Products of a linoleic hydroperoxide-decomposing enzyme of alfalfa seed

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Abstract Alfalfa seeds and seedlings contain an enzyme that catalyzes a reaction with the 13- and 9-hydroperoxides of linoleic acid to form 13-hydroxy-10-oxo-*trans*-octadecenoic acid and 9-hydroxy-12-oxo-*trans*-10-octadecenoic acid, respectively. When commercial lipoxygenase is used to generate the hydroperoxides, the above acids appear in about 2:1 proportions, respectively. The products of the action of the enzyme on the hydroperoxides were stabilized for analysis by reduction with H_2 and $LiAlH_4$. Trimethylsilyl derivatives of reduced products were analyzed by combined gas–liquid chromatography–mass spectrometry. Specific deuterium labeling permitted definite location of the oxo functions. $^{18}O_2$ labeling experiments showed that the oxygens of both the oxo and the hydroxyl functions were derived from the hydroperoxide. Retention of both oxygens suggests that the reaction proceeds through a cyclic epiperoxide followed by a ketohydroxy-forming rearrangement. No products of hydroperoxide isomerase were found in reactions catalyzed by the crude enzyme from alfalfa seeds.

Supplementary key words lipohydroperoxidase · lipohydroperoxide isomerase · linoleic acid metabolism · hydroperoxides · oxygenated fatty acids · combined gas–liquid chromatography–mass spectrometry

The products of seed enzyme degradation of 9- and 13-hydroperoxides of linoleic acid have received considerable attention in recent years. Zimmerman (1) reported a flaxseed enzyme, hydroperoxide isomerase, which catalyzed the formation of 12-oxo-13-hydroxy-*cis*-9-octadecenoic acid and 9-hydroxy-10-oxo-*cis*-12-octadecenoic acid from 13-hydroperoxy-*cis*-9-*trans*-11- and 9-hydroperoxy-*trans*-10-*cis*-12-octadecadienoic acids. Veldink, Vliegenthart, and Boldingh (2, 3) found only the 12-oxo-13-hydroxy-*cis*-9-octadecenoic acids as products of the degradation of linoleic hydroperoxides by flaxseed enzymes. In addition, the latter authors reported the incorporation of only one atom of ^{18}O into position 12 of that product when -^{18}O-^{18}OH-labeled linoleic hydroperoxide was used as substrate. Gardner (4) reported the occurrence of both the 12-oxo-13-hydroxy and 9-hydroxy-10-oxo isomers as products of a corn germ hydroperoxide isomerase. In addition, he identified other products, two of which are 13-hydroxy-10-oxo-*trans*-11-octadecenoic acid and (tentatively) the 9-hydroxy-12-oxo-*trans*-10-octadecenoic acid.

The identification of the latter two products as the major products of an alfalfa seedling enzyme by different methods is the subject of this report. In addition, we report here the results of incorporation of $^{18}O_2$ into the products of the reaction. We have adopted the name lipohydroperoxidase (5) for this enzyme, and we propose that two different enzymes are forming the Zimmerman (1) products and the products identified here.

MATERIALS AND METHODS

Seed germination

Alfalfa seeds (Ranger variety, purchased from Agway, Inc., State College, Pa.) were germinated on moistened filter paper for 4 days at 20°C. Petri dishes, 13.5 cm, seeded at the rate of 2 g of seed per dish, were used as the germination chambers.

Product preparation

The seedlings from 2 g of seed were homogenized in a Waring Blendor in 50 ml of 0.1 M phosphate buffer, pH 6.8. Homogenates were centrifuged at 12,000 *g* for 30 min at 0°C. The supernatant liquid (crude enzyme) was filtered through a glass wool plug and used immediately after preparation.

The substrate solution contained 16.1 μmoles of sodium linoleate (Supelco, Inc., Bellefonte, Pa.) and 15 μl of Tween 20 in 11 ml of water. The linoleate was oxidized to linoleate hydroperoxide by adding the substrate solution to 1 mg of crystalline soybean lipoxygenase (Worthington) dissolved in 39 ml of water. The solution of enzyme and

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Abbreviations: TLC, thin-layer chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; TMS, trimethylsilyl.

