The Journal of the American Oil Chemists' Society

Volume 28 JULY, 1951 No. 7

Analysis of Fat Acid Oxidation Products by Countercurrent Distribution Methods. III. Methyl Linolenate[1]

J. FUGGER,[2] J. A. CANNON, K. T. ZILCH, and H. J. DUTTON, Northern Regional Research Laboratory,[3] Peoria, Illinois

ALTHOUGH the autoxidation processes and products of both the oleates and linoleates (1, 2, 3, 4, 5, 6) have been studied extensively, the study of the oxidation of linolenates has been largely neglected. Farmer (7) and Bolland (5) assume that the linolenate oxidation mechanism follows the same general pattern as that established for the mono-olefins and non-conjugated diolefins. The mechanism involves hydroperoxide formation on an α-methylenic carbon followed by the rearrangements, polymerizations, and chain scissions characteristic of peroxidic decomposition. Because of a distinct lack of experimental evidence however it cannot be said that the exact nature of the linolenate oxidation process has been established (2, 3, 8, 9). The present experimental approach, involving the use of countercurrent distribution procedures, is believed to throw new light upon this important problem.

Use of the countercurrent distribution instrument greatly facilitates autoxidation studies since it permits an examination of all components of the original oxidation mixture. In addition, separation by virtue of differential solubility constitutes a particularly mild fractionation procedure. In this investigation methyl linolenate was oxidized to levels ranging from 0.1 to 1.1 moles oxygen per mole ester. The oxidized esters were subsequently fractionated between aqueous ethanol and hexane by countercurrent distribution techniques. The resulting fractions were then analyzed by the physical and chemical methods cited below.

Methods and Materials

The methyl linolenate used was free of conjugated isomers and had an iodine value of 260.0 (theory 260.4). It was obtained from the Hormel Foundation, where it had been prepared from hexabromstearic acid.

Oxidations were conducted in a manometric system of our own construction similar to that reported by Ogg and Cooper (10). The oxygen was contained in burets of 50 ml. capacity. Magnetic stirrers were used for agitation.

Fractionations were accomplished in our large (160 ml. per tube) 29-tube and small (16 ml. per tube) 25-tube countercurrent distribution equipment. Detailed procedure for operating the Craig distribution apparatus has been adequately described elsewhere (11, 12).

The general procedure in the experiments involved:

1. Measurement of the quantity and rate of oxygen adsorption by the methyl linolenate.
2. Plot of the weight distribution curve of the fractionated material after countercurrent distribution.
3. Determination of the ultraviolet absorption spectra of the reaction products.
4. Semi-micro chemical examination of the fractions. Hydrogen absorption, peroxide, hydroxyl, and acid number determinations were made on selected fractions. Molecular weight values were obtained in some instances.

The weights of dissolved substances in each tube were determined by evaporating the solvents from 2 ml. aliquots of each layer in a Parr weighing flask. Weights of residue obtained on a semimicro-balance were subsequently multiplied by the appropriate factor to give the content of the whole tube.

Ultraviolet absorption spectra were determined in absolute ethanol with the use of a Beckman quartz spectrophotometer and a Cary recording spectrophotometer. Dienoic and trienoic conjugation were calculated from the optical densities, using extinction coefficients ($E^{1\%}_{1\,\text{cm.}}$) of 1,150 at wavelength 2365 Å and of 1,850 at wavelength 2680 Å, respectively. These coefficients were determined in this laboratory (13) and are comparable after calculation for the acids with those published for 9-10 linoleic and alpha-eleostearic acids (14).

Hydrogen absorption was determined on a semimicro scale (15), using a palladium-barium sulfate catalyst (16). Hydrogenations were conducted at room temperature and atmospheric pressure with samples of 40-100 mg. dissolved in absolute ethanol.

Peroxide values were determined by HI reduction according to a procedure modeled after that of Bergström, Lea, and Lundberg (4, 17, 18). Samples weighing 40-100 mg. were dissolved in 10 ml. of a chloroform-acetic acid (1:2) solvent which had been deaerated with nitrogen. After adding 0.1 ml. of saturated aqueous potassium iodide and allowing the reaction mixture to stand at room temperature for 5 minutes (with swirling) under a nitrogen atmosphere, 1.0 ml. of aqueous 0.1 N potassium iodide was introduced. The liberated iodine was then immediately titrated in a nitrogen atmosphere with air-free 0.03 N sodium thiosulfate. Peroxide values as milliequivalents per kilogram were thus determined.

