Sequential enzymes of linoleic acid oxidation in corn germ: lipoxygenase and linoleate hydroperoxide isomerase

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ABSTRACT Linoleic acid oxidation catalyzed by lipoxygenase (lipoxidase) activity in extracts of defatted corn germ does not terminate in the product, linoleic acid hydroperoxide, unless the lipoxygenase is first partially purified. If purification is not attempted, the hydroperoxide product exists only as a barely detectable intermediate in the synthesis of three products. One of these was identified as 9-hydroxy-10-oxo-*cis*-12-octadecenoic acid formed from the hydroperoxide by the enzyme, linoleate hydroperoxide isomerase. Another product, 13-hydroxy-10-oxo-*trans*-11-octadecenoic acid, is believed to be formed by an isomerase also. The third product was the linoleate ester of one of the hydroxy-oxo-fatty acids, 9-(*cis*-9,*cis*-12-octadecadienoyl)-10-oxo-*cis*-12-octadecenoic acid. It is not known if the synthesis of the ester is enzyme-catalyzed.

When a mixture of 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid and 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid from soybean lipoxygenase oxidation of linoleic acid was used as a substrate, 13-hydroxy-12-oxo-*cis*-9-octadecenoic acid and 9-hydroxy-12-oxo-*trans*-10-octadecenoic acid were formed as the major products of catalysis by linoleate hydroperoxide isomerase(s) from corn. Smaller quantities of 9-hydroxy-10-oxo-*cis*-12-octadecenoic acid and 13-hydroxy-10-oxo-*trans*-11-octadecenoic acid were also formed.

SUPPLEMENTARY KEY WORDS linoleic acid hydroperoxide · 9-hydroxy-10-oxo-*cis*-12-octadecenoic acid · 13-hydroxy-12-oxo-*cis*-9-octadecenoic acid · 9-(*cis*-9,*cis*-12-octadecadienoyl)-10-oxo-*cis*-12-octadecenoic acid · 9-hydroxy-12-oxo-*trans*-10-octadecenoic acid · 13-hydroxy-10-oxo-*trans*-11-octadecenoic acid

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; IR, infrared; UV, ultraviolet; NMR, nuclear magnetic resonance.

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ALMOST ALL studies concerning lipoxygenase activity have used the oxidation of linoleic acid by soybean lipoxygenase as a model system (1, 2). The products of soybean lipoxygenase oxidation have been reported to be either a mixture of 70% 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid and 30% 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid (3), or 100% of the former isomer (4). Lipoxygenase in corn has been detected in the seed (5) and seedlings (5, 6). Wagenknecht (7) attributed the development of off-flavors in underblanched sweet corn to lipoxygenase activity in fractions containing germ. In none of these investigations of corn lipoxygenase were the oxidation products characterized.

Blain and Barr (8) presented evidence that linoleate hydroperoxides arising from lipoxygenase activity are subsequently decomposed by an enzyme in soybeans. A similar hydroperoxide-decomposing enzyme from alfalfa seedlings was reported by Gardner and Clagett (9). Zimmerman (10) characterized a flaxseed enzyme, linoleate hydroperoxide isomerase, which catalyzed the conversion of the hydroperoxides to fatty acids with the general formula $R'—CHOH—CO—CH_2—CH\overset{cis}{=}CH—R$. Zimmerman proposed that the structures were compatible with the two isomeric substrates, 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic and 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acids. On the basis of this reasoning, the product fatty acids would be a mixture of 13-hydroxy-12-oxo-*cis*-9-octadecenoic and 9-hydroxy-10-oxo-*cis*-12-octadecenoic acids.

The oxidation of linoleic acid by a sequential enzyme system in corn germ extracts is described here. The products accumulated after the sequential reaction of lipoxy-

genase–linoleate hydroperoxide isomerase are identified. In addition, another reaction is described in which linoleic acid is utilized in an acylation reaction, possibly catalyzed by an enzyme.[1]

MATERIALS AND METHODS

Preparation of Extracts

Crib-dried Bear[2] ×800 hybrid corn, *Zea mays*, was obtained from Bear Hybrid Corn Co., Decatur, Ill., and was used as the enzyme source.

Either hexane-defatted germ or whole germ was used as an enzyme source. Hexane-defatting was done by thorough grinding of the germ in cold hexane, followed by collection of the fine residue. Phosphate buffer (0.2 M, pH 6.9) was used to extract enzyme, 1 g of whole germ or 0.7 g of defatted germ per 10 ml of buffer. Endosperm extracts were prepared similarly in the proportion of 2 g/10 ml. The samples were thoroughly ground in buffer by a mortar and pestle. The resulting homogenate was centrifuged at 8000 *g* for 15 min. The supernatant contained the crude enzyme system.

Enzyme activities in the extracts were concentrated by $(NH_4)_2SO_4$ precipitation. Fractions were precipitated at 0°C for 30 min using a percentage of saturation nomogram for room temperature (11). One fraction was obtained at 40% of saturation, and another was collected between 40 and 50% of saturation.

The protein content of the enzyme preparations was determined by the biuret reaction (12). When whole germ was used, turbid solutions were encountered; these were clarified without affecting the color by the addition of about 5 μl of Triton X-100 per ml of solution.

Enzyme Assays

The Action of Sequential Enzymes. Linoleic acid was used as an initial substrate for the sequential enzyme system. The method used was a modification of the conditions described by Surrey (13) for the oxidation of linoleic acid by lipoxygenase. In the modified method the substrate was an aqueous solution of Tween 20 (0.5%, v/v) and linoleic acid (0.5%, v/v). Linoleic acid (99+% pure from The Hormel Institute, Austin, Minn.) was titrated to the potassium salt with 0.5 M K_2CO_3. Freshly prepared substrate was added to an equal volume of corn extract (usually 2–10 ml) and incubated at 25 ± 0.5°C while oxygenating the solution with a stream of O_2. The final substrate concentration was 8.0×10^{-3} M.

The progress of the reaction was followed by TLC analysis of the products (see below). Aliquots were removed at intervals during the course of the reaction. These samples were immediately acidified with 1 N HCl and extracted with $CHCl_3$–CH_3OH 2:1 (v/v).

Lipoxygenase. The conditions of reaction for lipoxygenase assay were identical to those described above for the sequential enzyme system.