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substrate (50 ml in a stoppered 250-ml volumetric flask) was purged with oxygen for 5 min and shaken on a wrist-action shaker for 30 min. The mixture of 9- and 13-linoleic hydroperoxides could also be prepared from lipoxygenase isolated from acetone powders of alfalfa seeds as described in an earlier report (6), but soybean lipoxygenase was used throughout this study. The linoleic hydroperoxide substrate was judged to be pure by TLC. If the hydroperoxide solution was allowed to stand, or if longer reaction times were used, impurities with both smaller and larger R_F values on a thin-layer chromatogram appeared.

Equal volumes of the hydroperoxide solution and the crude enzyme supernatant liquid were mixed and shaken for 15 min at room temperature. The reaction was stopped by careful addition of HCl to pH 3. The products were recovered by three sequential extractions with 100-ml portions of peroxide-free ethyl ether. The ether from the three extractions was combined, dried over anhydrous Na_2SO_4, and concentrated in a rotary evaporator at room temperature to 0.5 ml.

Hydroperoxide-decomposing activity may be assessed by following the decrease of 234-nm absorbance (linoleic hydroperoxide, ϵ_{max} = 24,500) in a cuvette containing a solution made by mixing 0.1 ml of the crude enzyme preparation, 0.1 ml of the above hydroperoxide preparation, and 0.8 ml of 0.1 M phosphate buffer, pH 6.8. If the enzyme preparation was heated to 100°C for 1 min and incubated with substrate, no decrease in absorbance at 234 nm was observed.

Product isolation

The products were applied as a streak to 20 × 20 cm preparative thin-layer plates of silica gel G (1 mm thick) (Brinkmann). Partial separation of the mixture of products was achieved with a developing solvent of ethyl ether–hexane–acetic acid 80:20:1 (v/v/v). Bands were visualized by charring a 1-cm strip with sulfuric acid on one edge and by spraying the opposite margin with 2,4-dinitrophenylhydrazine. Products were recovered by scraping the silica gel containing the bands from the plate and extracting the powder several times with peroxide-free anhydrous ethyl ether. The solvent was removed with a stream of dry nitrogen. In typical runs, 50–55% of dried ether extracts was recovered as compounds A_9 and A_{13} ($A_{9\ 13}$) (Fig. 1). See Results for explanation of compound designations. The remaining portion of the applied sample was located at the origin (10–20%), in a slower moving spot than A_{9+13} (10–15%), and in a spot on the chromatogram between $A_{9+\ 13}$ and unreacted lipohydroperoxide (5–10%).

Chemical reactions

The products thus isolated were hydrogenated in absolute ethanol in proportions of about 1 mg of PtO_2 to 10 mg of unsaturated fatty acid under a stream of H_2 at atmospheric pressure. In the case of compound A (see Results), the completion of the reduction could be determined by loss of α,β-unsaturated ketone absorption at 200 nm.

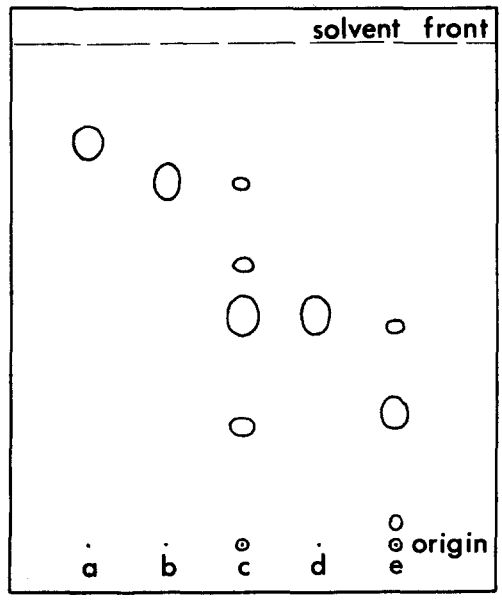

Fig. 1. Tracing of a thin-layer chromatogram showing major products of reactions studied. Lane a, linoleic acid; lane b, linoleic hydroperoxide (LHP_{9+13}); lane c, products of lipohydroperoxidase with LHP_{9+13} as substrate; lane d, isolated compounds A_{9+13}; lane e, compounds B_{9+13} (large spot). Solvent system: ether–hexane–acetic acid 80:20:1 (v/v/v) developed on a 0.2-mm silica gel G-coated plate; visualized after carbonization by heating a plate sprayed with sulfuric acid.