---

[1] A portion of this paper is based upon a thesis to be submitted by J. Fugger in partial fulfillment of the requirements for the Degree of Doctor of Philosophy at the University of Pittsburgh. This work was carried out under an agreement between the University and the U. S. Department of Agriculture. Presented at 118th National Meeting of the American Chemical Society, September 3-8, 1950, in Chicago, Illinois, and at the American Oil Chemists' Society Meeting in San Francisco, California, September 26-28, 1950.

[2] Fellow under the Research Internship program of the U. S. Department of Agriculture.

[3] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Report of a study made under the Research and Marketing Act of 1946.

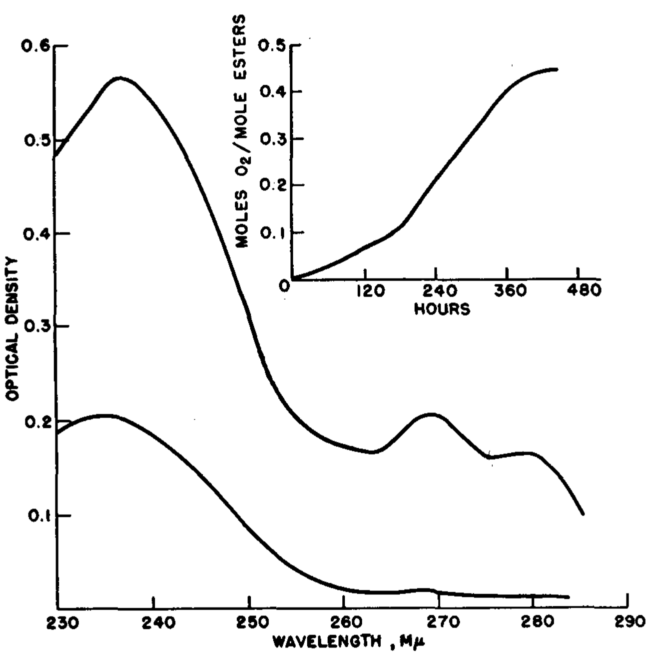

Fig. 1. Ultraviolet absorption spectra of two samples (1.75 mg. in 50 ml. absolute ethanol) of autoxidized methyl linolenate (Experiment I). Bottom curve: 0.1 mole O_2/mole ester. Upper curve: 0.448 mole O_2/mole ester. Inset: Rate of oxidation of methyl linolenate at 2°-4°C.

The percentage hydroxyl formed from the peroxides after hydrogenation of the oxygenated esters was determined on 10-20 mg. sample weights. A phthalic anhydride-pyridine esterification reagent was used and was that described in the method of Elving and Warshowsky (19). The modified micro procedure developed in this laboratory involved warming the sample and reagent in a sealed ampoule for 1 hour and then titrating the reaction mixture with 0.02 N alcoholic sodium hydroxide (20).

Acid number determinations (milligrams potassium hydroxide per gram sample) were made on material recovered after hydrogenating the peroxides. Alcoholic solutions of 5 to 12 mg. quantities of material were titrated with 0.02 N alcoholic sodium hydroxide.

Molecular weights were determined by the isothermal distillation procedure of Zigner using diethyl ether as solvent (21). A 10-day period was allowed for equilibration (temperature 25°C.). Accuracy of ± 0.7% has been obtained on standard compounds.

In all of the experimental work utmost care was used to avoid oxidation and decomposition during necessary laboratory manipulations. Transfers of methyl linolenate were conducted in an inert (purified nitrogen with oxygen content less than 0.0001%) atmosphere. Evaporations were conducted *in vacuo* at room temperature or below with the use of a stream of prepurified nitrogen. Solutions of oxidized ester were stored when necessary at 0°C. and after flushing with purified nitrogen.

Results and Discussion

Experiment I. Methyl linolenate (0.01 mole) was oxidized in the dark at 2°-4°C. with pure oxygen. After 500 hours 0.448 mole oxygen per mole ester had been absorbed. At this time oxygen uptake had practically ceased. Spectral data obtained at completion of the oxidation indicated that 18% of the ester had been conjugated. Dienoic and trienoic conjugated materials amounted to 81 and 19%, respectively, of the total conjugation observed.

Figure 1 illustrates both the rate of oxidation of methyl linolenate and the ultraviolet absorption spectra after 0.1 mole oxygen per mole ester had been absorbed and after 0.448 mole oxygen per mole ester had been absorbed.

Countercurrent distribution of the autoxidized ester was performed with the use of 80% aqueous ethanol and hexane-pentane (b.p. 37°-56°C.) as the immiscible solvent pair. Separation of oxygenated material from unoxidized ester was readily accomplished, as shown in Figure 2. Unoxidized methyl linolenate present in tubes 21 through 28 amounted to 44.5% of the original material. The remaining 55.5% was converted to the oxidation products found in tubes of 0 to 13 and must contain therefore 0.82 mole oxygen per mole ester.