Assay depended on measurement of the linoleic acid hydroperoxide by its UV absorption. At intervals a 1 ml portion was withdrawn from the reaction and immediately extracted with 10 ml isooctane (redistilled)–*n*-butanol 19:1 (v/v) after acidification with 1 ml of 1 N HCl. The absorbancy of the upper phase was determined at 234 nm against a blank prepared with unreacted substrate and extract.

Linoleate Hydroperoxide Isomerase. A solution of linoleic acid hydroperoxide was prepared for use as a substrate immediately before each experiment. 5 ml of linoleic acid–Tween 20 solution (0.5% linoleic acid as potassium salt and 0.5% Tween 20) was added to 50 ml of lipoxygenase solution (2.5 mg of soybean lipoxygenase [20,000 U/mg from Pierce Chemical Co., Rockford, Ill.] in 50 ml of 5.0 mM potassium borate buffer, pH 8.7). The mixture was incubated at 25°C for 40 min with magnetic stirring in a stoppered 1 liter flask flushed with O_2. At termination of the oxidation, the concentration of the linoleic acid hydroperoxide was calculated from the UV absorption of conjugated diene. The pure hydroperoxide is reported to have its λ_{max} at 234 nm with an ϵ_{max} of 24,500 (14, 15).

The isomerase reaction commenced upon mixing the hydroperoxide solution with an equal volume of the corn extract. Isomerase activity was measured by the initial rate of decrease in conjugated diene absorption at 234 nm using a Cary 14 recording spectrophotometer. The reaction proceeded in a 1.00 mm photometric cell placed in a constant temperature cell jacket at 25 ± 0.5°C. Activity measurements commenced from 5 to 15 sec after mixing the substrate and corn extracts.

Product Isolation

Products from the Action of Sequential Enzymes. Hexane-defatted germ extract was used to oxidize 0.9 g linoleic acid for 1 hr using the method described for assay of the sequential enzyme system. The reaction mixture (400 ml) was acidified with 1 N HCl and extracted with $CHCl_3$–CH_3OH 2:1 (v/v).

Since nearly half of the extracted lipid was Tween 20, hexane extraction was used to separate the products from the Tween. The lipid was emulsified in 10 ml of water

[1] The reaction products discussed in this study are designated by letters and are as follows: A_1, 9-hydroxy-10-oxo-*cis*-12-octadecenoic acid; A_2, 13-hydroxy-12-oxo-*cis*-9-octadecenoic acid; B, 9-(*cis*-9,*cis*-12-octadecadienoyl)-10-oxo-*cis*-12-octadecenoic acid; C_1, 13-hydroxy-10-oxo-*trans*-11-octadecenoic acid; C_2, 9-hydroxy-12-oxo-*trans*-10-octadecenoic acid.

[2] The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

and extracted twice with an equal volume of hexane. The hexane-extracted material was very slightly enriched in less polar products as assayed by TLC, but was free of Tween. A 0.64 g portion of the product mixture was separated by silicic acid column chromatography (see below).

Products from the Action of Linoleate Hydroperoxide Isomerase. A mixture of products resulted from the action of corn germ isomerase on linoleic acid hydroperoxides. The substrate was prepared by mixing 0.9 g of linoleic acid (as the potassium salt), 0.5 ml of Tween 20, and 100 mg of soybean lipoxygenase with a liter of 5.0 mM potassium borate buffer (pH 8.7). Good oxidation was assured by using an O_2 atmosphere, stirring, a large surface area, and a 1.3 hr reaction time. The resulting hydroperoxide solution was added to 500 ml of an extract prepared from hexane-defatted germ. The germ extract was 4.5 times more dilute than the crude germ extract and also was composed of 6.5×10^{-4} M dithiothreitol and 0.05% Tween 20. After a 30 min incubation, the mixture was acidified and extracted as above (sequential enzyme system).

A 0.55 g portion of the recovered product (including the Tween 20, which was also extracted) was dissolved in $CHCl_3$ and added to 2 g of silicic acid (Mallinckrodt 100 mesh). This mixture was evaporated to dryness on a rotary evaporator and then slurried in a minimum volume of hexane. The slurry was applied to a silicic acid column for separation of the products.

Structural Characterization of the Products

Spectral Analyses. The isolated fatty acids were prepared as liquid films on silver chloride plates for IR spectroscopy (Perkin–Elmer 621).

NMR spectra were recorded with a Varian Model HA-100. The samples were dissolved in $CDCl_3$ for analysis using 1% tetramethylsilane as an internal reference. The measurements were made at a frequency of 100 MHz, and at a sample temperature of 29°C. The peak positions were measured by first-order analysis.

UV spectra were obtained with a Cary 14 recording spectrophotometer; either spectral-grade hexane or ethanol was used.

Mass spectra were obtained with a Nuclide 12-90G mass spectrometer using a probe inlet at a probe and source temperature of 100°C.

Microchemical Techniques. An excess of $NaBH_4$ in 50% methanol reduced the ketones in 0.5–1 hr at room temperature. Traces of boric acid were removed from the product by evaporation of methyl borate through addition and evaporation of absolute methanol about six times.

$NaIO_4$ oxidation, as outlined by King (16), was used to cleave certain isolated products. The chloroform extract from the periodate oxidation mixture was divided into two portions. One portion was carefully evaporated and then dissolved in benzene or hexane for GLC analysis of volatile aldehydes. The other portion was esterified with BF_3–methanol (Applied Science Laboratories Inc., State College, Pa.) for GLC analysis of the carboxylic acids.

Permanganate–periodate oxidation was carried out in pyridine according to von Rudloff (17). The oxidation fragments were esterified with diazomethane (18) and analyzed by GLC.

The linoleic acid ester formed during the enzymatic reactions (B) was saponified with 0.5 N KOH in 50% methanol at 85°C for 10 min.

B was transesterified with 0.1 N KOH in absolute methanol for 30 min at room temperature. When it had been previously reduced by $NaBH_4$, a reaction time of at least 1.5 hr at room temperature was necessary for complete transesterification.

C_1 was hydrogenated by H_2 in $CHCl_3$ using 10% palladium on charcoal.

