On the Specificity of the Oxygenation of Unsaturated Fatty Acids Catalyzed by Soybean Lipoxidase*

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SUMMARY

The positional and stereochemical specificities of the soybean lipoxidase-catalyzed oxygenation of unsaturated fatty acids have been investigated. It was found that the oxygen function was introduced at position 6 in all of the fatty acids which reacted. In one case a small amount of the substrate was oxygenated in position 10.

The structural requirement for substrates is the presence of a cis,cis-1,4-pentadiene group with its methylene group located in position 8. The structure of the hydroperoxy acid formed from 8,11,14-eicosatrienoic acid was 15β-hydroperoxy-8(cis),11(cis),13(trans)-eicosatrienoic acid. By using sterースpecifically tritium-labeled 8,11,14-eicosatrienoic acids, it was found that the removal of hydrogen from the 6 methylene group is stereospecific. Thus, only the hydrogen of the configuration is removed by the enzyme during the conversion into 15β-hydroperoxy-8(cis),11(cis),13(trans)-eicosatrienoic acid. The conversion of [13C-3H,3H]8,11,14-eicosatrienoic acid is accompanied by an isotope effect, suggesting that the hydrogen removal occurs at the initial step of the reaction.

The enzyme lipoxidase catalyzes the oxygenation of unsaturated fatty acids by molecular oxygen. The reaction, which seems to be specific for fatty acids possessing methylene group-interrupted cis double bonds, results in the formation of unsaturated hydroperoxy fatty acids with one pair of conjugated double bonds (1). Earlier studies showed that aerobic incubation of sodium linoleate with lipoxidase yields two isomeric hydroperoxy fatty acids, viz. 9-hydroperoxy-10,12-octadecadienoic acid and 13-hydroperoxy-9,11-octadecadienoic acid (2, 3). This finding seemed to indicate that the lipoxidase-catalyzed oxygenation of unsaturated fatty acids is of a relatively unspecific nature; i.e. every pair of methylene group interrupted cis double bonds should give rise to two isomeric hydroperoxides. According to this view, the number of isomeric hydroperoxy fatty acids should be 2n - 2, where n equals the number of double bonds in the substrate molecule.

The present study involves characterization of the products formed by the action of soybean lipoxidase on a large number of unsaturated fatty acids. The results show that the enzyme-catalyzed oxygenation is very specific with respect to the position in the fatty acid that is oxygenated. It was also found, by using naturally occurring and synthetic fatty acid analogues, that the cis,cis-1,4-pentadiene grouping of the fatty acid, which was previously considered to be the only structural requirement (1), must be located with its methylene group in position 6 in order to secure reaction. We have studied the stereochemical features of the reaction by determining the absolute configuration of the carbon atom bearing the hydroperoxy group and the stereochemistry of the removal of the hydrogen from the methylene group. Data on isotope discrimination in the reaction also provide information on the initial step of the transformation.

Part of this investigation has been published previously in preliminary form.

MATERIALS AND METHODS

Lipoxidase—Crystalline soybean lipoxidase (20,000 units per mg) purchased from Fluka was used.

Unsaturated Fatty Acids—The substrates were purified by silicic acid chromatography prior to the incubations. The purity was checked by gas-liquid chromatography on a polyester column (8% ethylene glycol succinate S-X supported on Gas-Chrom P). The following fatty acids were gifts from the sources indicated: 15-methyl-8,11,14-eicosatrienoic acid from Dr. J. E. Pike, the Upjohn Company; 5,8,11-eicosatrienoic acid from Professor W. Stoffel, Cologne; 8,14-eicosadienoic acid from Dr. D. A. van Dorp, Unilever, Vlaardingen; and 8,11,14-docosatrienoic acid, 9,12,15-heneicosatrienoic acid, 9,12,15-docosatrienoic acid from Dr. D. Klenberg, Karolinska Institutet, Stockholm.