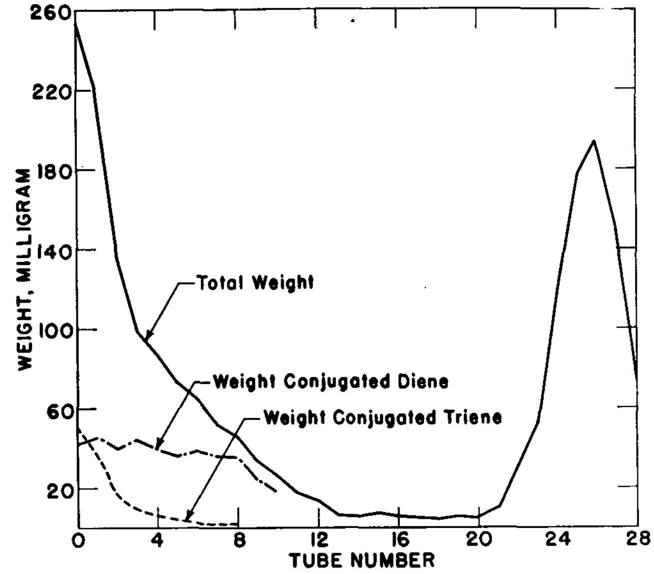

Fig. 2. Weight distribution curve following countercurrent fractionation of autoxidized methyl linolenate (Experiment I). Calculated diene and triene quantities expressed as weights of methyl linolenate converted to conjugated form.

Spectrophotometric analysis indicated that the oxidized material in tubes 0 to 13 was 46% conjugated and the ratio of diene conjugation to triene was 3.18. Since it can be calculated after countercurrent distribution that 25% of the ester subjected to oxidation was converted to a conjugated form, it is apparent that some change in conjugation occurred after oxygen uptake was stopped. The Cary spectrophotometric data illustrated in Figure 3 indicate the variations in quantity and type of conjugated material occurring in various selected tubes following fractionation. The maxima occurring at 2360 Å are characteristic of diene conjugated substances. Triene conjugation gives rise to maxima at 2590 Å, 2680 Å, and 2800 Å. The peak apparent in the absorption curve for tube 0 at a wavelength of 2480 Å has not yet been identified.

It is apparent that the material in tubes 0-13 of Figure 2 is a complex multicomponent system. No single theoretical curve or simple combination of theoretical curves can satisfactorily account for the experimental weight distribution. The oxygenated fraction can be divided however into two major types of

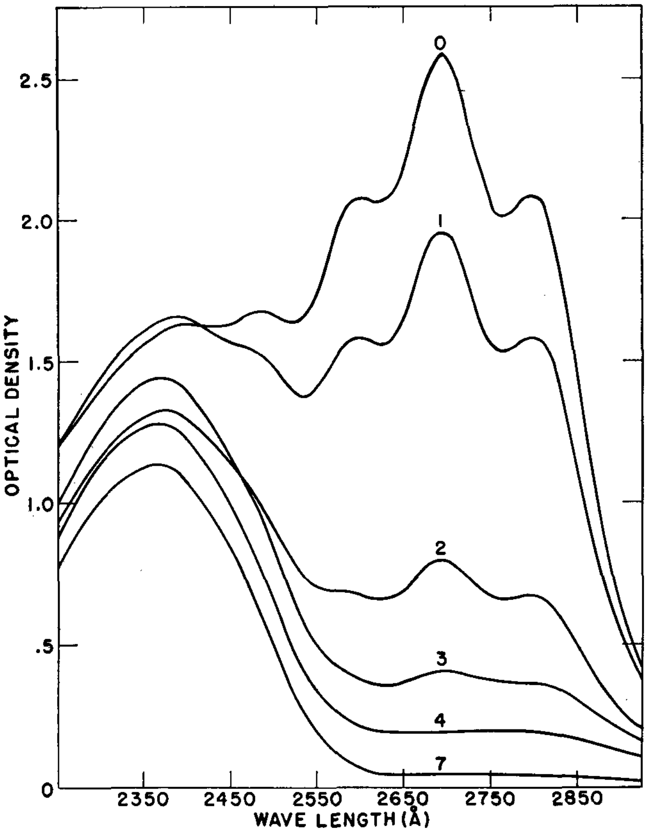

FIG. 3. Ultraviolet absorption spectra of selected tubes following countercurrent distribution of autoxidized methyl linolenate (Experiment I). Cary recording spectrophotometer used. Contents of tubes diluted 16.7 fold with absolute ethanol.