Column Chromtography

Columns (height, 21 cm; I.D., 2.5 cm) were packed with a slurry of 50 g Mallinckrodt silicic acid (100 mesh, analytical reagent) in hexane. The column was eluted in a combination stepwise-gradient fashion: the mixing chamber was filled with 70 ml of 10% anhydrous ether in redistilled hexane, and this solvent mixture was replenished from a reservoir filled consecutively with 200 ml 20% ether, 250 ml 30% ether, 250 ml 40% ether, and 600 ml 50% ether in hexane. Slight N_2 pressure was applied to the column, and the flow rate was then adjusted to 1.5–1.8 ml/min. 10-ml fractions were collected.

TLC

Analytical and preparative TLC plates were prepared as described previously (19). Dihydroxy fatty acids were best separated by isooctane (redistilled)–anhydrous ethyl ether–acetic acid 40:60:1 or 30:70:1 (v/v/v). For all other fatty acid separations, the plates were developed with isooctane–ether–acetic acid 50:50:1 (v/v/v).

The separated components were visualized with the aid of various reagents applied to separate lanes. Specific sprays, either 2,4-dinitrophenylhydrazine or KI-starch (20), were used to identify components with aldehyde, ketone, or peroxide functions. The dinitrophenylhydrazine spray reacted slowly with linoleic acid hydroperoxides presumably by an acid-catalyzed dehydration of the hydroperoxide group. All fatty acids were then detected by overnight exposure to I_2 vapors or by H_2SO_4-dichromate charring.

GLC

An Aerograph 1520 apparatus with an H_2 flame de-

tector was used for all separations. An 8 ft column ($^1/_8$ in. O.D.) was packed with 5% LAC-2R-446 polyester on 60–80 mesh Chromosorb W (acid-washed and treated with dimethyldichlorosilane) which was purchased from Applied Science Laboratories Inc. The temperature was programmed from 50°C to 190°C at the rate of 8°C/min. At 190°C the temperature was held constant until all the material was eluted.

The GLC standards dimethyl decanedioate, dimethyl nonanedioate, methyl hexanoate, and dimethyl dodecanedioate were from Applied Science Laboratories Inc.; methyl octanoate, methyl decanoate, and methyl linoleate were from The Hormel Institute; and hexanal was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. Methyl nonanoate-9-al was synthesized by periodate oxidation of 9,10-dihydroxystearic acid (Applied Science Laboratories Inc.) followed by BF_3–methanol esterification. Unknown fatty acids were identified by cochromatography with known standards. If specific standards were not available, plots of retention time vs. carbon number and functional groups were used to identify the unknowns. Often volatile compounds, e.g., hexanal, nonenal, methyl hexanoate, and methyl heptanoate, were found only as trace components unless the amount of solvent used in isolation was kept to a minimum.

RESULTS

Identification of the Products from Sequential Reactions

Linoleic acid added to a corn germ extract was converted to at least three different products, A_1, B, and C_1, by the reaction sequence. The preparation of the lipid extract was described above (see Product Isolation, Materials and Methods).

9-Hydroxy-10-oxo-cis-12-octadecenoic Acid (A_1). A_1 is shown separated by column chromatography in Fig. 1. The double peak was apparently an artifact; fractions 62–78 appeared to be homogenous as determined by TLC and IR spectra of individual fractions. Subsequent column separations of A_1 resulted in single peaks.

The structure of A_1 was determined by spectral methods and by oxidative chain cleavage techniques. Analyses by NMR and IR spectroscopy indicated that this fatty acid had the general formula R—CHOH—CO—CH_2—CH^{cis}=CH—R. The absorption features of the NMR (Table 1) and IR spectra from this type of fatty acid were reported by Zimmerman (10). UV molar absorptivity was low (Table 2). Chain cleavage of A_1 by periodate oxidation (Fig. 2) established the complete structure. No other cleavage product was detected from oxidation of the A_1 peak.

9-(cis-9,cis-12-Octadecadienoyl)-10-oxo-cis-12-Octadecenoic Acid (*B*). Fractions 26–29 (Fig. 1) were pooled for analyses of B. TLC showed that the pooled sample was essentially homogenous containing only minor impurities and no detectable linoleic acid.

TABLE 1 NMR PROTON ABSORPTIONS OF LINOLEIC ACID OXIDATION PRODUCTS

Assignment	δ Value		
	B	A_1, A_2	C_1, C_2
	ppm		
CH_2*—COOH	2.33	2.32	2.34
CH_2*—CO—CH=CH	—	—	2.56
CO—CH_2*—CH=CH	3.19	3.22	—
CH_2*—CH=CH	2.00	2.00	—
CH=CH—CH_2*—CH=CH	2.73	—	—
CH*=CH*—CH_2—CO (*cis*)	5.53	5.54	—
CH*=CH*—CH_2—CH=CH (*cis*)	5.30	—	—
CH*=CH—CO (*trans*)	—	—	6.80
CH=CH*—CO (*trans*)	—	—	6.28
CH*—OH	—	4.21	4.29
CH*—OR	5.00	—	—

*Denotes the proton in question.

TABLE 2 ULTRAVIOLET ABSORPTION OF LINOLEIC ACID OXIDATION PRODUCTS

Product	λ_{max}	Molar Absorptivity
	nm	ϵ_{max}
B (hexane)	<210	—
	229 (shoulder)	760
	285	140
A_1, A_2 (ethanol)	226	2900 ± 400
	277	1300 ± 200
C_1, C_2 (ethanol)	226	9900 ± 1100
	275	260 ± 30

Spectral analyses greatly aided the structural identification of B. An NMR spectrum of B (Fig. 3) showed the same features as superimposed spectra of linoleic acid (21) and A_1, except for the downfield displacement of the secondary (C—H) alcohol proton peak to 5.0 ppm because of its proximity to the acyl linkage with linoleic acid. Other assignments of the proton absorptions are found in Table 1. An IR spectrum showed clearly the ester carbonyl absorption at 1730 cm^{-1} and no hydroxyl absorption near 1100 cm^{-1} (secondary hydroxyl) or at 3460 cm^{-1}. The *cis* absorption at 3000 and 1650 cm^{-1} was evident with no *trans* being found at 970 cm^{-1}. The UV molar absorptivity of B (Table 2) was, as expected, small.

The complete structure of B was determined by the reactions summarized in Fig. 2. However, it was noted that the $NaIO_4$ oxidation of the postulated 9,10-dihydroxy-*cis*-12-octadecenoic acid did not result in a 100% conversion to nonenal and nonanoic-9-al as expected. Small amounts of two oxidation fragments were found in addition to nonenal and nonanoic-9-al.

