Thermal Decomposition of Individual Positional Isomers of Methyl Linoleate Hydroperoxide: Evidence of Carbon-Oxygen Bond Scission

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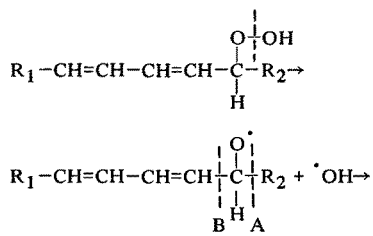
ABSTRACT

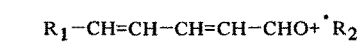
The methyl ester of individual positional isomers of linoleate hydroperoxide were prepared by an enzymic oxidation of linoleate. On injection onto a gas chromatographic column they were thermally decomposed and the resulting volatile components analyzed. The major (67-80% yield on a molar basis) cleavage products were found to be hexanal, methyl octanoate, 2,4-decadienal isomers, and methyl 9-oxononanoate. Both the 9 and 13 isomers of linoleate hydroperoxide gave rise to these same four compounds, an observation suggesting carbon-oxygen scission in their decomposition. This was confirmed by using very pure individual isomers obtained by high performance liquid chromatography. The involvement of an isomerization reaction of the hydroperoxides is discussed.

INTRODUCTION

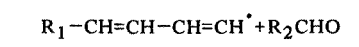
The concept that oxidative rancidity is caused by the oxidation of unsaturated fatty acid moieties whose subsequent decomposition gives rise to volatile compounds is now widely accepted. While the manner of oxidation of unsaturated fatty acids by enzymic oxidation and autoxidation to form either single isomers or mixtures of isomers of hydroperoxides is well established, the pathway by which they decompose to yield cleavage products is still poorly understood. It is generally contended (1-6) that the first step of the decomposition of an unsaturated fatty acid hydroperoxide is the homoytic cleavage of the oxygen-oxygen bond to yield an alkoxy and a hydroxy radical. The further decomposition of the alkoxy radical involving carbon-carbon bond scission forms a major pathway for the generation of cleavage compounds. As an example, the possible products that can be obtained from such decomposition of the 9 and 13 positional isomers of the hydroperoxides of methyl linoleate are illustrated in the following scheme:



A scission gives



B scission gives



For the 9-isomer $R_1 = C_5H_{11}$, $R_2 = C_7H_{15}COOCH_3$

For the 13-isomer $R_1 = C_7H_{15}COOCH_3$, $R_2 = C_5H_{11}$

Scission of the C-C bond on the side of the oxygen-bearing carbon atom away from the olefinic linkages (A scission) will result in the formation of an aldehyde and an alkyl radical, while scission of the C-C bond between the vinyl function and the carbon atom bearing the oxygen atom (B scission) gives rise to a vinyl radical and an aldehyde. Thus, for the 9-hydroperoxide the theoretically derived carbonyl compounds of low mol wt are 2,4-decadienal and methyl 9-oxononanoate and for the 13-hydroperoxide, hexanal and methyl 13-oxotrideca-9,11-dienoate. Other products may be formed by further reaction of the radicals. Abstraction of a hydrogen atom by the alkyl radicals will give rise to pentane (from the 13-isomer) and methyl octanoate (from the 9-isomer). It has also been suggested (3,4,6) that reaction of a hydroxyl radical with an alkyl, a vinyl, or a 1,3 butadienyl radical gives rise to an alcohol, an alkanal, or a 2-alkenal, respectively, the aldehydes being formed via their enolic forms. However, despite the overwhelming evidence, some of which has been quoted above, the validity of the theory that carbonyl compounds are formed by the scission of monohydroperoxides has recently been questioned (7).