Incubation Procedure—Incubations on a preparative scale were performed at 0° and pH 9.0. The substrates (4 mg) were

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converted into their ammonium salts by the addition of \( \text{NH}_2\text{OH} \) (0.019 m, 1 ml). The enzyme was dissolved in a buffer solution (1 mg of lipoxidase per ml of 0.1 m sodium borate buffer, pH 9.0). Incubations were started by the addition of 2.5 ml of enzyme solution. After 15 min an additional 0.5 ml of enzyme solution was added. Measurements of the absorbance at 234 \( \text{m}\alpha \) of aliquots of the incubation mixture removed every 5 min showed that the conversion was almost complete after 30 min. After this period of incubation, 15 ml of ethanol were added. The solution was immediately diluted with water, acidified to pH 3, and extracted with ether. The ether was washed with water until the reaction was neutral, and was evaporated at room temperature under reduced pressure. Incubations were also performed on an analytical scale at room temperature. The substrates (10 to 50 \( \mu\)g) were transferred into quartz cuvettes and were converted into their ammonium salts by the addition of NH\( _2\text{OH} \). After addition of 2 ml of borate buffer, pH 9.0, the incubations were started by the addition of enzyme solution. The absorption at 234 \( \text{m}\alpha \) was followed with a Zeiss PMQ II spectrophotometer.

Reduction with Sodium Borohydride—The compounds (4 to 100 mg) were dissolved in methanol (1 to 10 ml), cooled to 0\(^\circ\), and reduced with sodium borohydride (10 to 150 mg). After 20 min at 0\(^\circ\) and 40 min at room temperature, the solution was acidified and extracted with ether.

Catalytic Hydrogenation—For complete hydrogenation, the unsaturated compounds (1 to 4 mg) were dissolved in 2 ml of ethanol and were hydrogenated under a stream of hydrogen gas with 10 mg of PtO\( _2 \) as catalyst. For selective hydrogenation, the compounds (10 to 20 mg) were dissolved in 10 ml of methanol, and 50 mg of a modified Lindlar catalyst and 50 \( \mu\)l of quinoline were added. The solution was stirred under a stream of hydrogen gas.

Oxidation with Chromic Acid—The compounds (1 to 3 mg) were dissolved in 1 ml of glacial acetic acid and added to 1 ml of 95% acetic acid containing 3.4 mg of CrO\( _3 \). After 40 min at 37\(^\circ\), methanol was added. The solution was acidified and extracted with ether.

Oxidative Ozonolysis—Prior to ozonolysis, the unsaturated hydroxy acids were esterified by treatment with diazomethane and acetylated with acetic anhydride in pyridine. These derivatives (2 to 4 mg) were dissolved in 4 ml of chloroform, cooled to -15\(^\circ\), and treated with an excess of ozone. After 10 min at room temperature, the chloroform was evaporated at reduced pressure. The ozonides were oxidized with 0.2 ml of 30\% \( \text{H}_2\text{O}_2 \) in 1 ml of glacial acetic acid. After 18 hours at 50\(^\circ\), the solution was taken almost to dryness at 40\(^\circ\) under reduced pressure.

Mass Spectrometry—Mass spectrometry was performed in combination with gas chromatography as described by Ryhage (4) and the LKB 0000 instrument. A column of 1\% SE 30 on Chromosorb P, operated at 100-190\(^\circ\) at a pressure of 3 kg per cm\(^2\), was used. In one case, the samples were introduced through the direct inlet of the mass spectrometer. This was necessary for the analysis of hydroperoxy esters, which could not be passed through the gas chromatograph without degradation.

Chromatographic Methods—Silicic acid chromatography was carried out on silicic acid (Mallinkrodt, 100 mesh) activated at 115\(^\circ\). The columns were eluted with increasing concentrations of ether in hexane.

Two solvent systems were used for thin layer chromatography. Glass plates coated with Silica Gel G were developed with ether-pentane, 50:50 (5). For the development of glass plates coated with Silica Gel G-silver nitrate (25:1), the organic layer of the following system was used: ethyl acetate-2,4-trimethylpentane-water (75:75:100). The compounds were detected with an ultraviolet lamp after spraying with 2',7'-dichlorofluorescin.