$LiAlH_4$ (Metal Hydrides Inc., Beverly, Mass.) reduction was carried out by first dissolving the compound to be reduced in dry peroxide-free ether (kept over sodium) in a ratio of about 0.1 mg to 10 ml of solvent. A freshly prepared saturated ethereal solution of $LiAlH_4$ (7) was added to the compound dropwise until further addition yielded no evolution of H_2. Care was taken not to add a large excess of $LiAlH_4$ because of competing side reactions such as dehydration.

Reduced and hydrogenated compounds were repurified by preparative TLC using the same conditions as for the initial isolation. Compounds eluted from the silica gel were derivatized with bis(trimethylsilyl)acetamide (Supelco) and were chromatographed with a Varian Aerograph 1740 GLC apparatus on a 150-cm glass column containing 1% SE-30 on 80–100 mesh Celite with a flow rate of 25 ml/min of helium at 200°C.

Combined gas–liquid chromatography–mass spectrometry was performed with an LKB 9000 instrument with a 150-cm column containing 1% SE-30 on 80–100 mesh Celite at an ionization potential of 20 eV and an ion source temperature of 290°C.

Preparation of ^{18}O-labeled linoleic hydroperoxide

^{18}O-labeled linoleic hydroperoxide was prepared by substituting $^{18}O_2$ (92% enriched in ^{18}O; 80% enriched in ^{18}O-^{18}O; Miles Laboratories) for $^{16}O_2$ in the oxida-

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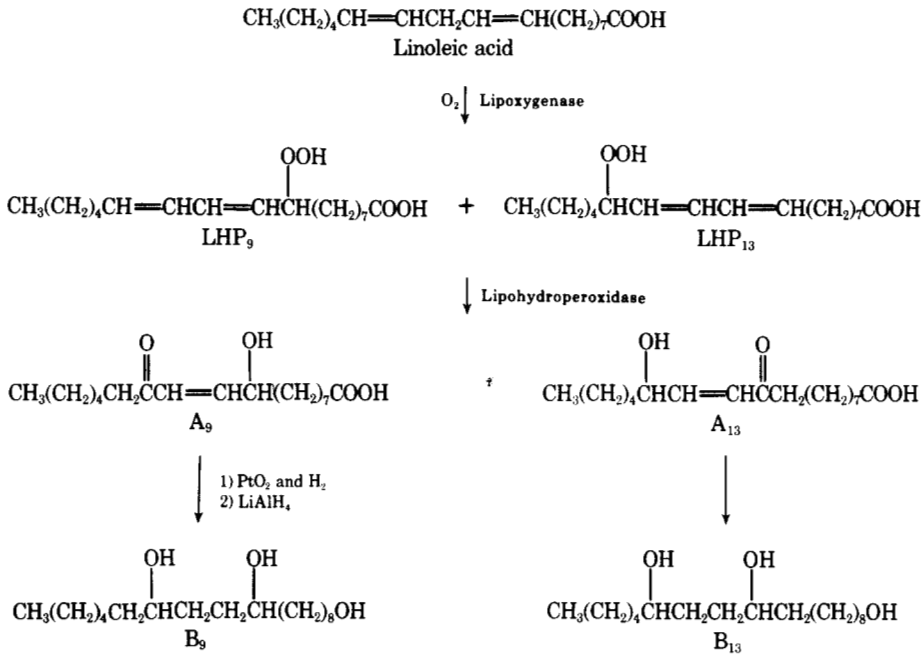
tion of linoleic acid with soybean lipoxygenase. A closed reaction vessel was designed, and it was connected to a Teppler mercury displacement pump for gas transfer. The reaction conditions were identical with those described above. The hydroperoxide reaction with crude enzyme, product recovery, and preparation of derivatives for GLC–MS analysis were identical with the methods outlined above.