In previous studies (4,6,8,9) in this area, autoxidized fats or unsaturated fatty acid derivatives have been used. In an autoxidized fat, the number of peroxidic species are invariably large owing to the presence of different unsaturated fatty acids that are subject to oxidation. In a model system such as autoxidized linoleate, two major positional isomers of hydroperoxides are present. On the other hand, enzymic oxidation can produce a single positional isomer of linoleate hydroperoxide. To investigate the system in its simplest form, the decomposition of the methyl esters of single positional isomers of hydroperoxides of linoleic acid obtained by lipoxygenase oxidation was studied in this investigation. The hydroperoxides were decomposed in situ by injection onto a heated precolumn section of the gas chromatographic column used in the analysis of products. This procedure is similar to that used by Evans et al. (10) for the analysis of pentane and pentane precursors in oil samples and ensures that product analysis is carried out immediately after decomposition of the hydroperoxides.

EXPERIMENTAL PROCEDURES

Materials

Unsaturated fatty acids (99% pure), their methyl esters, and lipoxygenase (lipoxidase type I) were obtained from Sigma Chemical Co. (St. Louis, MO). Silica CC-4 (Mallinkrodt) and Kieselgel H (Merck) were supplied by Camlabs (Cambridge, U.K.). Materials used for packing the column used in gas chromatography were supplied by J.J.'s (Chromatography) Ltd. (Kingslynn, U.K.).

Methyl 9-Hydroperoxy-10-trans-12-cis-octadecadienoate

The method of Galliard and Phillips (11) was used with minor adaptations for preparative work. Linoleic acid was purified by chromatography on Silica CC-4 in hexane:ether.

Potato extracts were obtained by homogenizing each of four portions (50 g) of diced potato tuber in water (100 ml). The extracts were centrifuged for 25 min at 15,000 x g and the supernatant filtered through two layers of cheesecloth. Immediately prior to incubation, the potato extracts were combined with 0.5 M acetate buffer (100 ml, pH 5.5).

The ammonium salt of linoleic acid was made by adding ammonium hydroxide (2M, 40 ml) followed by Tween 80 (1.35% w/v 400 μl) to the purified linoleic acid (700 mg) in a 1 liter round bottom flask. The linoleic acid was dispersed using a glass rod and the flask swirled to obtain an even dispersion. Excess ammonia was displaced from the flask by a stream of argon until the pH of the solution was 9-9.5. The incubation (at 25 C) was then started by adding the buffered potato extracts, and a slow stream of oxygen was passed through the mixture for a 60 sec duration at 5 min intervals. After 30 min, the incubation was stopped by acidification to pH 3 using 2M HCl. The aqueous emulsion was then extracted with ether (3 x 400 ml), centrifugation being used to separate the layers when necessary; the ethereal extracts were washed with water (2 x 400 ml) and dried with anhydrous $MgSO_4$. Solvent volume was then reduced to 100 ml and the products methylated with diazomethane. The products were purified on a column (2.5 cm x 12 cm) of Kieselgel H (20 g). Stepwise elution was used (5% ether in hexane [200 ml] followed by 7.5% ether in hexane). Fractions (10 ml) were taken and monitored for products by UV absorption and ferrous thiocyanate spot test.

Fractions 58-67 contained methyl 9-hydroperoxy-10-*trans*-12-*cis*-octadecadienoate (the "9-hydroperoxide," 205 mg). The product was homogeneous on thin layer chromatography (silicic acid, developed with 30% ether in hexane). Its peroxide content (assayed by iodometric titration [12]) was 2.91 mmoles/g, 95% of theoretical), and its molecular extinction coefficient at 234 nm was 26,000. Assay of the isomeric composition of the product by the mass spectroscopic method described below showed that the product was a 95:5 mixture of 9 and 13 isomers.

Methyl 13-Hydroperoxy-9-cis-11-trans-octadecadienoate

This was obtained by oxidation of ammonium linoleate using commercial soybean lipoxygenase (lipoxidase type I) in borate buffer (0.1M pH 10.5). The incubation, isolation, methylation, and purification procedures were identical with those described above, except that the enzyme (150 mg) was added in three aliquots during the incubation, which was carried out at 20 C for 30 min. The yield of the "13-hydroperoxide" was 316 mg. Its 9 to 13 isomeric composition was 7:93.

Hydroxydienes and Oxodienes

The methyl esters of the hydroxy compounds corresponding to the hydroperoxides (hydroxydienes) were obtained by sodium borohydride reduction (in methanol) followed by chromatography on Kieselgel H.

