Mechanism of Lipoxidase Reaction. I. Specificity of Hydroperoxidation of Linoleic Acid[1]

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ABSTRACT

Linoleate hydroperoxides from autoxidation of methyl linoleate and from lipoxidase oxidation of linoleic acid are compared. Data indicate an equal amount of methyl 9- and 13-hydroperoxyoctadecadienoate produced by autoxidation of methyl linoleate, and the exclusive formation of 13-hydroperoxyoctadeca-9,11-dienoic acid from the incubation of lipoxidase with linoleic acid. As a result of these findings, a specific mechanism for the reaction of lipoxidase with linoleic acid is postulated.

INTRODUCTION

IN 1945–46 PRODUCTS from the autoxidation of linoleic acid were reported to be the same as those from the oxidation of linoleic acid by lipoxidase (1–3). Enzymatic oxidation of linoleic acid was thought to follow the same general course as ordinary metal-catalyzed autoxidation. The quantitative determination and identification of the 9- and 3-hydroperoxides were based on comparison of melting points (mp). Since it was subsequently shown (4) that different isomers of hydroxystearates can form eutectic systems, this identification based on mixed melting points was questionable.

In the latest review on lipoxidase (5), the mechanism of reaction is thought to involve the formation of a free radical on C-11, which explains the possibility for random formation of the 9- and 13-hydroperoxide isomers. This mechanism is strongly supported by other evidence like the presence of free radical intermediates during lipoxidase-catalyzed oxidation of linoleic acid (6–8). Lipoxidase is highly specific for oxidation of *cis, cis*-1,4-pentadiene-containing fatty acids (9) and formation of optically active conjugated *cis,trans* diene hydroperoxides. Presumably the isomerized double bond is the one converted to the *trans* configuration.

During the preparation of this manuscript, other workers pointed out (10) that lipoxidase-catalyzed hydroperoxidation of unsaturated fatty acids is relatively specific with regard to carbon atom attacked. They demonstrated that during lipoxidase-catalyzed oxidation of linoleic acid, 70% of the products formed is 13-hydroperoxyoctadecadienoic acid and only 30%, 9-hydroperoxyoctadecadienoic acid. Our work shows an even higher specificity of lipoxidase; i.e., the exclusive formation of 13-conjugated hydroperoxide by lipoxidase. Consequently, a specific mechanism for the reaction of lipoxidase with linoleic acid is justifiable and is postulated.

EXPERIMENTAL

Preparation of Hydroxystearates

From Incubation Product of Lipoxidase. The incubation of lipoxidase with linoleic acid was done in a Parr 3910 hydrogenation apparatus connected to an oxygen tank. Linoleic acid (180 mg, The Hormel Institute, 99.8% pure) was dissolved in 10.0 ml 95% ethanol and added to 80.0 ml 0.05 M borate buffer, pH 9.0. Two drops of Antifoam B (Dow Corning Corporation) was added and eliminated foaming completely. The enzyme (10.0 mg, crystalline lipoxidase[4] Nutritional Biochemical Corporation) was supended over the reaction mixture in a small glass basket while the flask was evacuated and flushed several times with oxygen. Pressure was then adjusted to 1 atmosphere of 100% oxygen at 24C. Starting the shaker released the enzyme basket which dropped into the solution. After 60 min of incubation with continuous shaking, the reaction vessel was removed from the apparatus. A more complete description and analysis of

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[4] The crystalline lipoxidase preparation employed in this study was completely specific for the 13 position of linoleic acid. However, other crystalline enzyme preparations from the same supplier showed no apparent specificity oxidizing both the 9 and 13 carbons. These observations suggest that even crystalline lipoxidase may be a mixture of two or more enzymes.

this incubating procedure will be included in a future publication.

Hydroperoxide was reduced to alcohol by the addition of 100 mg $NaBH_4$ to the reaction mixture at room temperature with magnetic stirring (11). After 60 min, excess $NaBH_4$ was decomposed by acidifying the reaction mixture to pH 3.0 with concentrated HCl. During the reduction a flow of N_2 was maintained over the reaction mixture.