Reversed phase partition chromatography was carried out as described by Norman and Bjövall (6). The solvent systems used were methanol-water, 129:171 (moving phase), and chloroform-isooctyl alcohol, 15:15 (stationary phase).

Gas-liquid chromatography was performed with an F and M Biomedical gas chromatograph, model 400. Part of the effluent was led into an ionization chamber via a stream splitter, thus permitting continuous measurement of radioactivity in combination with gas chromatography. The phases used were silicone grease (15\% on 100 to 120 mesh Celite), SE-30 (1\% on Gas-Chrom P), and ethylene glycol succinate S-X (8\% on Gas-Chrom P).

Measurement of Radioactivity—A Frieseke Hoepfner FH 90A gas flow counter operated in the proportional range was used for the assay of radioactivity in chromatographic fractions. A Packard Tri-Carb model 4225 liquid scintillation counter was used for the determination of the \( ^{3}\text{H}:^{13}\text{C} \) ratio.

Sterespecifically Tritium-labeled Fatty Acids—The preparations of [13\%\text{H}, 3\%\text{C}]8,11,14-eicosatrienoic acid and [13\%\text{H}, 3\%\text{C}]8,11,14-eicosatrienoic acid are described in an accompanying report (7). The reactions used for the preparation of [15\%\text{H}, 3\%\text{C}]8,11,14-eicosatrienoic acid are also described in an accompanying report (8).

Method Used for Analysis of Products—The substrate specificity of the enzyme was examined by incubating fatty acids differing in chain length and in location and degree of unsaturation. The hydroperoxy acids were reduced with sodium borohydride, yielding the hydroxy acids. These were subjected to silicic acid chromatography. The unsaturated hydroxy acids (eluted with ether-hexane, 2:8) were subjected to catalytic hydrogenation. The saturated hydroxy acids were esterified by treatment with diazomethane and were subsequently oxidized with chromic acid, yielding methyl esters of saturated keto acids. The position of the keto group of these esters could be determined by mass spectrometry in combination with gas chromatography. Since there is no rearrangement of the hydroperoxy group, the position of the keto group in the keto esters is the same as of the hydroperoxy group initially introduced by the enzyme. In the case when mass spectrometry indicated the presence of two isomeric keto esters (giving one single peak on gas-liquid chromatography; cf. Reference 9), the two corresponding isomeric hydroxy esters were separated by preparative thin layer chromatography on silica gel not containing silver nitrate. The compounds were scraped off, eluted from the silica gel, and subjected to gas-liquid chromatography. This procedure permitted determination of the percentage composition of the hydroxy ester mixture.

RESULTS

Positional Specificity—A series of 14 unsaturated fatty acids was incubated with lipoxidase, and the position of the hydroperoxy group in the products was determined as described above. The results are summarized in Table I. The mass
spectra recorded for the keto esters derived from the products of 9,12-octadecadienoic acid, 8,11,14-eicosatrienoic acid, and 4,7,10,13,16,19-docosahexaenoic acid are given in Figs. 1, 2, and 3, respectively. For comparison the mass spectra of methyl 9-ketostearate, methyl 13-ketostearate, and methyl 11-ketoarachidate are given in Figs. 4, 5, and 6, respectively. The mass spectra of the keto esters derived from 9,12-octadecadienoic acid (Fig. 1) obviously represents a mixture of methyl 9-ketostearate and methyl 13-ketostearate. The following ions are due to methyl 9-ketostearate: m/e 281 (M - 31), 200 (M - 112; β cleavage with loss of CH=CH-(CH2)7-CH3), 185 (M - 127; α cleavage with loss of -(CH2)5-CH3), 170 (M - 142; β cleavage with loss of CH—CH—(CH2)9-COOCH3), and 168 (M - 144; 200 - 32). The ions formed from methyl 13-ketostearate are 281 (M - 31), 256 (M - 56; β cleavage with loss of CH2=CH-CH2-CH3), 241 (M - 71; α cleavage with loss of -(CH2)5-CH3), and 199 (M - 113; β cleavage with loss of -(CH2)5-C-(CH2)9-CH3). The relative amounts of the two isomers were determined by thin layer chromato-graphic separation (solvent, ether-pentane) of the two saturated hydroxy esters and subsequent quantitative determination by gas-liquid chromatography on SE-30.