As determined by GLC the two additional compounds were indicative of decanoic-10-al and an "octenal." The octenal is believed to be an artifact arising from a prior $NaBH_4$ reduction shown in Fig. 2. This was substantiated by a small amount of octenal obtained through $NaIO_4$ oxidation of $NaBH_4$-reduced A_1. It was concluded that the major fragments, nonenal and nonanoic-9-al, are those indicative of the actual structure of B.

13-Hydroxy-10-oxo-trans-11-octadecenoic Acid (C_1). C_1 was isolated by preparative TLC more easily than by column chromatography. A crude fraction was obtained by development in isooctane–ether–acetic acid 50:50:1 (v/v/v). The crude fraction was finally purified by multiple development (3×) in chloroform–acetic acid 100:1 (v/v) on a preparative plate.

NMR and IR spectra of C_1 were essentially identical to those shown in Fig. 4. The NMR spectrum is indicative of a fatty acid with the moiety —CO—CH=CH—CHOH— in the carbon chain. Table 1 lists the assignments of the proton absorptions. The prominent features of the IR spectrum are the absence of *cis* absorptions at 3000 and 1650 cm^{-1}, the presence of a *trans* absorption at 973 cm^{-1}, an alcohol absorption at 1070 cm^{-1} (secondary hydroxyl) and at 3460 cm^{-1}, and finally an absorption at 1617 cm^{-1} due to stretching of a carbon double bond which is conjugated to a carbonyl. The UV absorbances of C_1 (Table 2) are characteristic of α,β-unsaturated carbonyls.

Periodate–permanganate oxidation of C_1 resulted in three major products, hexanoic acid, nonanedioic acid, and decanedioic acid. Nonanedioic acid probably was derived from the 10-oxo group, which enolized in the alkaline oxidation medium. The decanedioic acid to nonanedioic acid ratio increased from 2:1 to more than 9:1 by employing shorter oxidation times.

To prove that C_1 had 10-oxo and 13-hydroxyl groupings, a derivative of C_1 was prepared for mass spectral analysis. C_1 was hydrogenated, and the methyl ester was formed. The product was isolated by TLC using isooctane–ether 1:1 (v/v) as the developing solvent. A mass spectrum of the saturated ester yielded all of the mass peaks expected from methyl 13-hydroxy-10-oxo-octadecanoate, based on the findings of others (22, 23). The peak at m/e 328 (molecular ion, M) confirmed the expected molecular weight, and an intense peak at m/e 310 (M–18) was due to loss of H_2O. Other mass peaks were chiefly due to fragmentation about the 13-hydroxyl and 10-oxo groups (Table 3).

Characterization of the Reaction Sequence

The occurrence of sequential reactions in corn germ extracts starting with the addition of linoleic acid was indicated by anomalous results obtained using an assay method for lipoxygenase. The method measured absorbance of the expected product, linoleic acid hydroperoxide, at 234 nm. Assays of crude extracts from defatted germ resulted in extremely small absorbance values at 234 nm, which reached a maximum after 5 min of incubation and, thereafter, remained relatively constant at only 2.5% of the theoretical absorption for 100% conversion to the hydroperoxide. The hydroperoxide did not amount to more than a trace throughout the time of reaction as indicated by TLC analyses. Three other products,

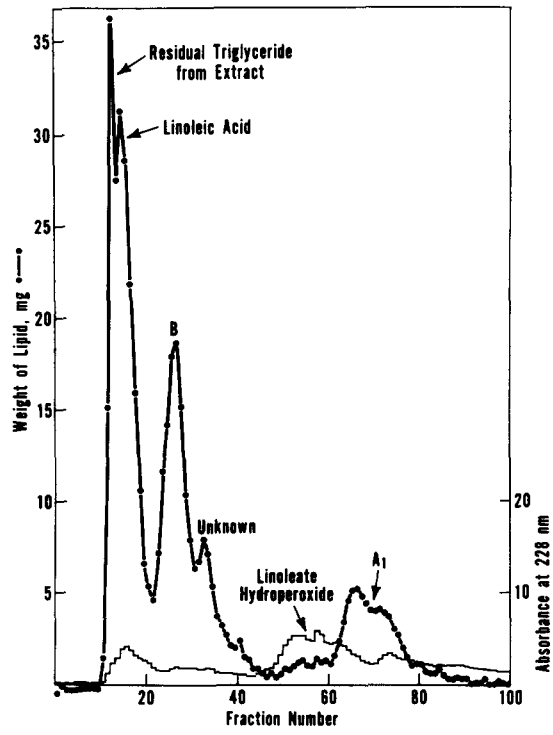

FIG. 1. Silicic acid column chromatography of a mixture resulting from sequential action of corn germ enzymes on linoleic acid.

TABLE 3 MASS SPECTRAL ANALYSIS OF METHYL 13-HYDROXY-10-OXO-OCTADECANOATE DERIVED FROM C_1

m/e	Relative Intensity	Possible Ion Structure	Possible Origin
M–129	11	$[CO(CH_2)_8CO_2CH_3]^+$	Cleavage at 10-oxo
M–129–32	100	—	Loss of CH_3OH from M–129
157	40	$[(CH_2)_7CO_2CH_3]^+$	Cleavage β from 10-oxo
		or	or
		$[CH_3(CH_2)_4CHOH-(CH_2)_2CO]^+$	Cleavage at 10-oxo
M–71	11	$[CHOH(CH_2)_2CO(CH_2)_8-CO_2CH_3]^+$	Cleavage at 13-hydroxyl
M–71–32	20	—	Loss of CH_3OH from M–71
M–71–18	6	—	Loss of H_2O from M–71
214	9	$[CH_2{=}COH(CH_2)_8-CO_2CH_3]^+$	Cleavage β from 10-oxo with rearrangement