The product was extracted three times with 50 ml diethyl ether, residual water in extract was removed with Na_2SO_4 and extract evaporated to about 25 ml under reduced pressure in a rotatory evaporatory at room temperature. After methylation in diethyl ether with diazomethane, the solvent was evaporated under a stream of N_2 and the residue removed under reduced pressure. Hydrogenation of the products took place in a microhydrogenator with absolute ethanol as solvent and 5% palladium on carbon as catalyst.

From Autoxidized Methyl Linoleate. Methyl linoleate (4.3 g and 99.6% pure) prepared by counter double current distribution (12) was placed in a 20-cm long 0.5 cm inside diameter test tube kept at 40C with oxygen bubbling through at a low rate. After the peroxide value (PV) reached a level of 2400, 300 mg of the autoxidized methyl linoleate was dissolved in 10 ml 95% ethanol, 80 ml 0.05 M borate buffer, pH 9.0, was added and the hydroperoxide was converted to an alcohol by reducing it with $NaBH_4$. The mixture was acidified, extracted with diethyl ether and hydrogenated catalytically.

Separation of Products

Column chromatography on high-grade silica gel (Adsorbosil, Applied Science Laboratories) was used. The quantitative separation between methylstearate, methyl keto stearates, methyl 9-hydroxystearate and methyl 13-hydroxystearate has been described previously (13). The identity and purity of the last two compounds were verified by comparing the analysis with that of known pure isomers by gas-liquid chromatography (GLC), thin-layer chromatography (TLC), infrared (IR), mass spectroscopy and melting points. GLC was performed on a Pye argon gas chromatograph equipped with a 4 ft × ¼ in. glass column packed with 14% EGSS-X on Gas-Chrom P, 100/200 mesh (Applied Science Laboratories) and with a radium D ionization detector. Column temperature was 170C and the argon gas flow 100 ml/min. The area under each curve was determined from

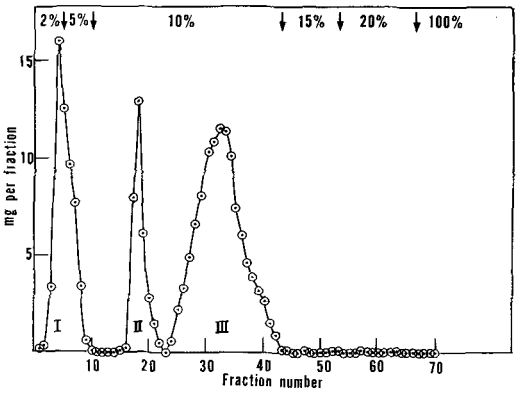


FIG. 1. Separation of lipoxidase hydroperoxidation products of linoleic acid. Methyl stearate (I), methyl keto stearate (II) and methyl 13-hydroxystearate (III) on Adsorbosil, 202 mg applied and collected in 70 fractions of 2 ml eluate each (98.2% recovery). Percentage indicated refers to concentration of diethyl ether in *n*-hexane.

an electronically integrated curve. Glass plates (20 × 20 cm) spread with a 0.2-mm layer of Silica Gel G (Brinkmann Instruments, Inc.) and activated for 30 min at 110C were used for TLC analysis. Hexane: diethyl ether (7:3) was the developing solvent. Infrared analysis was performed in a Model 621 Perkin-Elmer spectrophotometer. The mass spectra were measured on a Nuclide 12–90 G mass spectrometer equipped with an all-glass inlet; inlet

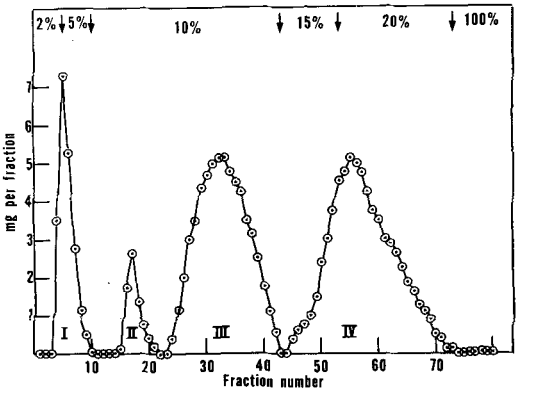


FIG. 2. Separation of autoxidation products of methyl linoleate. Methyl stearate (I), methyl keto stearate (II), methyl 13-hydroxystearate (III) and methyl 9-hydroxystearate (IV) on Adsorbosil, 155 mg applied and collected in 80 fractions of 2 ml eluate each (98.7% recovery). Percentage indicated refers to concentration of diethyl ether in *n*-hexane.