The mass spectrum of the keto ester derived from 8,11,14-eicosatrienoic acid (Fig. 2) shows ions at m/e 309 (M - 31), 284 (M - 56; β cleavage with loss of CH2=CH—CH=CH—CH3), 269 (M - 71; α cleavage with loss of -(CH2)5-CH3), 252 (M - 88; 284 - 32), 237 (M - 103; 269 - 32), 227 (M - 113; β cleavage with loss of -(CH2)5-C-(CH2)9-CH3), and 195 (M - 145; 227 - 32). By comparing this mass spectrum with the mass spectrum of methyl 11-ketoarachidate (prepared as described in an accompanying report (8)), the absence of the 11-

### Table I

Structures of keto esters derived from products obtained on incubation of unsaturated fatty acids with soybean lipoxidase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Saturated keto ester(s) derived from product</th>
</tr>
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<tbody>
<tr>
<td>9,12-Octadecadienoic acid</td>
<td>Methyl 13-ketostearate (92%)</td>
</tr>
<tr>
<td>9,12,15-Octadecatrienoic acid</td>
<td>Methyl 9-ketostearate (8%)</td>
</tr>
<tr>
<td>9,11,14-Eicosatrienoic acid</td>
<td>Methyl 13-ketostearate</td>
</tr>
<tr>
<td>5,8,11,14-Eicosatetraenoic acid</td>
<td>Methyl 13-ketostearate</td>
</tr>
<tr>
<td>5,8,11,14,17-Eicosapentaoenoic acid</td>
<td>Methyl 15-ketoarachidate</td>
</tr>
<tr>
<td>9,12,15-Heneicosatrienoic acid</td>
<td>Methyl 16-ketoheneicosanate</td>
</tr>
<tr>
<td>10,13,16-Docosatrienoic acid</td>
<td>Methyl 17-ketodocosanate</td>
</tr>
<tr>
<td>4,7,10,13,16,19-Docosahexaenoic acid</td>
<td>Methyl 17-ketodocosanate</td>
</tr>
</tbody>
</table>

Fig. 1. Mass spectrum recorded for the keto esters derived from 9,12-octadecadienoic acid.
Fig. 2. Mass spectrum recorded for the keto ester derived from 8,11,14-eicosatrienoic acid.
Fig. 3. Mass spectrum recorded for the keto ester derived from 4,7,10,13,16,19-docosahexaenoic acid.

The mass spectrum of the latter compound, given in Fig. 6, shows ions at m/e 309 (M - 31), 228 (M - 112; β cleavage with loss of CH=CH—CH=CH—CH3, -CH2, 213 (M - 127; α cleavage with loss of -(CH2)5-CH3), 196 (M - 144; 228 - 32), 171 (M - 160; β cleavage with loss of -(CH2)5-C-(CH2)9-CH3), 170 (M - 170; β cleavage with loss of CH=CH—CH=CH—COOCH3, and 155 (M - 185; α cleavage with loss of -(CH2)9-COOCH3). These ions either are absent in the mass spectrum of the keto ester derived from incubated 8, 11, 14-eicosatrienoic acid, or, if present, can be ascribed to methyl 15-ketoarachidate. The absence of the 8- and 12-keto isomers was established similarly by the absence of ions which, by applying the same type of fragmentation as described above,
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FIG. 4. Mass spectrum of methyl 9-ketostearate

FIG. 5. Mass spectrum of methyl 13-ketostearate

FIG. 6. Mass spectrum of methyl 11-ketoarachidate

could be predicted to be specific for these keto esters (10). The mass spectrum of the keto ester derived from the product obtained by the action of lipoxidase on 8,11,14-eicosatrienoic acid is thus in accordance only with that of methyl 15-ketoarachidate.