components: A, the highly polar, alcohol-soluble substance represented by tubes 0 through 3; and B, the less polar, alcohol-soluble material present in tubes 4 through 12. Of the methyl linolenate present in component A 40% is in a conjugated form; the diene/triene ratio being 1.5. Component B contains 62% of conjugated esters, almost all the conjugation being dienoic. Although the diene/triene conjugation ratio varies greatly in the two components, the number of ethylenic bonds actually in conjugation averages about 1 ethenoid bond per mole ester in each case. The data are summarized in Table I while the characteristics of the combined tubes 0-13 are listed in Table IV (Exp. I).

TABLE I

Analyses of Oxidized Methyl Linolenate Fractions (Experiment I)[a] After Countercurrent Distribution

	Tube numbers	
	(A) 0 through 3	(B) 4 through 10
Ester conjugated—%	40	62
Conjugation as diene—%	60	95
Conjugation as triene—%	40	5
Diene/triene	1.5	19
Av. number double bonds in conjugation per mole ester	0.98	1.28

[a] 0.448 mole oxygen absorbed per mole ester at 2°-4°C.

Results of chemical analysis performed upon material from selected tubes are presented in Table II. For convenience of interpretation all the analytical calculations are based on the molecular weight of unoxidized methyl linolenate, 292.4.

TABLE II

Analyses of Oxidized Methyl Linolenate Fractions (Experiment I)[a] After Countercurrent Distribution

	Tube number			
	1	2	3	4 through 10
PV	3980	3840	3630	3900
Moles peroxide oxygen/mole ester	0.64	0.62	0.58	0.63
Moles H_2 absorbed/mole ester	2.01	1.68	1.32	1.91
Moles OH/mole ester	0.96	0.78	0.78	0.94
OH—%	5.2	4.2	4.2	5.1
Acid No	25	29	34	6
Mol. wt.	....	....	521	425

[a] 0.448 mole oxygen absorbed per mole ester at 2°-4°C.

Of the 0.82 mole oxygen known to be present in the total oxygenated portion, 75% could be accounted for by the peroxide determination. Since the peroxide values of the tubes analyzed were found to vary by not more than 10%, differences in polarity of A and B must be attributable to functional groups not reducible by HI.

Neither component A or B absorbed more than 2 moles of hydrogen per mole methyl linolenate. Since both components contained approximately 1 mole of hydroxyl per mole ester following hydrogenation and since at least 1 mole of hydrogen per mole ester necessarily was required to saturate the ethylenic bonds in conjugation in each case, it appears doubtful that non-conjugated double bonds are present to any extent. This assumption gains additional support from one experiment in which material of tubes 1 through 5 was combined and subjected to hydriodic acid reduction. The isolated product retained its original conjugation and, upon catalytic hydrogenation, absorbed only 1 mole hydrogen per mole ester, yielding a product in which the original conjugation was destroyed and in which the hydroxyl content amounted to 3.5%. In interpreting the above results however, it must be kept in mind that peroxide isomerization in presence of a catalyst, as mentioned by Farmer (7), no doubt occurs to a certain extent.

Acidic scission products are concentrated in component A because of their polarity. The average acid number of A indicates that about 9.5% of this fraction could theoretically consist of C_9 acids. The work of Erdmann, *et al.* (22), has indicated some of the free acids are quite possibly formed during the course of linolenate oxidation.

The two molecular weight values of 521 and 425 given in Table II are of interest as indicating the high degree of dimerization which must have occurred even under the very mild conditions of oxidation which we employed.

Analytical evidence did not indicate the presence of any significant amount of monomeric linolenate monohydroperoxide in this experiment. The maximum in the weight curve of the hydroperoxide, if present, would have been in the vicinity of tube 8 of Figure 2. This observation is based upon countercurrent distribution data obtained for methyl ricinoleate, methyl hydroxy-stearate, and for the monohydroperoxides of methyl oleate (11) and linoleate (unpublished data).

Experiment II. Pure methyl linolenate (0.01 mole) was oxidized in the dark at 4°C. with pure oxygen. The oxidation was stopped after 176 hours when 0.102 mole oxygen per mole ester had been absorbed. A preliminary separatory funnel extraction of half of this oxidized ester was carried out in four separate funnels in countercurrent fashion similar to the procedure described by Craig (23). Equal 75-ml. portions of 60% aqueous ethanol and hexane-pentane were used and the distribution was continued until four hexane fractions had been passed through the funnels and collected. The solvent was removed from the combined hexane extracts at 0°C. *in vacuo* with a stream of nitrogen. The residue was then fractionated in the 25-tube countercurrent distribution instrument with 60% ethanol and hexane-pentane as the immiscible solvents. Figure 4 reveals that an oxidation product, designated as component C, was separated from the large quantity of unoxidized methyl linolenate. Substance C, present in tubes 6 through 18, amounts to 8% of the original ester subjected to oxidation and represents one-half of the methyl linolenate actually oxidized. The other more polar half of the oxygenated linolenate remained in the aqueous ethanol layer during the preliminary separation mentioned above.