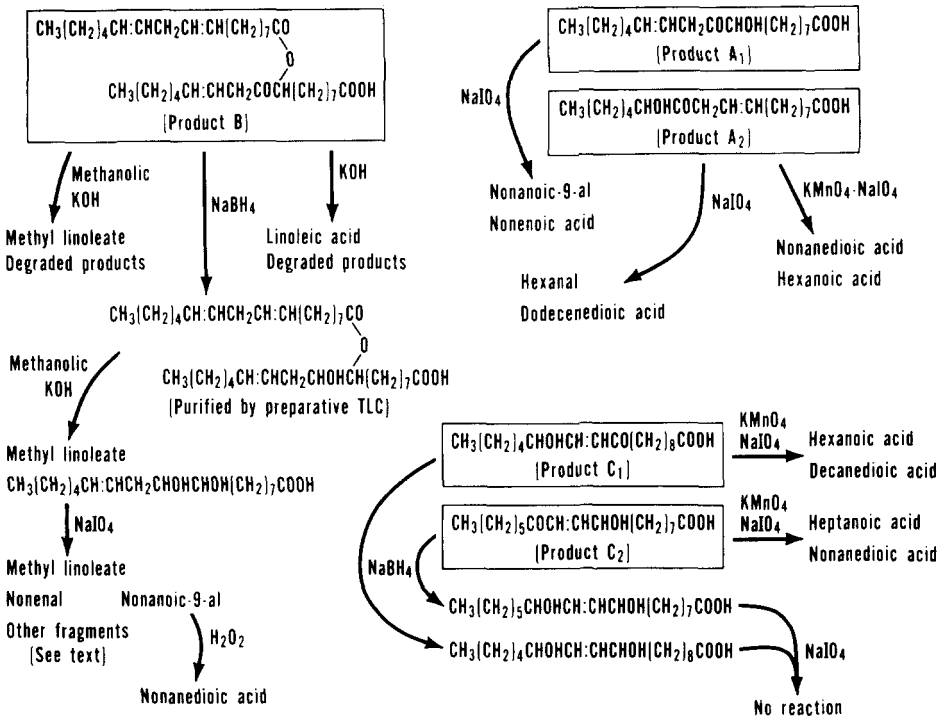

FIG. 2. Flow chart showing microchemical reactions used to determine the structures of A_1, A_2, B, C_1, and C_2.

A_1, B, and C_1, accumulated. Their separation on TLC is shown in Fig. 5.

The relative percentages of A_1, B, and C_1 varied very little during the time of reaction, and thus it appeared that none of the three was being formed directly from any of the other two. A_1 and B were the two major products (Fig. 1), and C_1 was a small percentage of the total product mixture.

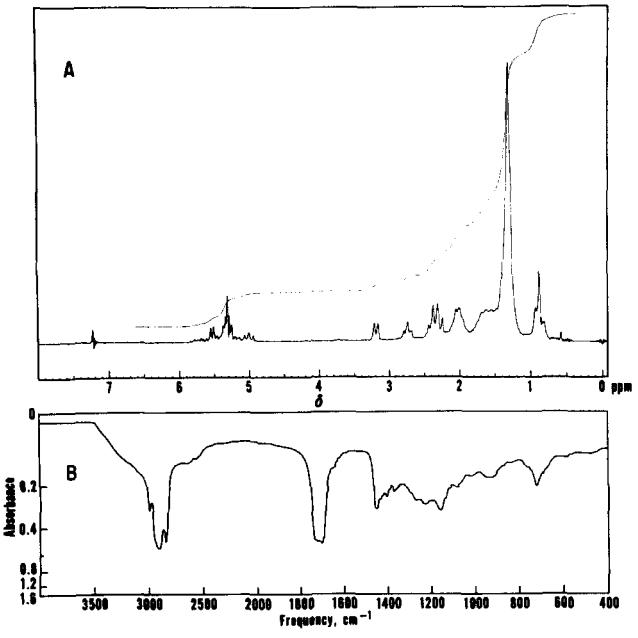

FIG. 3. NMR (*A*) and IR (*B*) spectroscopy of B. The concentration of B was 20% for NMR analysis.

Small amounts of a compound presumed to be a conjugated oxo-diene, oxo-octadecadienoic acid (20), always migrated slightly forward and mixed with linoleic acid hydroperoxide on silicic acid columns and TLC. The compound was tentatively identified by its UV absorption at 277 nm and its immediate reaction with 2,4-dinitrophenylhydrazine on TLC plates.

Corn endosperm was a poor source of the sequential enzyme system as indicated by Fig. 5. Endosperm extracts were not used further in this study.

In order to clarify the reaction sequence of linoleic acid oxidation, each reaction step in the series was studied individually.

Purification and Properties of Corn Lipoxygenase

The initial reaction step in the conversion of linoleic acid to A_1, B, and C_1 was assumed to be lipoxygenase oxidation. A simple salt precipitation of defatted germ extract with $(NH_4)_2SO_4$ between 40 and 50% of saturation concentrated most of the lipoxygenase activity, and removed most of the other enzymes involved in this study. Lipoxygenase activity assays of the purified fraction by the conventional method of following the increase in absorbance at 234 nm showed that it had much more apparent activity than the crude extract. Absorbance increased in the reaction mixture until, after 30–40 min, the total absorbance accounted for as much as 72% of the

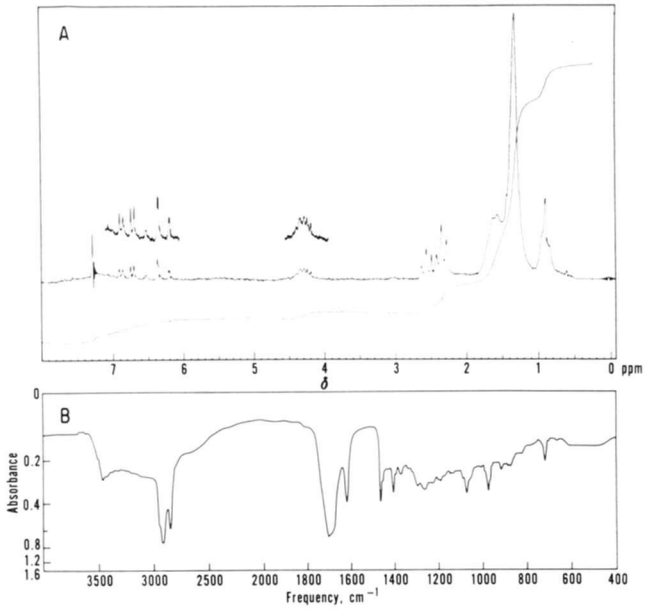

FIG. 4. NMR (*A*) and IR (*B*) spectra of a mixture of C_1 and C_2. The concentration of the mixture of C_1 and C_2 was 10% for NMR analysis.

