distilled water. Turn on the instrument at least 15 min. before use.

Place a 50-mm. cell containing the CCl_4 in the cell compartment. Set the instrument to the desired wavelength, using the wavelength knob. Turn the sensitivity knob to position "1" and, with the cell aperture closed, adjust the dark current to give a reading of zero. Open the cell aperture and adjust the slit to 0.1 mm. With the continuous sensitivity control adjust the reading to 100%. Again check the dark current and readjust if necessary. Close the cell aperture. Fill a second cell with the sample, which must be clear and brilliant. Adjust the temperature of the oil to 85°F. ±5°F. Place in the instrument.

Open the cell aperture and read the absorption as shown by the meter. Close the cell aperture and read the absorption as shown by the meter. Close the cell aperture and change the wavelength setting to the next desired wavelength with CCl_4 in the light beam. Readjust the dark current and sensitivity control to give 0 and 100% readings. Again put the sample in the light beam. Make all of the desired readings following these procedures.

All absorbance values should fall between 0.3 and 0.8 except on the Cary instrument, where values up to 3.0 are acceptable. Use the maximum cell size to give the desired values.

Calculations

p.p.m. chlorophyll, using Beckman "DU" =

$$\frac{A_{670} - \frac{A_{630} + A_{710}}{2}}{0.1016\ L}$$

p.p.m. chlorophyll, using Beckman B with red sensitive tube =

$$\frac{A_{670} - \frac{A_{630} + A_{710}}{2}}{0.0964\ L}$$

p.p.m. chlorophyll, using Cary =

$$\frac{A_{670} - \frac{A_{630} + A_{710}}{2}}{.1086\ L}$$

L = length in cms.

This method of determining chlorophyll is not applicable to hydrogenated oils, deodorized oils, and finished products since the chlorophyll absorption no longer occurs at 670 millimicrons.

The Coleman Jr. Spectrophotometer can be used for determining chlorophyll in amounts above 0.1 p.p.m. Readings are made in the 25-mm. cuvette against CCl_4 at 630 and 670 millimicrons.

$$\text{p.p.m. chlorophyll} = \frac{A_{670} - A_{630}}{0.0668}$$

Products of the Lipoxidase-Catalyzed Oxidation of Sodium Linoleate[1]

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ALTHOUGH considerable progress has been made in elucidating the mechanism whereby soybean lipoxidase catalyzes the oxidation of polyunsaturated fatty acid compounds, the reactions are sufficiently complex so that a number of details await further clarification. Bergström (2) found that the monohydroxystearic acids and other hydroxylated compounds present in the hydrogenated products from autoxidation and lipoxidase-catalyzed oxidation of linoleate were quite similar. On the basis of this and other evidence he deduced that the principal products of the lipoxidase-catalyzed oxidation of linoleate were 9 and 13 hydroperoxides.

Subsequent studies led to the belief by some investigators (1, 8) that the lipoxidase-catalyzed and autoxidation reaction mechanisms were both chain reactions, the lipoxidase serving primarily to create free radicals for the chain mechanism. However Tappel *et al.* (15, 16) cast doubt on the role of lipoxidase as an initiator of reaction chains of the autoxidative type and reported that the lipoxidase oxidation exhibited many of the characteristics of ordinary types of enzyme reactions.

Earlier studies by Bergström and Holman (3) have led to an apparent molecular extinction coefficient of 31,400 for the products of the lipoxidase oxidation of sodium linoleate at 0°C. with a pure lipoxidase preparation. Since this was considerably higher than the molecular extinction coefficient observed for the products of autoxidation, it appeared at that time that the lipoxidase oxidation led to a higher proportion of conjugated diene hydroperoxides (3). More recent studies (4, 11) have revealed that at least 90% of the hydroperoxides formed in the low temperature autoxidation of methyl linoleate are also conjugated dienes; the lower molecular extinction coefficient for the products of autoxidation could be attributed to the presence of predominantly *cis, trans* isomers. On this basis it might appear that the higher molecular extinction coefficient observed quite consistently for the products of lipoxidase-catalyzed oxidation could be attributed to the formation of *trans, trans* conjugation (or possibly *cis, cis* conjugation since the molecular extinction coefficient of the latter has not been determined). However, although crude lipoxidase preparations were used in our work, this possibility appeared to be very unlikely in view of the results of the present investigation in which it was found that the diene conjugation existed predominantly in the *cis,trans* configuration when the oxidation was conducted under mild conditions. Presented herewith is a detailed study of the products formed in the lipoxidase-catalyzed oxidations conducted under relatively mild conditions to obtain more information on the nature of the reaction.

[1] Hormel Institute publication No. 127 and Paper No. 3342 in the scientific journal series of the Institute of Agriculture.