Fig. 3 shows the mass spectrum of the keto ester prepared from the product of 4,7,10,13,16,19-docosahexaenoic acid. The identification of the keto ester as methyl 17-ketodocosanate is based on the mass spectrum, which showed ions at m/e 337 (M - 31), 312 (M - 56; β cleavage with loss of CH₃=CH–CH₃), 297 (M - 71; α cleavage with loss of -CH₃), 280 (M - 88; 312 - 32), 265 (M - 103; 297 - 32), 255 (M - 113; β cleavage with loss of CH₃–C–(CH₃)–CH₃, 223 (M - 145; 255 - 32), and 222 (M - 146; β cleavage with loss of CH₃–C–(CH₃)–CH₃ plus CH₃OH).

In one case, viz. the product from 8,11,14-eicosatrienoic acid, more complete structural work was performed. Oxidative ozonolysis of the methyl ester and acetate of the compound formed by sodium borohydride reduction of the hydroperoxy acid yielded two short chain acids. These were identified, after esterification by treatment with diazomethane with the use of mass spectrometry in combination with gas chromatography, as methyl 2-acetoxyheptanoate and dimethyl suberate (the mass spectra of the compounds were identical with those of the authentic compounds). Therefore, the parent hydroperoxy acid was 15-hydroperoxy-8,11,13-eicosatrienoic acid. This structure is independently supported by the structure of the derived saturated keto ester, methyl 15-ketoarachidate. The presence of one pair of conjugated double bonds is in agreement with the presence of a strong absorption band with λmax 225 nm. The molecular extinction coefficient was 30,000 at this wave length. This value is somewhat higher than that reported earlier, viz. 20,000 (11).

The geometrical configuration of the double bonds of 15-hydroperoxy-8,11,13-eicosatrienoic acid has also been determined. Selective hydrogenation of the Δ⁷ double bond of methyl 15-hydroxy-8,11,13-eicosatrienoate yielded methyl 15-hydroxy-8,13-eicosadienoate. This compound was isolated by preparative thin layer chromatography on silica gel containing silver nitrate. The structure of the compound was determined by oxidative ozonolysis performed on the acetate of the compound. Gas-liquid chromatographic analysis of the ozonolysis product treated with diazomethane showed the presence of three main compounds. These were identified, by means of gas-liquid chromatography and mass spectrometry, as dimethyl glutarate, methyl 2-acetoxyheptanoate, and dimethyl suberate. The infrared spectrum of methyl 15-hydroxy-8,11,13-eicosatrienoate showed absorption bands at 10.13 and 10.56 μ. The presence of these bands indicates that the two conjugated double bonds of methyl 15-hydroxy-8,11,13-eicosatrienoate are cis/trans (12). Since there was no absorption at 10.3 μ, the Δ⁷ double bond is cis. The infrared spectrum recorded for methyl 15-hydroxy-8,11,13-eicosadienoate showed an absorption band at 10.31 μ, suggesting the presence of an isolated double bond. This is therefore the Δ⁷ double bond. The structure of the hydroperoxy acid formed on incubation of 8,11,14-eicosatrienoic acid with lipoxidase is therefore 15-hydroperoxy-8(βα), 11(βα), 13(δα)-eicosatrienoic acid. The identity of this acid with a hydroperoxy acid was confirmed by mass spectrometry of the methyl ester of the compound. The mass spectrum showed ions of high intensity at m/e 334 (M - 18; loss of H₂O) and 318 (M - 34; loss of H₂O). Ions of lower intensity were present at m/e 335 (M - 17; loss of OH), 319 (M - 33; loss of OOH), 303 (M - 49; loss of H₂O + OCH₃), and 287 (M - 65; loss of H₂O + OCH₃).