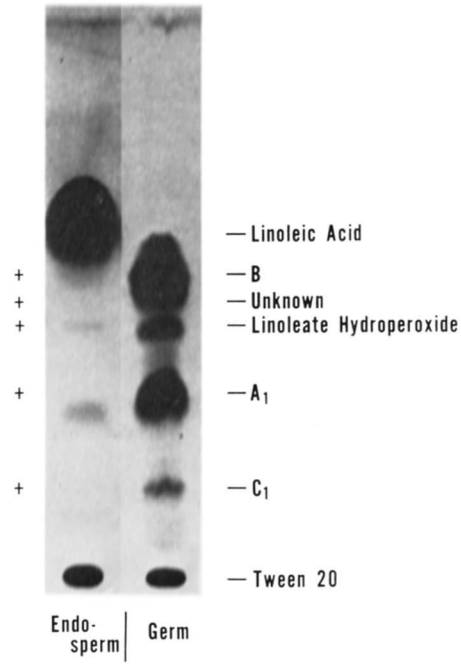

FIG. 5. TLC separation of products resulting from the action of corn germ extracts on linoleic acid by a sequence of enzymes. Linoleic acid was oxidized with undiluted extracts from defatted germ or endosperm for 30 min. The + sign denotes a positive reaction with 2,4-dinitrophenylhydrazine spray. The spots were detected by I_2 vapor.

added linoleic acid converted to linoleic acid hydroperoxide. The λ_{max} at 232–234 nm also aided in identifying the hydroperoxide. The product was isolated and then separated by TLC. The major spot reacted positively to KI-starch spray and chromatographed with linoleic acid hydroperoxide formed by soybean lipoxygenase.

By using $(NH_4)_2SO_4$-purified lipoxygenase, the pH optimum of the enzyme was determined to be about 6.5 with essentially no activity at pH 9.

Studies of Linoleate Hydroperoxide Isomerase from Corn

Another enzyme involved in the reaction sequence was assumed to be linoleate hydroperoxide isomerase. The formation of A_1 from linoleic acid indicated that the isomerase was involved in the reaction sequence. To study this enzyme and its products directly, the substrate, linoleic acid hydroperoxide, had to be prepared conveniently. Purified soybean lipoxygenase was employed for the purpose of producing the hydroperoxide. Soybean lipoxygenase is known to produce primarily 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid with smaller amounts of the 9-hydroperoxide. Introduction of the 13-hydroperoxide as a substrate resulted in the formation of A_2 and C_2, which are positional isomers of A_1 and C_1.

Identification of 13-Hydroxy-12-oxo-cis-9-octadecenoic Acid (A_2). A_2 was isolated by column chromatography (Fig. 6). A_2 was free of A_1 in the middle of the peak as indicated by periodate oxidation (Fig. 2). The spectral properties of A_2 were indistinguishable from those of A_1 (Tables 1 and 2), thus demonstrating the structural relationship with A_1. The periodate oxidation established the 13-hydroxy-12-oxo- groups, and permanganate–periodate oxidation indicated that the double bond was at carbon 9 (Fig. 2).

Identification of 9-Hydroxy-12-oxo-trans-10-octadecenoic Acid (C_2). C_2 was obtained from column chromatography as shown in Fig. 6 by pooling fractions 101–110. Oxidation by permanganate–periodate (Fig. 2) gave the expected cleavage products of nonanedioic and heptanoic acids, thus indicating that the functional group was between carbons 9 and 12. However, the appearance of smaller quantities of decanedioic acid and hexanoic acid demonstrated that C_2 was mixed with a small amount of C_1. Spectral analyses (Fig. 4 and Table 2) completed the proof of the structure. The spectra were interpreted in the same manner as for C_1. One other possible structure, 12-hydroxy-9-oxo-*trans*-10-octadecenoic acid, is possible from the data. However, it was assumed that the functional moiety would have the opposite configuration as C_1 relative to the carboxylic acid end of the molecule, since this is usually true of products from the 9- and 13-hydroperoxide substrates.

Distribution of Isomeric Products. The use of 9- and 13-hydroperoxides as substrates for corn linoleate hydroperoxide isomerase determined the formation of two isomeric products based on the structure of the two substrates. For example, the A peak shown in Fig. 6 was

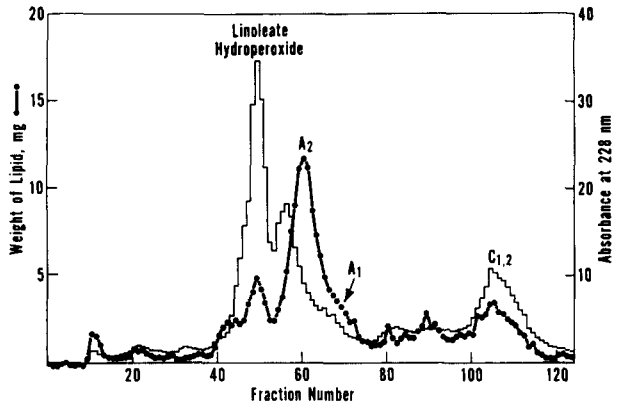

FIG. 6. Silicic acid column chromatography of the products resulting from action of corn linoleate hydroperoxide isomerase upon linoleic acid hydroperoxide formed by soybean lipoxygenase.

composed of A_1 and A_2. A_1 was found in the trailing edge of the peak and was barely distinguished by a slightly lower R_f value with TLC. The elution sequence of A_1 and A_2 was demonstrated by analyzing various fractions over the peak by periodate oxidation.

Using periodate oxidation, the percentage of each isomer can be calculated. Aliquot portions of each fraction from the A peak were pooled for periodate oxidation. Subsequent GLC analysis established the weight percentage of the esterified fragments, which was used to calculate the composition of the peak, to be 18% A_1 and 82% A_2.

The distribution of C_1 and C_2 was almost identical to that of A_1 and A_2. On the basis of the ratio of nonanedioic to decanedioic acids obtained from permanganate–periodate oxidation, the percentage composition was calculated as 85% C_2 and 15% C_1.