[2] Supported in part by a grant from the Hormel Foundation.

Materials and Methods

Linoleic Acid. Two preparations of *cis, cis* linoleic acid were used. For most of the work the substrate was prepared from relatively pure linoleic acid obtained by urea fractionation and low temperature crystallization of the fatty acids of safflower seed oil. Preliminary experiments were conducted with a 60% concentrate of linoleic acid obtained from fractionally distilled methyl esters of corn oil fatty acids.

Cis-9, trans-12-linoleic Acid. This acid was isolated from the nonconjugated fraction of dehydrated castor oil fatty acids by low temperature crystallization from acetone, essentially by the procedure described by Jackson *et al.* (7). Analytical data were as follows: m.p. −3.5° to −3.0°C.; $k_{232} = 0.302$ (no maximum); $n_D^{30°C.} = 1.4645$; I. V. (Wijs) = 156.0. The compound exhibited a single strong infrared absorption band at 10.33 microns, characteristic of isolated *trans* double bonds.

Methyl Cis-9, trans-11-linoleate. The conjugated fraction of the methyl esters of dehydrated castor oil acids which was presumed to consist primarily of methyl *cis*-9, *trans*-11-linoleate (7, 10) was separated by fractional distillation, and the desired compound then isolated by low temperature crystallization from acetone, using the procedure described by Jackson *et al.* (7) for the isolation of methyl *trans*-10, *cis*-12-linoleate. Recrystallizations were conducted until no further purification could be effected as evidenced by ultraviolet and infrared spectra.

Analytical data were as follows: k at 233 mμ = 87.2; $n_D^{30} = 1.4697$; I. V. (hydrogenation) = 171.3. Melting point of the free acid was 7.5 to 8.6°C. Infrared spectra of acid and ester showed double absorption at 10.55 and 10.18 microns characteristic of *cis, trans* diene conjugation. Although the specific extinction coefficient of the ester at 233 mμ was lower than 97.7 reported by Jackson *et al.* (7) and 93.2 by Nichols *et al.* (10) for methyl *trans*-10, *cis*-12-linoleate, this may be due in part to the difference in the position of the double bonds with respect to the ester group.

Linoelaidic Acid. Highly purified methyl linoleate was elaidinized by the oxides of nitrogen according to the method of Kass *et al.* (6). The crude elaidinized esters were converted to acids from which relatively pure linoleaidic acid was obtained by repeated crystallizations from acetone. Melting point of the final product was 27.5–27.6°C.; $n_D^{30} = 1.4650$. The infrared spectrum of this compound showed a strong band at 10.33 microns, approximately double the intensity of that observed with elaidic acid at this wavelength.

Lipoxidase. Crude lipoxidase preparations obtained by the aqueous extraction of defatted soybean meal were used in this study. In general, the following extraction procedure was employed: soybean flour, 150 g. (Archer-Daniels-Midland "Nutrisoy") was suspended in 1,000 ml. of acetate buffer at pH 4.5. The mix was stirred for 1 hr. and filtered. The filtrate containing the enzyme was adjusted to pH 7 with 1 N sodium hydroxide and filtered again. Extracts prepared in this manner usually contained from 30 to 50 units of lipoxidase activity per ml. as measured by the assay method of Theorell *et al.* (18, 1).

Preparation of Substrate. The sodium soaps were made by slowly adding the calculated amount of 1 N sodium hydroxide to neutralize a known weight of fatty acid. During the addition of sodium hydroxide and subsequent dilution with distilled water, the solution was stirred vigorously to bring about complete solution of soaps. Enough distilled water was added to give a 5% solution in terms of fatty acid concentration. The final step in the preparation of the substrate was to dilute the soap solution with two parts by volume of 1 N ammonium hydroxide-ammonium chloride buffer at pH 9.0. The soap solution was usually made one day before its use in oxidation studies and stored under an atmosphere of purified nitrogen. The buffer was added just prior to the start of each experiment.

Oxidation Procedure. Usually the oxidations were conducted with 300-ml. batches of substrate (containing 5 g. of neutralized linoleic acid) and varying amounts of enzyme. The enzyme extract and the substrate were saturated with oxygen before being mixed together, and during the oxidation oxygen was passed over or through the solution. The mixture was stirred vigorously by means of a magnetic stirrer to insure a good supply of dissolved oxygen at all times. Oxidations to a peroxide value of about 600 m.e./kg. required from less than 1 min. up to 1 hr., depending on the concentration of the enzyme.