RESULTS AND DISCUSSION

Evidence is presented in this report for the structures of the products of a linoleic hydroperoxide-destroying enzyme, lipohydroperoxidase. The two isomeric linoleic hydroperoxides (6, 8) designated LHP_9 and LHP_{13} are the substrates for the enzyme. Products (and derivatives) of the isomers are designated by the subscript 9 or 13 according to their hydroperoxide derivation. The products of the enzymic reaction and chemical derivatives are shown below.



A typical TLC separation of the products of lipohydroperoxidase reaction is shown in Fig. 1, lane c. A_9 and A_{13} appear as one orange-red spot with acidic 2,4-dinitrophenylhydrazine spray (indicative of an oxo function) and as one black spot with a sulfuric acid-char spray (lanes c and d). There are two other minor products above and below the main product A (lane c). The two minor products were not completely identified, but neither has the properties (spectral or mass spectral) of the compound reported by Zimmerman (1) and by Veldink et al. (2, 3). The reaction between linoleic hydroperoxide and heated enzyme preparation (100°C for 1 min) does not produce any degradation products detectable by TLC.

A_{9+13} isolated from preparative TLC plates had UV, IR, and NMR spectra identical with those reported by Gardner (4) for compounds isolated from corn endosperm enzymic reactions. These spectra are not discussed here except to note that A_{9+13} has a strong UV absorption ($\epsilon_{20} = 12{,}800$) at 220 nm, indicative of an α,β-unsaturated oxo function, and a 10.3-μm absorption in the IR region, indicative of a *trans* double bond. Attempts in this laboratory to completely separate by chromatography the isomers LHP_{9+13} and A_{9+13} were unsuccessful. Thus, it was necessary to analyze A_{9+13} as a mixture of two positional isomers.

When A_{9+13} was completely hydrogenated with PtO_2 and H_2 followed by reduction with $LiAlH_4$, one major spot (B_{9+13}) was evident on TLC plates (Fig. 1, lane e). Side products were always present at the origin, and, unless the reductions were carried to completion, partially reduced compounds appeared with greater R_F values than the major product. The 10.3-μm IR absorption, which is present in A_{9+13}, disappeared completely upon hydrogenation with PtO_2 and H_2.

The TMS derivatives of B_{9+13} were prepared by the addition of bis(trimethylsilyl)acetamide. Subsequent GLC analysis yielded a major peak at 6 min on a 3% SE-30 column operated at 200°C with a helium flow rate of 25 ml/min. Mass spectral analysis of the GLC peak using the combined GLC–mass spectrometer gave the results shown in Fig. 2*A*. The structures of the compounds are shown in Table 1, with the major observed fragmentation depicted by a dashed line and an m/e value.