The oxodienes were obtained as a by-product of the enzymic oxidation and subsequent methylation. They were isolated as a distinct band (fractions 45-53) separated from the hydroperoxides in the final purification on Kieselgel chromatography.

2,4-Decadienals

A mixture of 2-*trans*-4-*cis* and 2-*trans*-4-*trans*-decadienals was prepared according to the method of Ohlof and Pawlak (13).

Methyl 9-Oxononononoate (Azelaaldehydate)

This was prepared by reductive ozonolysis of methyl oleate.

Mass Spectral Analysis of Isomeric Ratios

The 9 to 13 ratio of isomers present in a sample of hydroperoxide was assayed by reducing 2 mg of the compound with sodium borohydride (2 mg) in methanol (2 ml) followed by hydrogenation using Adam's catalyst. The resultant mixture of hydroxystearates was purified by chromatogtaphy on a column (4 mm x 4 cm) of Kieselgel H and the fractions containing the hydroxystearate pooled and the mass spectrum determined. The 9 to 13 isomeric ratio is taken as the ratio of the peak intensities at m/e 155 and 211.

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) on Partisil-10 (50 cm x 4.6 mm) was used to separate and analyze mixtures of hydroperoxides as described previously (14). Apart from the equipment already described, a Jobling Pump was used to deliver solvent at 3.7 ml/min and the effluent monitored by a Cecil 272 spectrophotometer with a 30 μl flow cell.

For preparation of the very pure (> 99%) 9 and 13 hydroperoxides, samples (200 μg) containing predominantly the 9 or 13 isomers obtained from enzymic oxidation of linoleate were repeatedly injected and the separate components collected. The solutions containing the pure isomers were then concentrated and the isomeric composition redetermined by HPLC. The determination was repeated immediately prior to injection of the sample onto the gas chromatogram. Both determinations showed that the purity of the components was > 99%).

Gas Chromatography

A Pye series 104 model 84 gas chromatograph fitted with flame ionization and electron capture detectors was used. A standard glass column (1.55 m x 4 mm ID) was packed with 10% w/w of Carbowax 20M on diatomite C (100-120 mesh, acid washed and DMCS treated). The precolumn (7 cm) was packed with 3% w/w of methyl silicone gum (General Electric E30) on a heavily sintered and siliconized support (80-100 mesh chromosorb G [Johns-Manville], acid washed and DMCS treated). The stationary phase for the precolumn was chosen because of its thermal stability and chemical inertness, the support for its small surface area (compared with diatomite preparations) and robustness. This minimized the fracture of particles and exposure of fresh adsorptive and catalytically active sites upon repeated injection or mechanical handling. Moreover, the high density of the support and hence its high thermal capacity minimized the temperature drop caused by evaporation of the sample upon injection.

The precolumn was entirely surrounded by a tubular injection heater. Unless otherwise stated, this was kept at 160 C for the injection of samples. The flow rate of carrier gas was 40 ml/min and the oven temperature was programmed from 70 C to 200 C at 4 C/min and then held isothermally at 200 C. The injection heater was switched off when the oven temperature reached 200 C. To purge the column of any late running material, it was held at the maximum temperature for at least 7 hr after each chromatogram. A routine sequence of timed operations was used to reduce the column temperature from its maximum (200 C) to the initial temperature of the programmed chromatogram prior to each injection.

Samples (200 μg in 8 μl hexane) were injected into the precolumn using a syringe fitted with an 11 cm long needle. As the sample was injected 2 cm below the top of the pre-