Recovery of Products. When the desired amount of oxidation had occurred, an equal volume of cold alcohol (0°C. or less) was added to stop the reaction. The entire mixture was then cooled to 0°C. or below, acidified with cold 10% aqueous HCl, and extracted four times with approximately equal volumes of cold (0°C.) ethyl ether. The ether extract was washed with distilled water until free of HCl, as determined by testing the wash water with methyl orange indicator.

At this point the other extracts from several batch oxidations performed under the same conditions were usually combined, and most of the solvent was removed by evaporation under reduced pressure. The concentrate was dissolved in Skellysolve F, the separated water was removed in a separatory funnel and then diluted to volume in a 250-ml. volumetric flask.

Usually by this procedure more than 95% of the original weight of fatty acid was recovered. After aliquots were taken for analyses, the products of the oxidation were separated from the unoxidized fraction by a countercurrent extraction method (12).

Methods of Analyses

Analytical Distillation. Reduced peroxide concentrates were esterified with diazomethane at 0°C. in ethyl ether and were fractionated into monomer and polymer fractions in a micromolecular still similar to that described by Paschke *et al.* (14).

Infrared Absorption. All infrared spectra reported in this study were obtained with a Perkin-Elmer Model 21 double beam instrument. Measurements were made on a 10% solution of the compounds in carbon disulphide.

Molecular Weight. The cryoscopic method of Wilson *et al.* (19) was used. Anhydrous cyclohexanol required as solvent was obtained by fractional distillation of cyclohexanol (Eastman) through a Podbielniak Hyper-cal column. The progress of the fractionation was followed by measurements of refractive index. The best fractions (m.p. 25.0–25.2°C.) were

sealed under vacuum in glass and stored at —18°C. until used.

Optical Rotation. Measurements were made at room temperature in a Schmidt and Haensch polarimeter, using a 5-cm. cell; dioxane was used as a solvent in some measurements in order to diminish the effects of association.

The hydroperoxide concentrates were reduced with stannous chloride; the reductions were carried out with a five-fold excess of a 1% alcoholic solution of the reagent at room temperature.

Details of the methods for the determination of peroxide value, hydroxyl value, iodine value by hydrogenation, and ultraviolet spectra have been described in other publications from this laboratory (11, 13).

Results

The original plan of the study was to conduct oxidations under a few selected conditions based on results of previous kinetic studies, separate the oxidized fraction as quantitatively as possible by countercurrent extraction, and make detailed chemical and spectral analysis of the products of the reaction. This was eventually accomplished, but only after many false starts had been made and many supplementary experiments performed in which it was found that the analytical results could be profoundly influenced by relatively slight variations in the oxidation techniques and by chemical changes that could occur during the handling of the products. The following results were obtained in experiments where the effects of spurious factors were successfully eliminated.

Infrared Spectra. In Figure 1 in the infrared spectra of a typical linoleic acid peroxide concentrate [3] (C) and a reduced methyl ester of linoleic acid peroxide concentrate (D) obtained from a lipoxidase-catalyzed oxidation of sodium linoleate are compared with corresponding samples of *cis*-9, *trans*-11-octadecadienoic acid (A), and methyl *cis*-9, *trans*-11-octadecadienoate (B). Clearly, the conjugated diene products of the oxidation were predominantly *cis*, *trans* and further there was no evidence of any isolated *trans* double bonds. Curves similar to those in Figure 1 were obtained in oxidations conducted either in daylight or dark at either 0° or 26°C. and in oxidations carried to a peroxide value as high as 2600 m.e./kg. of acid (Experiment 2, Table I).

Analysis of Peroxide Concentrates. Table I summarizes some of the results from a number of typical oxidations, analyses of concentrates of oxidation products, and esterified and reduced concentrates. Also in Table I, the results of one experiment (five)

[3] Strictly speaking, the terms "linoleic acid peroxide" and "linoleate peroxide" are incorrect; their use is justified on the grounds of convenience in the belief that most readers of this paper will understand what is meant.