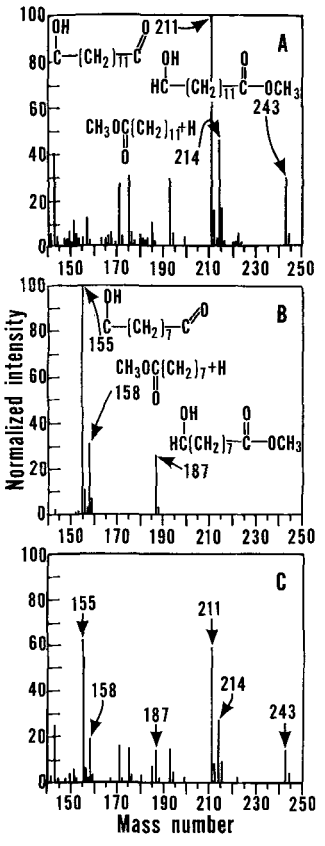


FIG. 3. Mass spectra fragmentation patterns. A is the constituent of peak III, methyl 13-hydroxystearate; B is the constituent of peak IV, methyl 9-hydroxystearate; and C is a mixture of both.

temperature, 170C; source temperature, 225C; and 70 v electron energy.

RESULTS AND DISCUSSION

Figures 1 and 2 show the separation of the different reduced products of lipoxidase oxidation and autoxidation of linoleic acid on the silica gel column. Only peaks III and IV had a characteristic hydroxy band at 3646 cm^{-1} as determined by IR. Both had the same retention time on GLC and it corresponded to the retention time of methyl 9-hydroxystearate (13). The respective mp's of products corresponding to peaks III and IV were 50.0–50.5C and 47.5–48.0C.

The mass spectra of the isomeric methyl hydroxystearates show characteristic fragmentation patterns that result from cleavage of the carbon-carbon bonds on each side of the carbon atom to which the hydroxy group is attached. This fragmentation pattern for known pure methyl 8-, 9-, 10, 12, and 13-monohydroxystearates is consistent with the interpretation of the methyl 8- and 10-monohydroxystearates given by Ryhage and Stenhagen (14). The various mass peaks are interpreted in Figure 3. The fragmentation pattern of the constituent of peak III (Fig. 3A) has a mass base peak 211, whereas the constituent of peak IV (Fig. 3B) gives a mass base peak 155. The mass spectrum of methyl 9-hydroxystearate from the hydrogenation of methyl dimorphecolate was identical with that of peak IV. Therefore, the constituents of peaks III and IV are the 13- and 9-hydroxystearates, respectively. The mass spectra of the methyl hydroxystearates from the reduction of the product of autoxidation of methyl linoleate are identical with the mass spectra of the mixture of both isomers. The fragmentation patterns of the isomers are quite different, but each is consistent and can be easily distinguished. Contamination of one of the isomers with the other can be clearly detected from the mass spectra of the mixture.

The quantitative analysis of the autoxidation products of methyl linoleate and lipoxidation products of linoleic acid is presented in Table I. As previously shown (15), hydroperoxides produced by the autoxidation of methyl linoleate form about equal amounts of the 9- and 13-isomers. Completely unexpected were the findings from the analysis of the lipoxidase oxidation products of linoleic acid. In several different incubations of lipoxidase with linoleic acid only the methyl 13-hydroxystearate was isolated, and there was no evidence of methyl 9-hydroxystearate. Possibly the 9-isomer might have been lost during the separation procedure, but autoxidation products of methyl linoleate carried through the same procedure yielded the expected 9- and 13-hydroxystearates.