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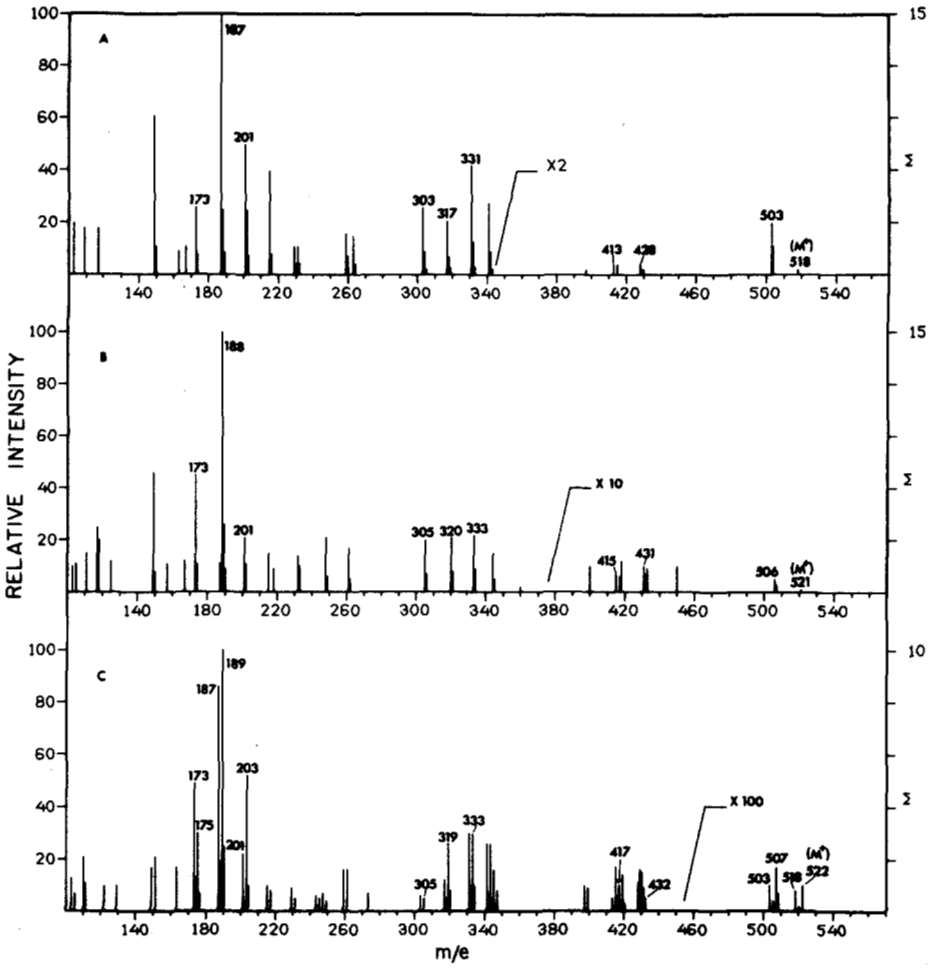

Fig. 2. Comparison of partial mass spectra of OTMS-dihydroxyoctadecanols. *A*, OTMS derivative of B_{9+13}. See Table 1, A for structure and cleavage assignments. *B*, OTMS derivative of B_{9+13} containing ^{2}H (see Table 1, B). *C*, OTMS derivative of B_{9+13} containing ^{18}O (see Table 1, C).

The general characteristics of mass spectral breakdowns of hydroxylated octadecanols derived from hydroxy fatty acids have been described in detail in an earlier report (9). The molecular weight (M) in Fig. 2*A* (cf. Table 1, A) is 518, and M−15 (*m/e* 503) and M−15−90 (*m/e* 413) are characteristic of this type of compound. The compounds reported here have similar breakdown patterns except that in this case there are spectra of two superimposed compounds with the same molecular weight. For example, in Fig. 2*A* the major peaks in the mass spectrum represent cleavages adjacent to ÇHOTMS groups on the chain of the molecule, as shown in Table 1, A. Fragments *m/e* 173 and *m/e* 187 represent cleavages at carbons 13 and 12, respectively, and the *m/e* values 303 and 317 correspond to cleavages at carbons 9 and 10 to include the 9- or 10-OTMS and the terminal -OTMS groups.

The exact nature or number of $LiAlH_4$-reducible groups cannot be determined from this spectrum alone. For example, reduction of an epoxy group (thought to be present in earlier studies) would give a hydroxyl group, and reduction of a ketone, which is known to be present from UV and chemical (2,4-dinitrophenylhydrazine) data, would also give a hydroxyl group. This problem was solved by specific deuterium labeling after reduction with $LiAl^{2}H_4$.