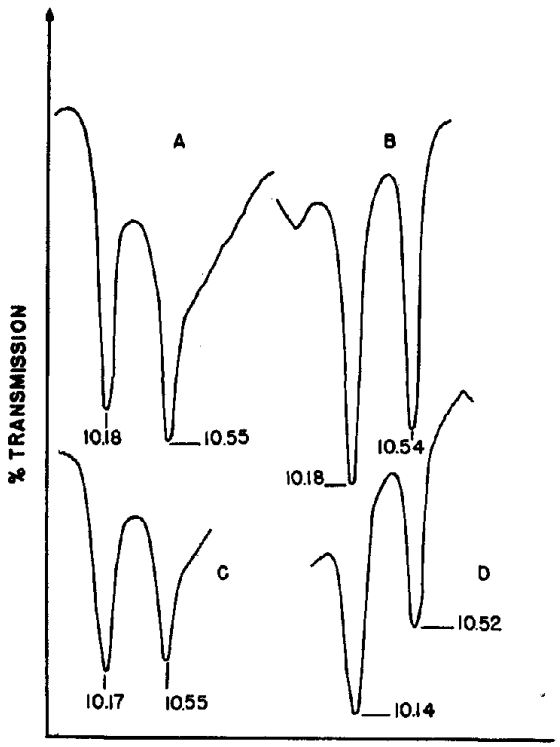

FIG. 1. Infrared Spectra
A. *Cis*-9, *trans*-11-octadecadienoic acid
B. Methyl *cis*-9, *trans*-11-octadecadienoate
C. Linoleic acid peroxide concentrate
D. Methyl linoleate peroxide concentrate

TABLE I

Lipoxidase-Catalyzed Oxidation of Sodium Linoleate [a]

Experiment:	1	2	3	4	5
Oxidation and Recovery of Acids					
1. Units of enzyme per gram of linoleic acid	15	200	50	50	15
2. Temperature of oxidation	0°C.	0°C.	20°C.	20°C.	0°C.
3. P.V. of soaps m.e./kg. (calculated on basis of wt. of linoleic acid)	632	2600	775	780	632
4. k_{234} of soaps (calculated on basis of wt. of linoleic acid)	8.0	38.0	9.74	10.6	8.0
5. P.V. of recovered acids m.e./kg.	636	2625	754	774	282
6. k_{234} of recovered acids	8.4	35.6	9.6	10.4	3.68
Countercurrent Extraction					
7. Weight of recovered acids, grams	46.88	23.45	37.93	35.6	45.67
8. Peroxide content of recovered acids (calculated as monohydroperoxide from lines 5 and 7), grams	4.66	9.58	4.45	4.31	2.0
9. Weight of oxidized fraction recovered by extraction, grams	4.99	10.91	5.30	5.20	4.17
10. P.V. of recovered oxidized fraction, m.e./kg.	5960	5660	5600	5440	2660
11. Peroxide content of recovered oxidized fraction (calculated as monohydroperoxide from lines 9 and 10), grams	4.64	9.63	4.63	4.41	1.73
12. k_{234} of recovered oxidized fraction	76.6	73.7	71.5	69.1	36.0
13. Calculated k_{234} for oxidized fraction (calculated from lines 6, 7, and 9)	78.8	76.4	68.7	71.2	40.2
Reduced and Esterified Oxidized Fractions					
14. P.V., m.e./kg.	132	78.2	75.5	173.0	58
15. k_{234}	76.6	71.6	71.7	71.1	35.0
16. OH, percent by weight	5.13	5.23	4.94	4.43	5.0
17. Iodine value (by hydrogenation)	140	136	140	140	112
18. Monomer percent by weight	92.0	87.5	90.5	91.1	91.5
19. Polymer percent by weight	8.0	12.5	9.5	8.9	8.5

[a] Crude lipoxidase preparation obtained by aqueous extraction of defatted soybean meal.

are reported in which approximately one-half of the alcoholic-poisoned substrate from Experiment 1 was allowed to stand at room temperature for three days after deaeration and acidification with an excess of HCl before being extracted with ethyl ether. Some difficulty was experienced in obtaining a good recovery of the liberated linoleic acid after oxidation by conventional extraction procedures, as has been previously reported (5), but it was found that by keeping the solutions cold, 0°C. or slightly lower, the linoleic acid was readily recovered in four or five extractions with ethyl ether. The low extraction temperatures also were desirable to avoid decomposition of the peroxides when the free acids were obtained from the soaps.

It is evident from the peroxide values and diene conjugation (k_{234}) shown in lines 3, 4, 5, and 6 of Table I (before and after recovery of the acids) that little, if any, decomposition or alteration of the peroxides occurred when the recovery of the oxidized acids was carried out as described. Also, no appreciable alteration of the peroxides or of the spectral absorption of the products at 234 millimicrons took place during the countercurrent extraction, and recovery of the peroxides was quite complete (lines 8 and 11, and lines 12 and 13, Table I).

The peroxides, in fact, appeared to be unusually stable in the alcoholic solution used as the hypophase in the countercurrent extraction. Samples that were allowed to stand in this solution under an oxygen-free atmosphere for six days at room temperature, either in daylight or in the dark, showed little change in their peroxide values, diene conjugation, or infrared spectra.

Also, no great changes appeared to take place in peroxide content or the ultraviolet and infrared spectra when, after the oxidation was stopped by the addition of alcohol and the solution purged with N_2, the solution of soaps was allowed to stand for six days at room temperature.