Unless two different specific oxidative enzymes are present in soybean lipoxidase (8,16), one specific for the formation of the 9-hydroperoxide isomer and the other specific for the 13-isomer, the formation of both isomers in unequal amounts is difficult to explain (10). Explanations for the differences between our study and Hamberg and Samuelsson's might cite various factors, such as the differences in the enzymes used, different incubation and analytical procedures, and the possibility of interference by autoxidation that would produce a significant amount of the 9-isomer and obscure specificity.

TABLE I

Quantitative Analysis of Autoxidation Products of Methyl Linoleate and Lipoxidation of Linoleic Acid

Compound	Lipoxidase oxidation of linoleic acid		Autoxidation of methyl linoleate	
	Separation on column[a]	GLC determination[b]	Separation on column[a]	GLC determination[b]
Methyl stearate	26.7	24.1	13.4	11.8
Methyl keto stearate	16.3	19.1	4.8	5.6
Methyl 9-hydroxystearate			39.5	
Methyl 13-hydroxystearate	57.0	56.8[c]	42.2	82.4[c]
	100.0	100.0	99.9	99.8

[a] Percent weight of recovered product.

[b] Relative percent area.

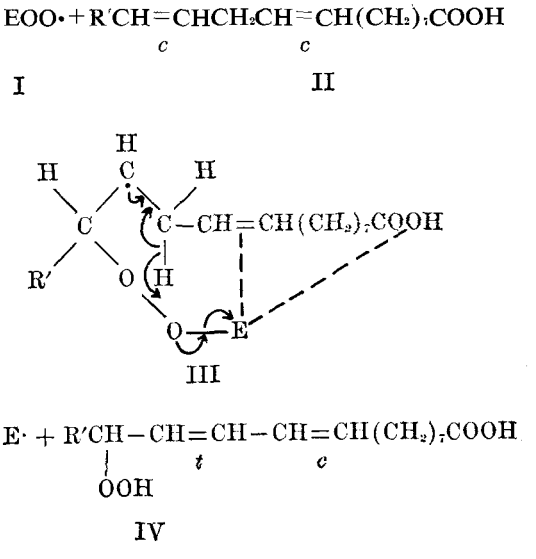
[c] The 9- and 13-hydroxystearate isomers gave one common peak on GLC.

Lipoxidase was previously demonstrated to be specific for the substrate, i.e., it attacks only a *cis,cis*-1,4-pentadiene system (9). The formation of an optically active isomer from lipoxidase hydroperoxidation (17) versus the lack of any optical activity in autoxidation products, also points out the high specificity of the enzymatic reaction.

Based on these new findings it might be well to reconsider the sequence of the reactions involved, as given in Scheme 1. From a purely mechanistic point of view one has two alternatives in explaining the synthesis of only one isomer. If one accepts the conventional mechanism proposed for the reaction of lipoxidase (5), it is difficult to account for the blocking of the C-9 position following the abstraction of the methylenic hydrogen and formation of a free radical on C-11.

Although a free radical intermediate was shown to be present in the overall reaction, it was not established to be present only in a specific step in the sequence of reactions involved nor was it assigned to a specific carbon atom. Since several oxidative enzymes, including lipoxidase, were shown to initiate free radical intermediates (8) we feel that a new mechanism is worth consideration. As shown in the scheme, we propose that a free radical adds to the substrate rather than abstracts hydrogen from it. Activation of the enzyme by oxygen forms a free radical (I) which reacts with linoleic acid (II) by addition at C-13; i.e., the unsaturated carbon atom farthest from the carboxyl group. It appears probable that I is held in the proper position to facilitate this reaction by attachment to the substrate via the double bond at C-9 and/or the carboxyl group. A series of one-electron shifts, indicated by arrows, then takes place in intermediate III. These shifts result in establishment of a new *trans* double bond at C_{10}, transfer of a hydrogen atom to oxygen to form the hydroperoxide group, and liberation of the enzyme, perhaps as the free radical E° which could react with O_2 to regenerate I. The product is, therefore, exclusively a 13-hydroperoxy-9,11-octadecadienoic acid (IV).

Investigation into the origin of the oxygen molecule incorporated into the hydroperoxide



SCHEME 1. Postulated reaction sequence of lipoxidase, oxygen and linoleic acid.

by the use of $^{18}O_2$ is completed and will be reported shortly.

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