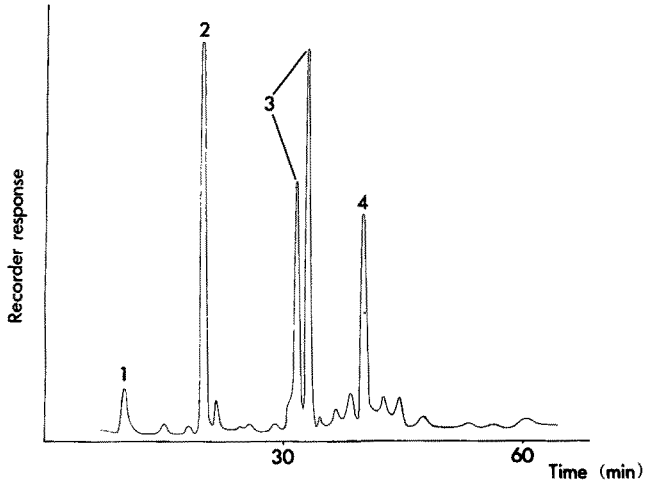


FIG. 1. Cleavage products from the decomposition of the methyl ester of linoleate hydroperoxide obtained from oxidation of linoleic acid by potato lipoxygenase. Major components: 1) hexanal, 2) methyl octanoate, 3) isomers of 2,4-decadienal, and 4) methyl 9-oxononanoate.

column packing, the carrier gas was preheated and there was no blowback from the vaporized sample. Before injection, the compounds were evacuated (10^{-4} τ) at 0 C for 1 hr to remove any possible interfering volatile compounds. Samples for injection were then made up by adding redistilled hexane. Quantitative estimateion of hexanal, methyl octanoate, 2,4-decadienals, and methyl 9-oxononanoate was carried out by co-injecting a sample of hydroperoxide with known amounts of authentic specimens and measuring the extent of peak area enhancement. The yields of each component were then calculated based on the amount (200 μg) of hydroperoxides injected and expressed both as percentage and molar yields.

Combined gas chromatography/mass spectrometry was carried out by attaching the chromatographic system to a GEC/AEI MS 902 mass spectrometer and the resultant data processed as previously described (15).

RESULTS

By injecting the hydroperoxide onto the heated precolumn consisting of a nonpolar and inert material, decomposition takes place under essential anaerobic conditions with minimal catalytic effect of metal ions. A typical chromatogram of the compounds resulting from the decomposition of a sample containing the 95:5 mixture of 9 to 13 isomers is shown in Figure 1. Owing to the use of solvents in the preparation of samples, very volatile compounds such as pentane chromatographed with the solvent and were not detected as products. The chromatogram shows only those products that appear within the first hour of the chromatogram (this includes 32 min of temperature programmed GC).[1] Thus only those volatile compounds that separate rapidly from the decomposing hydroperoxide are analyzed. Repeated identical injections gave rise to a maximal variation of 10% in the amounts of components produced while the relative proportions of the components did not vary by more than 5%. When the temperature of the precolumn was increased to 200 C for the injection, the yields and relative proportions did not vary beyond these limits. On the other hand, lowering the injection temperature to 100 C resulted in considerable reduction in the yield together with broadening of the peaks. These observations suggest that decomposition of the hydroperoxides is essentially complete at the temperature (160 C) at which these studies were made. The major components of the chromatogram were identified by GC/MS and by co-injection of authentic samples. They are, in order of increasing retention time (Figure 1 components 1 to 4), hexanal, methyl octanoate, isomers of 2,4-decadienal, and methyl 9-oxononanoate (azelaaldehydate).

The hydroxydiene and oxodiene analogues of the hydroperoxides (i.e., with hydroxyl and keto groups replacing the hydroperoxide function) were subjected to the same conditions of decomposition. No significant amounts of cleavage products were observed. The cleavage products are therefore obtained from the hydroperoxides without the intermediacy of these oxygenated linoleate compounds.

A sample of hydroperoxide obtained from soybean lipoxygenase oxidation of linoleate and predominating in the 13-isomer (9 to 13 isomeric ratio of 7:93) decomposed to give the same mixture of components. The quantities of each of the major components obtained in the above cases are expressed both as percentages by weight of hydroperoxide and as molar percentage yields in Table I.