On the other hand, precautions were necessary to prevent changes during neutralization of the soaps. In one supplementary experiment (Experiment 5, Table I), when the free acids were obtained with an excess of HCl and allowed to stand for three days at room temperature before being extracted with ethyl ether, approximately 55% of the peroxides decomposed. The ultraviolet absorption at 234 millimicrons indicated considerable destruction of conjugated diene, but there appeared to be little change in the *cis, trans* configuration of the conjugated diene that remained.

In spite of the foregoing observations that the peroxides were stable under suitable conditions, a larger amount of reaction product was obtained by countercurrent extraction than could be accounted for if all of the product were presumed to exist in the form of monohydroperoxide (lines 9 and 11, Table I). The analytical distillation of the reduced and esterified products of the recovered oxidized fractions indicated that polymeric products were formed in all oxidations. Some polymers were undoubtedly formed during distillation. However the polymer content varied consistently with variations in the conditions of oxidations, indicating that formation took place primarily during the enzymatic reaction.

In supplementary experiments no additional polymeric material was formed either when peroxides were allowed to decompose extensively under acid conditions or when the oxidized soaps were permitted to stand at room temperature for a prolonged period before recovering the free acids. These findings therefore also substantiated the belief that the polymers were formed mainly during the active oxidation of the substrate. Further, since the polymeric material exhibited a relatively high degree of optical activity (Table III), the enzyme appeared to be involved directly in its formation.

The polymer fractions generally had an average molecular weight between 600 and 800, indicating that they consisted mostly of dimers. Absorptivities at 234 mμ varied from 14.0 to 27.3 in the few samples that were analyzed, and, in contrast to the monomer fractions, no absorption maxima at 272 millimicrons were observed (Figure 2). The hydroxyl content averaged about 2.5%. Efforts to measure the degree of unsaturation by hydrogenation, using a palladium-on-charcoal catalyst and 95% alcohol as the solvent, were unsuccessful. The uptake of hydrogen was very slow and prolonged.

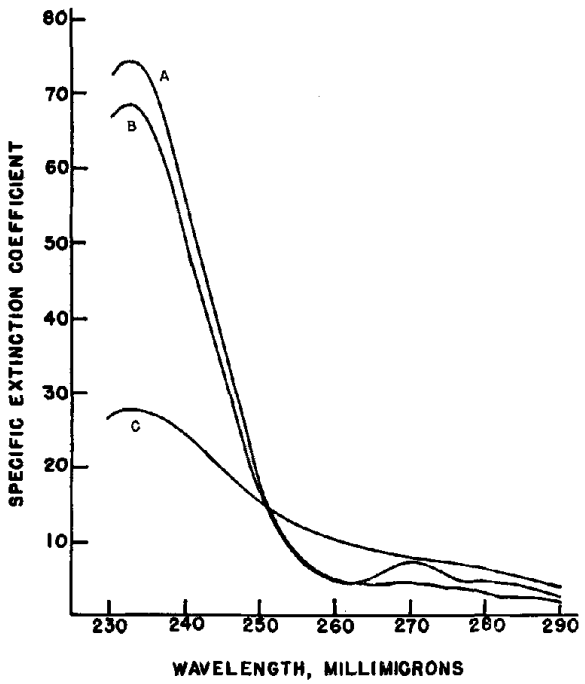

FIG. 2. Ultraviolet absorption spectra
A. Distilled monomer of reduced ester peroxide concentrate
B. Original reduced ester peroxide concentrate
C. Polymeric residue recovered from distilled reduced ester peroxide concentrate

It was also apparent that the monomeric fraction of the oxidation products did not consist entirely of diene monohydroperoxides. This was indicated chiefly by the iodine values and hydroxyl content (lines 16 and 17, Table I). The monomer fraction also generally exhibited a maximum in ultraviolet absorption at about 272 millimicrons (Figure 2).

Comparison of the ultraviolet spectra (Figure 2) of the original reduced peroxide concentrate with that of the monomer and polymer indicated that the substance responsible for the maximum at 270 mμ in the monomer might be produced during the distillation. However the maximum for reduced and unreduced hydroperoxides from this source at this wave-

length is generally much greater than the example shown. Although strong general absorption was produced on distillation, especially in the case of the polymer since there was no maximum produced in this fraction as compared with the monomer, it appeared that the substances in the original hydroperoxide concentrate responsible for the absorption in this region of the spectrum were monomeric.

Nonhydroperoxidic material may form to some extent by decomposition of the hydroperoxides during and subsequent to oxidation, but various evidences indicated that secondary products also formed directly during the oxidation. It is perhaps significant that the highest percentages of hydroperoxides and the lowest percentages of polymer and nonhydroperoxidic products were formed when low oxidation temperatures and minimal amounts of enzyme were used.

