **7**, 671–675
- May, H. E. & McCay, P. B. (1968) *J. Biol. Chem.* **243**, 2288–2295
- McDonald-Gibson, R. G. & Young, M. (1974) *Clin. Chim. Acta* **53**, 117–126
- Mead, J. F. (1976) in *Free Radicals in Biology* (Pryor, W. A., ed.), vol. 1, pp. 51–68, Academic Press, New York
- Morrison, W. R. & Smith, L. M. (1964) *J. Lipid Res.* **5**, 600–608
- O'Brien, P. J. & Rahimtula, A. (1975) *J. Agric. Food Chem.* **23**, 154–158
- Orrenius, S., Dallner, G. & Ernster, L. (1964) *Biochem. Biophys. Res. Commun.* **14**, 329–334
- Plaa, G. L. & Witschi, H. (1976) *Annu. Rev. Pharmacol.* **16**, 125–141
- Placer, Z. A., Cushman, L. L. & Johnson, B. C. (1966) *Anal. Biochem.* **16**, 359–364
- Poyer, J. L. & McCay, P. B. (1971) *J. Biol. Chem.* **246**, 263–269
- Rachelsperger, M. (1980) Doctoral Thesis, University of Graz, Graz, Austria
- Recknagel, R. O. & Glende, E. A. (1973) *C.R.C. Crit. Rev. Toxicol.* **2**, 263–297
- Reynolds, E. S. & Moslen, M. T. (1980) in *Free Radicals in Biology* (Pryor, W. A., ed.), vol. 4, pp. 49–90, Academic Press, New York
- Saslaw, L. D., Corwin, L. M. & Waravdekar, V. S. (1966) *Arch. Biochem. Biophys.* **114**, 61–66
- Schauenstein, E. (1967) *J. Lipid Res.* **8**, 417–428
- Schauenstein, E. & Esterbauer, H. (1979) in *Submolecular Biology of Cancer; Ciba Found. Symp.* **67**, 225–244
- Schauenstein, E., Esterbauer, H. & Zollner, H. (1977) *Aldehydes in Biological Systems: Their Natural Occurrence and Biological Activities*, Pion, London
- Schwartz, D. P. & Parks, O. W. (1963) *Microchem. J.* **7**, 403–406
- Seeber, S., Warnecke, P. & Weser, K. (1969) *Z. Krebsforsch.* **72**, 137–143
- Sinnhuber, R. O. & Yu, T. C. (1958) *Food Technol.* **12**, 9–12
- Slater, T. F. (1966) *Nature (London)* **209**, 36–40
- Slater, T. F. (1968) *Biochem. J.* **106**, 155–160
- Slater, T. F. (1972) *Free Radical Mechanisms in Tissue Injury*, Pion, London
- Slater, T. F. & Sawyer, B. C. (1971) *Biochem. J.* **123**, 805–814
- Tam, B. K. & McCay, P. B. (1970) *J. Biol. Chem.* **245**, 2295–2300
- Thiele, E. H. & Huff, J. W. (1964) *Arch. Biochem. Biophys.* **104**, 468–472
- Weddle, C. C., Hornbrook, K. R. & McCay, P. B. (1976) *J. Biol. Chem.* **254**, 4973–4978