Because of the ease with which hydroperoxides can undergo isomerization upon storage (16) and the difficulties encountered in assaying isomeric ratios using the mass spectral method (17), changes in the isomeric ratio of the hydroperoxides might have occurred between their isolation and decomposition without being detected. HPLC, which has been used successfully as a rapid method of separation and analysis of the hydroperoxides (14), was used to produce pure isomers to confirm the above results. This technique was used both to produce small quantities of very pure (> 99%) single isomers as well as to confirm their isomeric purity immediately prior to decomposition. Owing to the difficulties involved in obtaining quantities of the pure materials and in preventing isomerization before injection onto the GC, no attempt was made in these experiments to identify and quantitate the decomposition products by co-injection of authentic samples. Their identities were established by their retention times which were compared with those in chromatograms where the identities of the components had been established. Results obtained from the decomposition of the pure 9 and 13 isomers are expressed as percentage peak areas based on the total peak area of the four major components (Table II).

[1] Owing to the difficulty of analyzing accurately components that separate during the temperature programmed GC as well as peaks that appear after several hours of chromatography at maximum temperature, components with very low volatility, e.g., methyl 13-oxotrideca-9,11-dienoate, are not included in the analysis. Since yields are calculated in *molar* terms, the components that are analyzed comprise the major part (67-80%) of the injected hydroperoxide.

TABLE I

Cleavage Products from the Decomposition of Linoleate Hydroperoxides

Component	Percentage (w/w) yield		Molar percentage yield	
	9-Hydroperoxide	13-Hydroperoxide	9-Hydroperoxide	13-Hydroperoxide
1. Hexanal	1.7	8.25	5.5	26.9
2. Methyl octanoate	5.0	4.3	10.3	8.9
3. 2,4-Decadienals	20.0	12.5	42.9	26.8
4. Methyl azelaaldehydate	10.5	15.2	18.4	26.6

DISCUSSION

Under the conditions employed for the decomposition of hydroperoxides, a large proportion (Table I) of products are cleavage compounds. The total yield of these products is affected by the possibility that methyl octanoate and 2,4-decadienal can arise from the same hydroperoxide molecule. However, the molar percentage yields of carbonyl compounds alone (67-80%) are nonetheless considerably higher than those observed in previous related studies (6,9) for which corresponding figures of 10-20% were obtained. The high proportion of cleavage compounds formed in the present instance is possibly a consequence of the anerobic conditions of the decomposition, which are substantially different from those used previously. The use of purified hydroperoxides rather than a mixture of hydroperoxides in excess unreacted linoleate also means that reactions involving unoxidized material are excluded. Since the volatile products are analyzed immediately after the in situ decomposition, their further reactions are also avoided. The results obtained by studying the thermal decomposition of linoleate hydroperoxides in isolation allow two major conclusions to be drawn. These are discussed separately below.

The major cleavage products obtained are those expected from a simple carbon-carbon bond scission adjacent to a carbon-oxygen bond at both positions 9 and 13. The composition of species with C-O bonds at these positions at the point of decomposition is the subject for detailed analysis below. Of the four major components, three (the aldehydes) are derived by a single cleavage of an alkoxy radical, while the formation of the fourth (methyl octanoate) requires the abstraction of a hydrogen atom. Remarkable is the absence of 2-alkenals (especially 2-heptenal and 2-nonenal), which have been found previously in substantial quantities in the decomposition of autoxidized methyl linoleate, trilinolein, and linoleate containing fats (5,9). Both heptenal and nonenal were shown to be stable to the gas chromatographic system used in this study by injection of authentic samples, and their absence in substantial quantities was further confirmed by examination of the mass spectra of the minor components. These are not sufficiently distinctive to allow identification of the minor products; they are, however, markedly different from the spectra of the authentic 2-alkenals. It has previously been observed in the decomposition of autoxidized linoleate systems (9,18) that high temperature causes an increase in the yield of 2,4-decadienals without affecting the yield of alkenals. The 2-alkenals are therefore a result of low temperature reactions, and the present case of anaerobic conditions and short high temperature exposure may be regarded as an extreme case so that 2,4-dienals are the only unsaturated aldehydes produced. The previously observed formation of 2-alkenals may be due to (a) further reaction of a hydroperoxide before cleavage, (b) further reaction of cleavage products, and (c) reaction between a hydroperoxide or products derived therefrom with unreacted linoleate residues. All of these alternatives, which may involve the further participation of oxygen, are minimized or excluded in the conditions used in the present investigation, which shows distinctly that 2-alkenals are not formed as a result of anaerobic pyrolysis of hydroperoxides. The overwhelming proportions of products are derived from cleavage of the hydrocarbon skeleton adjacent to the peroxide linkage, as theoretical considerations have predicted.