Purification of Hydroperoxides. By further countercurrent fractionation of oxidized fractions similar to that of Experiment 1, Table I, relatively pure samples of hydroperoxides were obtained as shown in Table II. Sample 1 was obtained by countercurrent separation of the hydroperoxide rich fractions of an acid oxidized fraction. Sample 2 was obtained by a similar fractionation of an esterified oxidized fraction. Esterification was accomplished with diazomethane in ethyl ether at 0°C. and proceeded without any apparent side reactions as determined by chemical, ultraviolet, and infrared analyses.

TABLE II
Analyses of Hydroperoxide Concentrates

	Peroxides 1	2
P.V., (m.e./kg.)	6350 (acid)	6060 (ester)
k_{234}	78.0	79.2
Reduced Esters		
P.V., (m.e./kg.)	174.0	75.7
k_{234}	77.0	84.3
OH, moles/mole of ester	1.02	1.01
Hydrogen uptake, moles/mole of ester	1.82	2.01
Monomer, percent	94.4	96.0
Polymer, percent	5.6	4.0
k_{234}, monomer	82.6	86.6
OH monomer, moles/mole of ester	1.03	1.01
Hydrogen uptake monomer, moles/mole of ester	1.92	2.03

Although the chemical analyses indicated that these peroxide concentrates consisted of relatively pure hydroperoxides, the analytical distillation of the reduced esters suggested that they contained some polymeric material. As indicated earlier, a portion of the polymeric material could have originated from polymerization occurring during the distillation. The difficulty of separating all of the polymer by countercurrent extraction makes it appear that the polymers, like the monomeric peroxides, are quite highly polar.

Polarographic Analysis. In accord with the results of the chemical and other analyses, polarographic analysis showed that the principal products of the lipoxidase reaction were hydroperoxides (curve A, Figure 3). However polarographic analysis also showed that unless every precaution was taken in handling the peroxide concentrates, they were converted to products having quite different half-wave potentials. A sample of peroxide concentrate which was not protected by refrigeration showed a change in half-wave potential to 0.00 volts (curve B, Figure 3).

Although some changes occur at the same time in the physical and chemical values that have been measured, none of the changes appears to be sufficiently marked to account for the remarkable change in half-wave potential during storage of the peroxides at room temperature. The nature of the chemical change is therefore unknown as yet.

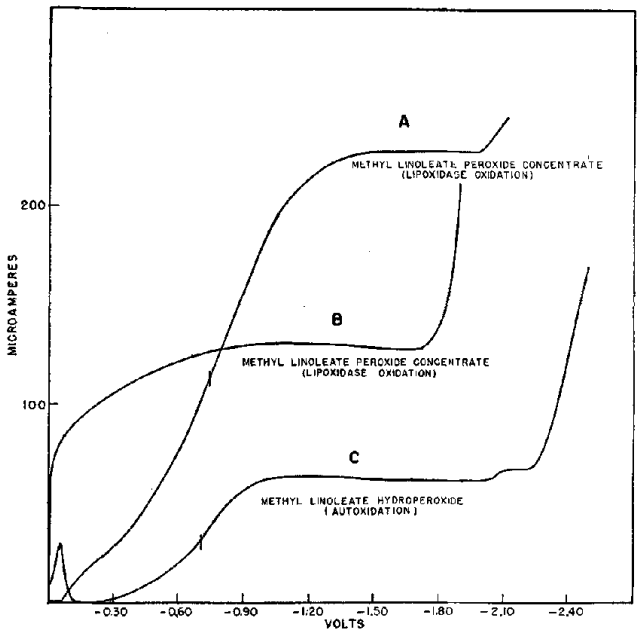

FIG. 3. Polarographic analysis
A. Lipoxidase-produced hydroperoxide concentrate
B. Peroxide concentrate produced on prolonged exposure of hydroperoxides to room temperatures
C. Hydroperoxide concentrate isolated from autoxidized methyl linoleate

Optical Rotation. If the lipoxidase-catalyzed oxidation of sodium linoleate occurs by a more or less typical type of enzymatic process, as postulated by Tappel *et al.* (15), one may expect that the reduced hydroperoxides would be optically active and would give about the same degree of rotation as methyl ricinoleate. The reduced hydroperoxides obtained from lipoxidase catalyzed oxidations did, in effect, give rotations approximating that for methyl ricinoleate (Table III, lines 5 and 6) whereas the hydroperoxides and corresponding reduced products isolated from autoxidized methyl linoleate were optically inactive (lines 1 and 2). The specific rotations were always higher for the reduced than for the unreduced peroxide concentrates from the lipoxidase reaction. Although a real difference may exist, part of the difference may have been due to experimental difficulties imposed by the darker and more viscous nature and the instability of the peroxide concentrates.