The complete structures of the hydroperoxy fatty acids obtained on incubation of the remainder of the substrates with lipoxidase have not been determined. However, there seems to be no reason why the general mechanism of lipoxidase ca-
tytysys involving isomerization of one double bond should not hold true for these substrates also.

**Absolute Configuration of Methyl 13-Hydroxyoctadec-2-
Hydroxyheptanoic Acid Derived from 9,12-Octadecadienoic Acid**

The product obtained on a large scale incubation of 9,12-octadecadienoic acid with lipoxidase was subjected to catalytic hydrogenation. The hydroxy acid fraction obtained on silicic acid chromatography was esterified by treatment with diazomethane and was subjected to preparative thin layer chromatography on silica gel not containing AgNO₃. Material obtained on elution of the silica gel was subjected to silicic acid chromatography and was subsequently crystallized from pentane, yielding methyl 13-hydroxyoctadecanoate. The purity of the compound was checked by gas-liquid chromatography and analytical thin layer chromatography. The compound exhibited a plain positive optical rotatory dispersion curve; \([\alpha]_{D0} +0.8, [\omega]_{D150} +2.3\) (c, 5.0, in methanol). Methyl 13-hydroxyoctadecanoate derived from coriolic acid gives a plain negative optical rotatory dispersion curve \([\alpha]_{D150} -0.83\) (c, 2.5, in methanol) (13) and has been assigned the \(S\) configuration by comparison of the optical rotatory dispersion spectrum with that of methyl 12-hydroxyoctadecanoate. Therefore, methyl 13-hydroxyoctadecanoate derived from the product obtained on incubation of 9,12-octadecadienoic acid with lipoxidase should have the \(R\) configuration.

Conclusive evidence on the stereochemistry was furnished by the optical rotation of 2-hydroxyheptanoic acid obtained on oxidative ozonolysis of the unsaturated hydroxy ester fraction formed from 9,12-octadecadienoic acid. The optical rotatory dispersion spectrum \([\alpha]_{D0} +38, [\omega]_{D25} +390\)

\[
[3-^{13}C,13L-^{3}H]8,11,14-eicosatrienoic~acid
\]

\[\Delta H/\Delta C = 1.81 \quad (100\%)\]

\[
[3-^{13}C,13D-^{3}H]8,11,14-eicosatrienoic~acid
\]

\[\Delta H/\Delta C = 1.69 \quad (93\%)\]

\[
[3-^{13}C,13L-^{3}H]8,11,13-eicosatrienoic~acid
\]

\[\Delta H/\Delta C = 1.38 \quad (100\%)\]

\[
[3-^{13}C,13D-^{3}H]8,11,13-eicosatrienoic~acid
\]

\[\Delta H/\Delta C = 0.14 \quad (10\%)\]

**Fig. 7.** Stereochemistry of hydrogen removal in the conversion of 8,11,14-eicosatrienoic acid into 15L-hydroperoxy-8(cis),11(cis),13(trans)-eicosatrienoic acid. The labeled acids \((250,000\) cpm, 0.5 mg) were dissolved in \(50\) μl of acetone, and \(0.3\) ml of \(0.1\) M NaOH was added. After cooling to 0°, 1 ml of \(0.1\) m borate buffer containing 75 μg of lipoxidase was added. After an incubation period of 30 min \((80\) to \(90\%) conversion), ethanol was added, and the solution was acidified and extracted with ether. The product obtained after evaporation of the ether was reduced with NaBH₄. The 15-hydroxy-8,11,13-eicosatrienoic acid was purified by silicic acid chromatography, treated with diazomethane, and subjected to preparative thin layer chromatography on silica gel not containing AgNO₃.