The GLC–MS analysis of A_{9+13} reduced with $LiAl^{2}H_4$ instead of $LiAlH_4$ is shown in Fig. 2*B*. It is evident from the shift in mass of the compounds (3 atomic mass units) that three deuterium atoms were incorporated into the molecules. The M peak, *m/e* 518, shifted to 521, and the M−15 peak, *m/e* 503, shifted to 506. Two of the incorporated deuterium atoms are at C-1 due to the reduction of the carboxylic acid function, and the other deuterium is elsewhere on the carbon chain. Since it is known that there is an α,β-unsaturated ketone in the original material, the one deuterium that is incorporated in the chain must be incorporated at the position of this oxo function. The second hydroxyl group on the final product must be an unreducible group in the starting material, namely, a hydroxyl group. The possibility of an epoxy

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TABLE 1. TMS derivatives of B_9 and B_{13} showing the major mass spectral cleavages

Structures and Cleavages	Mass Spectrum in:
A. $CH_3(CH_2)_4\underset{\underset{\text{OTMS}}{|}}{CH}CH_2CH_2\underset{\underset{\text{OTMS}}{|}}{CH}CH_2(CH_2)_8OTMS$ (173 / 201 \ 317)	Fig. 2*A*
$CH_3(CH_2)_4CH_2\underset{\underset{\text{OTMS}}{|}}{CH}CH_2CH_2\underset{\underset{\text{OTMS}}{|}}{CH}(CH_2)_8OTMS$ (187 / 331 \ 303)	
B. $CH_3(CH_2)_4\underset{\underset{\text{OTMS}}{|}}{CH}CH_2CH_2\underset{\underset{\text{OTMS}}{|}}{C^2H}CH_2(CH_2)_7C^2H_2OTMS$ (173 / 201 \ 320)	Fig. 2*B*
$CH_3(CH_2)_4CH_2\underset{\underset{\text{OTMS}}{|}}{C^2H}CH_2CH_2\underset{\underset{\text{OTMS}}{|}}{CH}(CH_2)_7C^2H_2OTMS$ (188 / 333 \ 305)	
C. $CH_3(CH_2)_4\underset{\underset{^{18}\text{OTMS}}{|}}{CH}CH_2CH_2\underset{\underset{^{18}\text{OTMS}}{|}}{CH}CH_2(CH_2)_8OTMS$ (175 / 347 \ 203 \ 319)	Fig. 2*C*
$CH_3(CH_2)_4CH_2\underset{\underset{^{18}\text{OTMS}}{|}}{CH}CH_2CH_2\underset{\underset{^{18}\text{OTMS}}{|}}{CH}(CH_2)_5OTMS$ (189 \ 333 \ 217 \ 305)	

The numbers adjacent to the dashed lines are the *m/e* values calculated for the indicated fragmentation.

group is eliminated by the absence of the addition of a second deuterium in the molecule. Reduction of 9,10-epoxy stearic acid with $LiAl^2H_4$ indicated that an epoxide is reduced to a hydroxy with incorporation of a deuterium on the adjacent carbon.

The position of the deuterium (and hence the oxo function) in the chain can be determined by examining the remainder of the mass spectrum in Fig. 2*B*. The main cleavage shifts are *m/e* 187 to 188, *m/e* 303 to 305, *m/e* 331 to 333, and *m/e* 317 to 320. The *m/e* values 173 and 201 remain unchanged. Examination of these shifts (remembering that two deuterium atoms are at C-1) leads to the conclusion that deuterium is incorporated in the C-10 and C-12 positions. The ketone function in A_9 must be in the C-12 position, and the hydroxyl must be in the position of the original hydroperoxide groups, C-9. Likewise, in the isomer A_{13}, derived from the C-13 hydroperoxide (LHP_{13}), C-13 contains a hydroxy group and C-10 contains a keto group that can be reduced by $LiAlH_4$ (one deuterium incorporated). The mass spectrum in Fig. 2*B* supports the structure of A_9 and A_{13} proposed above.

Mass chromatograms of compound B were obtained by taking mass spectra between *m/e* 275 and *m/e* 400 at 5-sec intervals during the elution of the material from the GLC column. Mass spectral intensities of *m/e* 317 (compound B_{13}) and *m/e* 303 (compound B_9) were plotted with time exactly as described in a previous report (6). The two curves obtained were integrated, and the mixture was found to be 65% 13-hydroxy-10-oxo compound (B_{13}) and 35% 9-hydroxy-12-oxo compound (B_9). This procedure was shown to be quantitative (6), and its application to the reduced and hydrogenated hydroperoxides produced from soybean lipoxygenase yielded a distribution of 30% 9- and 70% 13-hydroperoxy linoleic acids.