The large difference between two samples of the same material (lines 6 and 7) was probably due to association of the hydroxyl groups. The levorotation of the polymeric fraction in contrast to the dextrarotation of the monomers was not surprising in the light of the correlations between the direction of rotation and radical size described by Levine and Marker (9).

Specificity of Lipoxidase. In Table IV the rates of oxidation of solutions of sodium soaps of natural linoleic acid and the *cis*-9, *trans*-12, and *trans*-9, *trans*-12 isomers are compared. The conditions used in this experiment were identical with those outlined in the assay method of Theorell *et al.* (18, 1). Whereas the *cis, cis* linoleate was oxidized rapidly, as evidenced by a marked increase in absorptivity at 234 millimi-

TABLE III

Optical Activity Observations

Sample No.	Compound	Source	Specific rotation[a] $[\alpha]_D^{\text{room temp.}}$
1	Peroxide concentrate, P.V. 6100 (no solvent)	Autoxidized methyl linoleate	zero
2	Distilled monomer peroxide concentrate (no solvent)	Autoxidized methyl linoleate	zero
3	Methyl ricinoleate[b]	Castor oil	+5.2
4	Peroxide concentrate, P.V. 4470 (no solvent)	Lipoxidase-catalyzed oxidation	+2.84
5	Reduced and esterified peroxide concentrate (25% solution in dioxane)	Lipoxidase-catalyzed oxidation	+4.65
6	Distilled monomer reduced and esterified peroxide concentrate (25% solution in dioxane)	Lipoxidase-catalyzed oxidation	+5.3
7	Distilled monomer reduced and esterified peroxide concentrate (no solvent)	Lipoxidase-catalyzed oxidation	+13.3
8	Polymeric residue from molecular distillation (25% solution in dioxane)	Lipoxidase-catalyzed oxidation	−5.5

[a]Assuming a density of 0.9 for the peroxides and reduced derivatives.
[b]A. Haller Compt. rend. 144, 462 (1907).

crons, there was no indication of any oxidation of the other isomers. These results are in accord with the general conclusion (1) that the necessary substrate structure for attack by lipoxidase is a *cis, cis* 1,4-pentadiene system.

TABLE IV

Relative Rates of Oxidation of Linoleic Acid Isomers (crude soybean lipoxidase)

Reaction time	*Cis*-9, *cis*-12-octadecadienoic acid absorptivity	*Cis*-9, *trans*-12-octadecadienoic acid absorptivity	*Trans*-9, *trans*-12-octadecadienoic acid absorptivity
(minutes)	(k_{234} mμ)	(k_{234} mμ)	(k_{234} mμ)
0	0.30	0.23	0.10
1	21.4	0.36	0.13
2	44.4	0.30	0.21
5	57.5	0.59	0.30
10	67.8	0.88	0.69

Discussion

Several of the results obtained in this study shed further light on the mechanism involved in lipoxidase-catalyzed oxidations of unsaturated fatty acids and the character of the products formed. The analytical data show conclusively that the principal initial products formed from linoleates under ordinary circumstances are *cis, trans* conjugated monomeric monohydroperoxides. It is evident however that various secondary products always are produced but that the quantities of these may be decreased by using a low concentration of lipoxidase and a low temperature of oxidation.

Inasmuch as the principal products are *cis, trans* conjugated dienes, it is not to be anticipated that a molecular extinction coefficient greater than 28,000 would be observed. The reason for the significantly higher molecular extinction coefficient, 31,400, previously calculated on the basis of oxygen absorbed in a system involving a pure lipoxidase preparation (3) is not immediately apparent. One possibility that would lead to this high coefficient would be the formation of virtually pure *trans, trans* conjugated hydroperoxides with the pure lipoxidase preparation; it appears unlikely however that a pure lipoxidase preparation should give all *trans, trans* and a crude lipoxidase preparation virtually all *cis, trans* as we found under the mild conditions of oxidation employed in our study. With the crude lipoxidase preparation, appreciable quantities of *trans, trans* material are obtained only under conditions which give rise to appreciable secondary reactions and secondary products, such as higher lipoxidase concentrations, higher temperatures, and higher levels of oxidation; our evidence suggests that the *trans, trans* materials are formed in secondary reactions.

The suggestion that lower coefficients are obtained with crude lipoxidase preparations because of enzymes other than lipoxidase, or other contaminants, in the crude preparations appears untenable, not only because a *cis, trans* hydroperoxide would not be expected to exhibit a coefficient above 28,000, or thereabouts, but because a pure lipoxidase preparation (although it had undergone appreciable deterioration at the time it was used) was reported by others to give a molecular extinction coefficient not in excess of 27,400 under conditions similar to those we have employed (15).