Assay of Linoleate Hydroperoxide Isomerase. Linoleic acid hydroperoxide was prepared by oxidation of linoleic acid by soybean lipoxygenase, and served as the substrate in the assay of linoleate hydroperoxide isomerase from corn germ. The substrate was prepared as needed, and thereafter remained unchanged for several hours. About 89 ± 7.5% yields of hydroperoxide were obtained under the proper conditions. This amounted to 0.651 ± 0.055 mM substrate concentration; this is 90% of enzyme saturation calculated from the K_m value. The components remaining in the substrate other than linoleic acid hydroperoxide were mostly small amounts of linoleic acid and oxo-octadecadienoic acid. High yields of hydroperoxides depended on the pH, O_2 tension, and amount of lipoxygenase used. The best results were at pH 8.7–9.0, and with sufficient lipoxygenase to complete the reaction in 40–60 min. The molarity of the pH 8.7 buffer used was kept low so that addition of the substrate solution to the corn extracts (buffered at pH 6.9 with 0.2 M phosphate) would raise their pH to only 7.0.

Isomerase activity was assayed directly by observing the initial rate of decrease in conjugated diene absorbance at 234 nm in the presence of the substrate prepared as described above. If the reaction was allowed to proceed to completion, the absorbance finally reached a constant value greater than zero. The absorbance did not approach zero because of product absorbance, which reproducibly accounted for about 13% of the original after the reaction was complete. Decrease in substrate absorbance could be calculated from this percentage and the experimental curves using the following equations:

$$\text{Product abs.}_{t_m} = k(\text{Observed abs.}_{t_o} - \text{Observed abs.}_{t_m})$$

$$\text{Substrate abs.}_{t_m} = \text{Observed abs.}_{t_m} - \text{Product abs}_{t_m}$$

(t, time; m, number of min; abs., absorbance; and k, $0.13^1 + 0.13^2 + 0.13^3 + \ldots = 0.149$)

The specific activities of linoleate hydroperoxide isomerase in germ extracts were calculated from assay data. The value for whole germ extracts was 2.4 μmoles/min per mg of protein. The specific activity of extracts prepared from defatted germ was exactly half that of extract prepared from whole germ. This partial inactivation of the enzyme was often tolerated so that no interference with residual lipids was encountered during isolation of the products. Precipitation of the active factor by $(NH_4)_2SO_4$ at 40% of saturation resulted in a 2.3- and 1.4-fold increase in purification of the activity in whole germ and defatted germ extracts, respectively (based on the specific activity of whole germ, 2.4 μmoles/min per mg of protein). The specific activity in endosperm extracts (0.18 μmoles/min per mg of protein) was comparatively low.

Characterization of the Enzyme. The action of linoleate hydroperoxide isomerase(s) produced two general classes of fatty acids, A (A_1 or A_2) and C (C_1 or C_2). A and C appeared to be formed from the hydroperoxide in parallel as determined by TLC analyses throughout the time of reaction. The ratio of A and C produced was fairly constant regardless of whether crude or partially purified germ extracts were used. The quantity of A formed was always greater than C as can be seen in Fig. 6. Isolated A or C added to germ extracts remained essentially unchanged showing that A and C were not interconvertible. It is not known whether the same isomerase was involved in the formation of A and C.

The enzymic nature of the isomerase was demonstrated in a number of ways. Retention of activity after dialysis was indicative of the macromolecular nature of the catalyst. Boiling of crude extracts for 5 min terminated all isomerase activity. The protein denaturing reagent, guanidine hydrochloride (8 M) was equally effective in inactivation.

Isomerase activity dependence on pH and substrate concentration was determined. Dependence of activity on

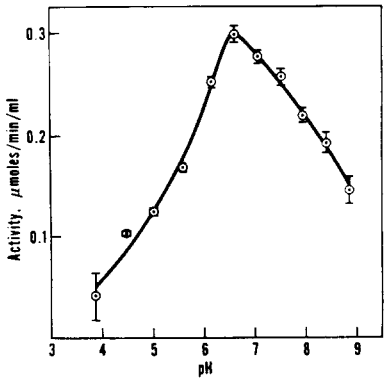

FIG. 7. Linoleate hydroperoxide isomerase activity as a function of pH. Defatted germ extract was fractionated with $(NH_4)_2SO_4$ (40% saturation), dialyzed, and lyophilized. The lyophilized residue was mixed with a solution of 0.2 M NaCl, 0.2% Tween 20, and 0.648×10^{-3} M dithiothreitol (10 mg/10 ml) for 2 hr. The mixture was centrifuged at 8000 *g* for 10 min, and the supernatant was mixed with an equal volume of the appropriate 0.2 M buffer. This enzyme solution was then added to an equal volume of substrate solution for assay.

pH is shown by Fig. 7. The optimum pH is about 6.6. Lineweaver–Burke plots showed that the Michaelis constant of linoleate hydroperoxide isomerase was 0.07 mM.

DISCUSSION

Linoleic acid was oxidized by corn germ extracts resulting in the accumulation of two major products, A_1 and B. Smaller quantities of C_1 and an unknown were also found. The structures of A_1, B, and C_1 were indicative of the action of several enzymatic reactions acting in series. These sequential enzymes, lipoxygenase, linoleate hydroperoxide isomerase(s), and a probable acylating enzyme, were studied separately. The partial reactions obtained by each study confirmed the position of the enzymes in the series.

The action of the first enzyme in the series, lipoxygenase, was not immediately apparent. The product, linoleic acid hydroperoxide, was never present in more than trace quantities unless lipoxygenase was first purified. Purification removed other enzymes in the series permitting linoleic acid hydroperoxide to accumulate in the reaction.

The properties of corn lipoxygenase, as well as its product, appear to differ from soybean lipoxygenase. The inaction of corn lipoxygenase at pH 9 distinguished the corn enzyme from soybean lipoxygenase, which is quite active at pH 9. The termination of corn lipoxygenase activity at about pH 8–9 indicated that there may be a strict dependence of the carboxylic acid moiety for binding to the enzyme. As explained later in the discussion, the oxidation of linoleic acid by corn lipoxygenase is believed to result in 9-hydroperoxy-octadecadienoic acid, while soybean lipoxygenase oxidizes linoleic acid to predominantly 13-hydroperoxy-octadecadienoic acid. Hamberg and Samuelsson (24) reported that in soybean lipoxygenase-catalyzed oxidation the reactivity depended on the size of the hydrocarbon end of the fatty acids. Hydrophobic binding of the hydrocarbon end of the molecule to lipoxygenase could be different in an oxidation specific for the 9-carbon, as proposed for corn lipoxygenase.

B, an ester of linoleic acid with the 9-hydroxyl of A_1, was only formed when linoleic acid was present in crude germ extracts. B was not found in excess of trace quantities when linoleic acid hydroperoxide was used as a substrate (Fig. 6).