The most striking discovery of the present investigation concerns, however, the derivation of individual volatile compounds from a single positional isomer of linoleate hydroperoxide. An examination of the figures for the percentages of cleavage products formed from essentially pure 9 and 13 hydroperoxides (Table I) readily reveals that the products cannot result from the decomposition of the 9 and 13 isomers as distinct species. For instance, the large proportion of 2,4-decadienals theoretically derived only from decomposition of the 9-hydroperoxide, obtained from the sample predominant in the 13-isomer, indicates a degree of mobility of the oxygen functionality at positions 9 and 13. By using very pure isomers of the hydroperoxides whose isomeric purity was checked immediately prior to decomposition, the above observations were confirmed; i.e., a single positional isomer of linoleate hydroperoxide can give rise to products normally attributed to the other isomer (Table II). In particular, it shows that the 13-isomer is a *precursor* (although not necessarily the immediate precursor) of 2,4-decadienals and that the 9-isomer is a precursor of hexanal. Indeed, comparable amounts of hexanal and decadienals are formed from the 13-hydroperoxide.

TABLE II

Decomposition of Pure (99%) Individual Isomers of Linoleate Hydroperoxides

Component	Percentage of total peak area of four major components	
	9-Isomer	13-Isomer
Hexanal	1	28
Methyl octanoate	37	24
2,4-Decadienals	51	33
Methyl azelaaldehydate	12	16

Although care has been taken to establish the isomeric purity of the hydroperoxides before injection, the above apparently anomalous results could be explained by the rapid isomerization of the individual isomers, after injection and prior to decomposition. We have recently shown that both the 9- and 13-isomers of linoleate hydroperoxide undergo isomerization readily at low temperatures (16). Thus, sufficient isomerization could have taken place at the point of injection to account for the results observed in this study. However, it should be noted that at the high temperatures employed, other processes as well as isomerization may be important. In particular, intermediates in the isomerization reaction as well as species derived from the hydroperoxides without cleavage of carbon-carbon bonds may also give rise to products. For example, after cleavage of the carbon-oxygen bond (at the carbon atom bearing the hydroperoxy group) which must take place during isomerization, a species may be generated which is capable of forming cleavage products without reforming a hydroperoxide.

Although detailed mechanistic questions can only be answered by further investigations, the results presented here show that not only the scissions of the oxygen-oxygen and carbon-carbon bonds take place during linoleate hydroperoxide decomposition, as is generally accepted, but also that carbon-oxygen bond scission occurs. This is clearly shown by the large quantities of 2,4-decadienals formed from the 13-isomer. Studies in the bond strengths of hydroperoxides have shown that the C-O bond in *saturated* organic hydroperoxides is considerably stronger than the O-O bond (19). The results reported here, as well as our previously reported results (16) on the isomerization of linoleate hydroperoxides, indicate that the C-O bond is much weaker when adjacent to a butadienyl group. It is therefore no longer purposeful to discuss the decomposition of linoleate hydroperoxide in terms of A & B scissions, since the point of reference (i.e., the C-O bond) is itself cleaved. The proportions of products formed are dependent on the kinetic parameters that govern these three bond scissions and are no longer solely related to the proportions of positional isomers in the starting mixture, as has been clearly demonstrated here.

The relative degrees of the three bond scissions and hence the distribution of cleavage products will vary with

the conditions of the decomposition of the hydroperoxides. Thus, the variation in the proportions of cleavage products observed in previous studies (6,20,21) is due, at least in part, to the varying degrees of carbon-oxygen bond scission that accompanied the carbon-carbon and oxygen-oxygen bond scissions.

Although the present study has been confined to linoleate, oleate hydroperoxides also undergo isomerizations (H.W.-S. Chan and G. Levett, unpublished data). Carbon-oxygen scission is also not necessarily confined to the decomposition of monohydroperoxides. These questions, as well as the mechanism of the isomerization reactions, are currently under investigation.

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