The higher coefficient may be explained only in part on the basis that a higher yield of hydroperoxide was produced with pure enzyme. Further increase in absorption very likely was due to nonhydroperoxide substances, notably polymers formed under the conditions used by Bergstrom and Holman, in which the enzyme apparently was used in relatively high concentrations.

In general, high enzyme concentrations give high yields of polymeric materials, especially when the reaction is allowed to proceed beyond the initial stages. Thus a combination of conditions as used by Bergstrom and Holman may well give a high molecular extinction coefficient even though *cis, trans* diene conjugated hydroperoxides are produced primarily. This situation appears to be far more probable than any explanation implying that a different mechanism is involved with the use of crude lipoxidase preparations.

Nothing conclusive can be ascertained about the structure of the polymers from the currently available data. The hydroxy content generally was found to be about 2.5%, that is, about one hydroxy group per molecule. On the other hand, the absorptivity at 234 mμ was quite variable.

The formation of less polymer at low enzyme concentrations and lower temperatures might superficially seem to support the autoxidative chain reaction theory of lipoxidase oxidation. In the autoxidative mechanism, reaction chains become shorter with increasing free radical concentrations and hence higher proportions of polymers are formed in chain termination reactions. However this view cannot be reconciled with the observation that the major products of the lipoxidase oxidation, even under conditions that would favor long reaction chains, are optically active monomeric hydroperoxides, and therefore different from the optically inactive hydroperoxides formed in autoxidation. There is no reason why, on the basis of a more normal type of enzymatic mechanism (15), polymeric products should not form. If polymers are formed by interaction of two enzyme-substrate complexes, for example, a higher proportion of polymers would be anticipated at higher enzyme concentrations and at higher oxidation temperatures. The observation that the polymer is optically active is further

evidence that the enzyme probably is involved in its formation.

The levorotation of the polymer in contrast to the dextrarotation of the reduced hydroperoxides may be explained on the basis of the observation of Levine and Marker (9) that when a large radical is attached to the asymmetric molecule in place of a relatively small group, direction of rotation may be reversed.

The probability that lipoxidase will attack only the *cis, cis* form of the 1,4-pentadiene system is further supported by the finding that *cis*-9, *trans*-12, and *trans*-9, *trans*-12-octadecadienoates were not oxidized. It is interesting to note, whether it be biologically significant or mere coincidence, that the *cis*-1, *cis*-4-pentadiene system for which lipoxidase is specific, is also essential to biological activity of the so-called essential fatty acids.

Summary

Studies of products formed in the lipoxidase-catalyzed oxidation of linoleates have been made by physical and chemical measurements directly on the oxidized substrate, on concentrates of the oxidized fraction, and on reduced and esterified products of the oxidized fraction. The following principal observations and conclusions have been reached:

a) The principal products obtained in the oxidation of aqueous solutions of sodium linoleate are optically active *cis, trans* conjugated monomeric monohydroperoxides. This was found true whether the oxidations were conducted at 0° or 26°C., in either daylight or darkness.

b) Other products of the enzymatic reaction were optically active polymers, and these were formed in greater proportions at higher concentrations of enzyme and at higher oxidation temperatures. The polymers contained added oxygen in an apparently constant proportion, irrespective of the conditions of oxidation, but conjugated diene which was also present in the polymer was more variable.

c) The formation of hydroperoxides was predominantly substantiated by polarographic analysis. It was further found however that when peroxide concentrates from the lipoxidase reaction were kept at room temperature for any appreciable time, they underwent a relatively marked change in their half-wave potential, the nature of which was not readily apparent.

d) The results further substantiate a previously proposed mechanism (15) for the lipoxidase reaction in which the enzyme is considered to be involved in the formation of each peroxide molecule. The mechanism may be extended to account for the formation of optically active polymeric products.

e) Lipoxidase was inactive with respect to the *cis*-9, *trans*-12, and *trans*-9, *trans*-12-isomers of natural linoleic acid.

Acknowledgments

The authors wish to express their sincere appreciation to C. Ricciuti of the Analytical, Physico-Chemical, and Physics Section of the Eastern Utilization Research Branch, U. S. Department of Agriculture, Philadelphia, Pennsylvania, for polarographic analyses of the peroxide concentrates. The authors are also indebted to D. H. Wheeler and Wesley Tolberg of the General Mills Research Laboratory, Minneapolis, Minnesota, for infrared analyses performed in the preliminary stages of this work, and to J. R. Chipault of the Hormel Institute staff, for the infrared absorption data used in the preparation of this paper.

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[Received March 14, 1955]

ABSTRACTS . . . . . R. A. Reiners, Editor

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