**Table II.** Enrichment of tritium in precursor during conversion of \([15L-^{3}H,3-^{14}C]8,11,14-eicosatrienoic~acid~into~[8-^{14}C]15L-18,11,14-hydroperoxy-8(cis),11(cis),13(trans)-eicosatrienoic~acid**

<table>
<thead>
<tr>
<th>Reaction time</th>
<th>Conversion</th>
<th>(%)</th>
<th>(\Delta H/\Delta C) in remaining 8,11,14-eicosatrienoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>20</td>
<td>1.51</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>46</td>
<td>2.03</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>59</td>
<td>2.74</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>69</td>
<td>3.44</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>81</td>
<td>4.64</td>
<td></td>
</tr>
</tbody>
</table>

The precursor, 1.4 mg \((700,000\) cpm, \(3^{14}C = 1.36)\), was dissolved in \(50\) μl of acetone, and \(0.3\) ml of \(0.1\) M NaOH was added. After cooling to 0°, 1 ml of \(0.1\) m borate buffer, \(pH 9.0\), containing 75 μg of lipoxidase, was added. The incubation mixture was shaken at 0° for 25 min. At the times indicated, 0.2 ml was withdrawn and added to 2 ml of ethanol. Sodium borohydride, \(20\) mg, was added to each sample. Remaining 8,11,14-eicosatrienoic acid was isolated by silicic acid chromatography (eluted with ether-hexane, 5:95), and the \(\Delta H/\Delta C\) ratio was determined. Every 5 min, \(30\) μl of the incubation mixture were withdrawn and added to 2 ml of ethanol. By using an extinction coefficient of 30,000 for the hydroperoxy acid, the percentage conversions of the precursor could be determined.

**Stereochemistry of Reaction**—The absolute configuration at C-13 of 15-hydroperoxy-8,11,13-eicosatrienoic acid has been determined. The products obtained on oxidative ozonolysis of 130 mg of methyl 15-acetoxy-8,11,13-eicosatrienoate were subjected to reversed phase partition chromatography. Treatment of the isolated 2-acetoxyheptanoic acid with \(0.1\) M NaOH for 18 hours at room temperature yielded \(18\) mg of 2-hydroxyheptanoic acid. After decolorization with Norit, the optical rotation of the sodium salt was measured. The experimentally found optical rotation corresponded to \([\alpha]_{D25}^{25} -9.0\) (c, 3.0, in \(n\) NaOH). According to Baker and Meister (14), the acid then has the \(L\) configuration (cf. also Reference 15).

**Absolute Configuration of Hydrogen Removed from C-13 of 8,11,14-eicosatrienoic Acid**—The absolute configuration of the hydrogen removed from C-13 of 8,11,14-eicosatrienoic acid has also been determined. Incubations were performed with \([13L-^{3}H,3-^{14}C]8,11,14-eicosatrienoic~acid\) and \([13L-^{3}H,3-^{14}C]8,11,14-eicosatrienoic~acid\). The \(\Delta H/\Delta C\) ratios of the derived hydroxymethyl esters were determined. Fig. 7 shows the percentage retentions of tritium in the products relative to the precursor. These experiments indicate that the \(13L\) hydrogen is selectively removed from the methylene group at C-13, whereas the \(13S\) hydrogen is retained. This removal is accompanied by a pronounced isotope effect. The magnitude of the isotope effect is shown in Table II.

**Incubation of [15L-^{3}H,3-^{14}C]8,11,14-Eicosatrienoic Acid**—The
fate of the hydrogen at C-15 of 8,11,14-eicosatrienoic acid during the conversion into 15L-hydroperoxy-8,11,13-eicosatrienoic acid seemed to be of interest since a carbon-oxygen bond is created at C-15. Therefore \( [15-^3H,3-^{14}C] \) 8,11,14-eicosatrienoic acid (8) was incubated with lipoxidase and the 15L-hydroperoxy-8,11,13-eicosatrienoic acid formed was isolated by silicic acid chromatography. As shown in Fig. 8, the tritium label is retained in 15-hydroperoxy-8,11,13-eicosatrienoic acid and in methyl 15-hydroxyarachidate. However, the tritium label is lost in methyl 15-ketoarachidate. Thus the hydrogen at C-15 of 8,11,14-eicosatrienoic acid remains in the same position during the conversion into 15L-hydroperoxy-8,11,13-eicosatrienoic acid.