When attempts were made to demonstrate the presence of an enzyme responsible for the acylation reaction, difficulties were encountered. For example, when stearic acid and A_1 were added to germ extracts, no esterification occurred. Linoleic acid and A_1 could also be added to extracts with no formation of B, provided the reaction was kept under nitrogen to prevent the oxidation of linoleic acid. However, B could be formed under nitrogen if linoleic acid and linoleic acid hydroperoxide were simultaneously added to germ extracts. The acylation reaction is more complex than the straightforward reaction which was anticipated. One mechanism which fits the data may be an acylation which occurs through a transitory intermediate as shown:

$$\text{Linoleic acid hydroperoxide} \rightarrow [x] \rightarrow A_1$$
$$\text{Linoleic acid} + [x] \rightarrow B$$

The final reaction in the series appears to be catalyzed by linoleate hydroperoxide isomerase. This enzyme in corn appears to be identical to the isomerase in flaxseed reported by Zimmerman (10). This study and the study by Zimmerman found that the major product of isomerase activity was of the general structure R—CHOH—CO—CH_2—CH=CH—R′, corresponding to products A_1 or A_2. However, a minor product of the general structure R—CO—CH=CH—CHOH—R′ was also formed in corn germ extracts. This structure corresponds to products C_1 or C_2, which also may be products of a linoleate hydroperoxide isomerase.

The final products of the sequential enzyme system, corn lipoxygenase and linoleate hydroperoxide isomerase, are A_1 and C_1. The structure of the major product, A_1, indicated that the intermediate in the reaction is 9-hydroperoxy-octadecadienoic acid. Thus it appears that corn lipoxygenase oxidizes the 9-position of linoleic acid specifically.

Soybean lipoxygenase is known to oxidize linoleic acid to predominantly 13-hydroperoxy-octadecadienoic acid (3, 4) and occasionally to some 9-hydroperoxy-octadecadienoic acid (3). If soybean lipoxygenase is used to generate linoleic acid hydroperoxide as a substrate for

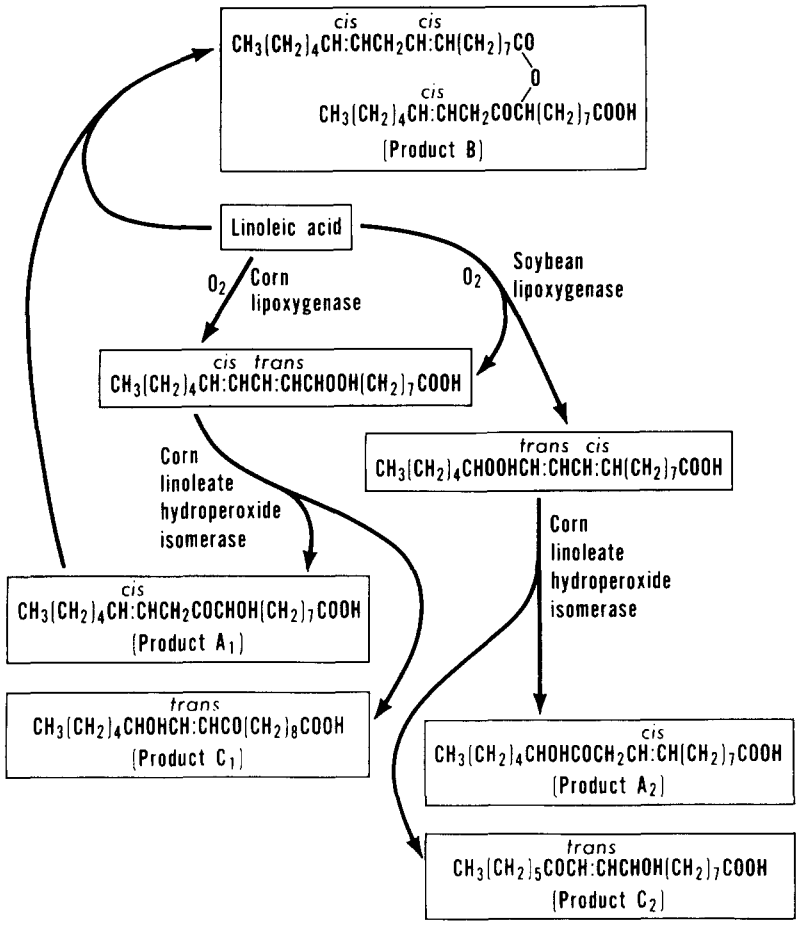

FIG. 8. Proposed scheme for the enzymic oxidation of linoleic acid.

linoleate hydroperoxide isomerase from corn, A_1, A_2, C_1, and C_2 are formed. A_2 and C_2 are the predominant isomers, amounting to about 83% of the total. By inference, the soybean lipoxygenase oxidized linoleic acid to 83% 13-hydroperoxy-octadecadienoic acid. However, at least part of the remaining 17% 9-hydroperoxy-octadecadienoic acid could have been derived through corn lipoxygenase oxidation of residual linoleic acid left unoxidized by soybean lipoxygenase.

The formation of C_1 and C_2 from the linoleic acid hydroperoxides requires some discussion because of the extensive rearrangement of the molecule which occurred. The results indicate that C_1 is derived from 9-hydroperoxy-octadecadienoic acid, and C_2 from 13-hydroperoxy-octadecadienoic acid. If these conversions proceed as proposed, rearrangement would require migration of a double bond and saturation of the position of the hydroperoxide substituent. The nature of this reaction needs to be characterized further.

Evidence obtained from study of the sequential enzyme system in corn germ extracts, as well as the partial reactions, enabled the construction of the pathway of linoleic acid oxidation (Fig. 8). A side reaction involving use of soybean lipoxygenase in one step of the sequence is also shown.

Although conditions for further metabolism of A_1, B, and C_1 are unknown, the oxidation and isomerizations described may be the initial reactions required for a chain cleavage and further oxidation of the fragments during corn germination.

The assistance of Dr. David Weisleder and Mr. Larry Tjarks for NMR spectra is gratefully acknowledged. Thanks are expressed to Mr. Charles Blessin for aid in selecting an optimum GLC program, Mr. R. L. Reichert for hydrogenation, and Dr. W. K. Rohwedder for the mass spectral analysis.

Manuscript received 28 July 1969; accepted 17 March 1970.

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