The ratio of the products B_9 (derived from A_9) to B_{13} (derived from A_{13}) agrees with the ratio of the hydroperoxides LHP_9 to LHP_{13} and supports the pathways shown above. Thus, the action of the hydroperoxide-decomposing enzyme on LHP_9 yields A_9, and the action on LHP_{13} yields A_{13}. This result requires the translocation of only one oxygen atom, whereas the rearrangement proposed by Gardner (4) ($LHP_{13} \rightarrow A_9$ and $LHP_9 \rightarrow A_{13}$) requires the translocation of both hydroperoxide oxygens. The positions of the two oxygen functions on these molecules raise the question of their origin. Do both oxygen functions originate from the hydroperoxide and hence from molecular oxygen? To answer this question, linoleic acid was incubated as described above with soybean lipoxygenase in the presence of $^{18}O_2$. The products LHP_9 and LHP_{13} were used as substrates for lipohydroperoxidase. The products were isolated, reduced with PtO_2/H_2 and $LiAlH_4$, and derivatized with bis(trimethylsilyl)acetamide. GLC–MS analysis is shown in Fig. 2*C*. The M and M−15 peaks have increased by 4 *m/e* units, indicating that both oxygens from molecular oxygen are retained by the products. The shifts from *m/e* 503 to 507 and from *m/e* 518 to 522 were not complete for two reasons: (*a*) the sample of oxygen contained only 80% ^{18}O-^{18}O, and (*b*) it was impractical to remove all of the endogenous $^{16}O_2$ from the reaction solution and from lipoxygenase.

All of the shifts of major fragments correlate closely with the expected shifts (2 *m/e* units) for fragments containing one ^{18}O; these are, for the B_9 compound, *m/e* 187 to 189, *m/e* 331 to 333, *m/e* 215 to 217, and *m/e* 303 to 305. For B_{13}, the significant shifts are *m/e* 173 to 175, *m/e* 345 to 347, *m/e* 201 to 203, and *m/e* 317 to 319. These show that each of the major oxygen-containing fragments other than C-1 contains ^{18}O and that there are two ^{18}O atoms in each molecule.

The mechanism of conversion of linoleic hydroperoxide to an oxo-hydroxy compound with retention of both atoms of oxygen probably would proceed through a cyclic epiperoxide followed by a ketohydroxy-forming rearrangement (10). The *trans* double bond that is present in the final product would, however, severely strain such a cyclic structure. More information is necessary to define the

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exact mechanism of the reaction, and evidence is being sought for this presently.

Attempts in our laboratory to purify the hydroperoxide-decomposing enzyme have shown that the enzyme is extremely unstable and its activity is not enhanced by addition of usual cofactors or metals. It is stabilized by the addition of mercaptoethanol or dithiothreitol. The instability of the enzymes involved in the metabolism of linoleic hydroperoxide probably accounts for some of the variation in the products obtained with various systems (1, 3, 4). However, the major differences appear to be due to inherent differences in the decomposing enzymes of alfalfa and flax seeds. None of the products from the alfalfa seed decomposing enzyme system yielded mass spectra comparable to those reported for the lipoperoxide isomerase from flax (2, 3). Comparison of the ^{18}O data for the two systems supports the conclusion that different mechanisms are involved. The data of Gardner (4) suggest that corn contains both the flax and the alfalfa types of enzymes.

The biological function of hydroperoxide formed in plant seeds is still in doubt. Although peroxidized unsaturated lipids have been suggested as precursors in the formation of the hormone ethylene (11, 12), 1-octen-3-ol (13), and other compounds in plants, no enzymatic mechanisms have been found. It is possible that one of the isomers, A_{13}, identified in this study could be an intermediate in the enzymatic formation of 1-octen-3-ol in soaked soybeans, which contain linoleic acid, lipoxygenase, and hydroperoxide-destroying enzymes. In addition, mushrooms, which are rich in linoleic acid, produce 1-octen-3-ol (14) as one of their main flavor constituents.

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