**DISCUSSION**

In the present investigation, the products formed by the action of soybean lipoxidase on a number of unsaturated fatty acids have been analyzed. The procedure involved conversion of the hydroperoxy acids into saturated keto esters, followed by characterization by mass spectrometry. This method permitted determination of the positions of the keto groups in the derivatives and thus the location of the hydroperoxy groups in the enzymatic products. These studies revealed a very high degree of specificity in the oxygenation with respect to the position attacked.

The results summarized in Table III show that in all of the acids that reacted, oxygen was introduced at the carbon in position \( \omega_6 \). In the case of 9,12-octadecadienoic acid, however, a small amount of the substrate was oxygenated in position \( \omega_{10} \). These findings seemed to indicate that the structural requirement for substrates in the lipoxidase-catalyzed oxygenation is the presence of a \( \omega_6 \text{-cis}, \omega_7 \text{-trans} \)-pentadiene group with its methylene group in position \( \omega_8 \).

In order to test this hypothesis further, acids with the methylene group of the pentadiene structure in positions \( \omega_{10} \) (8,11,14-docosatrienoic acid and 9,12,15-tricosatrienoic acid) or \( \omega_5 \) (5,8,11-eicosatrienoic acid) were tested. None of these acids reacted, nor did 15-methyl-8,11,14-eicosatrienoic acid, which contains a methyl group on the double bonded carbon atom which is oxygenated in the unsubstituted acid (Fig. 9).
The structure of 15n-hydroperoxy-8(cis),11(cis),13(trans)-eicosatrienoic acid was established by mass spectrometric analysis of the derived keto ester, oxidative ozonolysis, and partial hydrogenation, as well as by ultraviolet and infrared spectroscopy. The determination of the geometrical configuration of the double bonds is noteworthy, since it has earlier been assumed but never been shown that the isomerized double bond has the trans configuration. By determining the optical rotation of 2-hydroxyheptanoic acid formed on oxidative ozonolysis, it could be shown that C-15 has the \( L \) configuration.

Prostaglandin El, which is formed from 8,11,14-eicosatrienoic acid in a reaction having many features in common with the lipoxidase-catalyzed oxygenation (7), has also the \( L \) configuration (17).

The stereochemistry of the hydrogen removal has been studied with 8,11,14-eicosatrienoic acid as substrate. In this acid the transformation involves elimination of hydrogen from C-13 and introduction of oxygen at C-15. The stereochemistry of the elimination of hydrogen was determined by using [13\( n \)H, 3\( \delta \)C]- and [13\( n \)H, 3\( \delta \)C]8,11,14-eicosatrienoic acids. These studies showed that the 13\( n \)-tritio acid was transformed by practically complete loss of the \( \Pi \) label, whereas the 13\( n \)-tritio acid retained the label. The isotope data also showed a significant isotope effect in the removal of the tritium. Since enrichment of tritium was found in the precursor acid, the loss of hydrogen from C-13 most likely occurs as the initial step of the reaction, at least before a covalent bond has been established between the substrate and oxygen. After the \( \omega \)-H-hydrogen has been eliminated, \( \pi \) electrons of the \( \omega \)-6 and \( \omega \)-9 double bonds are redistributed to form the conjugated pentadienyl free radical. The biradical oxygen is then introduced stereospecifically, and the peroxy radical finally recaptures a hydrogen atom. The hydrogen attached to the carbon atom which is attacked by oxygen remains in the same position in the hydroperoxy acid.

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REFERENCES
On the Specificity of the Oxygenation of Unsaturated Fatty Acids Catalyzed by Soybean Lipoxidase
Mats Hamberg and Bengt Samuelsson


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