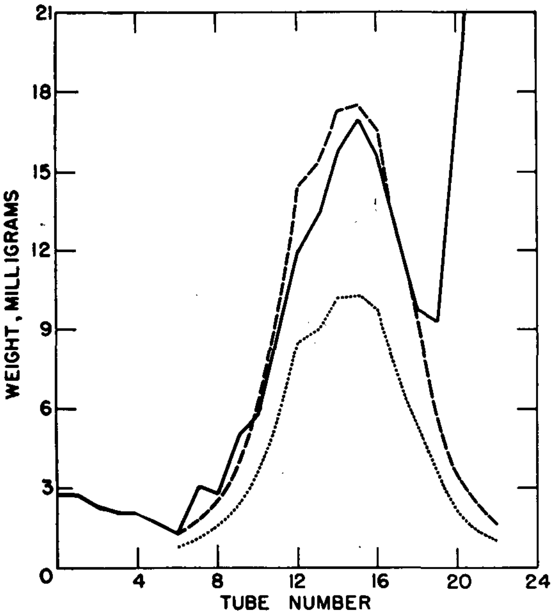

FIG. 4. Weight distribution curve following countercurrent fractionation of the hexane solubles of Experiment II: ...., calculated quantity of methyl linolenate converted to diene conjugated form; ———, experimental weight distribution; – – –, quantity of component C in the diene conjugated form calculated by the use of experimental molecular weight of 497.

Results of a spectrophotometric analysis of the material in the individual tubes of the Craig apparatus also are illustrated in Figure 4. The conjugation present in product C is thus seen to be almost completely dienoic. Of the methyl linolenate comprising component C, 62% was converted to a diene conjugated form. Tubes 10 through 17 were combined and provided the material for determining the analytical results summarized in Table III and Table IV.

TABLE III

Analysis of the Hexane-Soluble Fraction (Component C; Tubes 10 Through 17) from Oxidized Methyl Linolenate (Experiment II)[a] After Countercurrent Distribution

	Calculated as:	
	Methyl linolenate, mol. wt. 292.4	Linolenate polymer, mol. wt. 497
Moles diene conjugation	0.62	1.04
Moles H_2 absorbed/mole	3.10	5.15
Moles OH/mole	0.67	1.11

[a] 0.102 mole oxygen absorbed per mole ester at 2°-4°C.

TABLE IV

Oxygen Content and Degree of Conjugation of Fractions Obtained by Countercurrent Distribution of Oxidized Methyl Linolenate

	Exp. I (2°-4°C.)	Exp. III (28°-32°C.)	Exp. II (4°C.)
Moles oxygen abs./mole ester	0.448	1.022	0.102
Fract'n solvent: hexane+	80% EtOH	90% EtOH	60% EtOH
Tube numbers	0 through 13	0 through 10	6 through 18
Ester oxygenated—%	56	56	8
Moles oxygen per mole ester (calc'd)	0.82	2.02	....
Oxidized ester conjugated—%	46	49	62
Conjugation as diene—%	76	84	98.6
Conjugation as triene—%	24	16	1.4
Diene/triene	3.2	5.1	70

Dimerization was again evident in substance C since this oxidation product possessed a molecular weight of 497. A recalculation of the degree of conjugation of C on the basis of this molecular weight indicated that slightly more than 1 mole of diene conjugation per mole C was present (Figure 4). Substance C absorbed 3.1 moles of hydrogen per mole linolenate and its hydroxyl content, after hydrogenation, amounted to 3.6% or 0.67 mole per mole linolenate.

In our work it has been noted that C, stored in ether solution at room temperature, is partially converted to a more highly polar material and simultaneously develops a significant amount of triene conjugation. It is thus quite possible that substance C is the precursor of components A and B.

Experiment III. An autoxidation of a sample of methyl linolenate (0.01 mole) was conducted at room temperature (28°-32°C.) in diffused daylight. The oxidation was stopped at 70 hours after 1.022 moles oxygen per mole ester has been absorbed.

Fractionation of 0.008 mole of the autoxidized ester was performed, in this instance, in the 25-tube

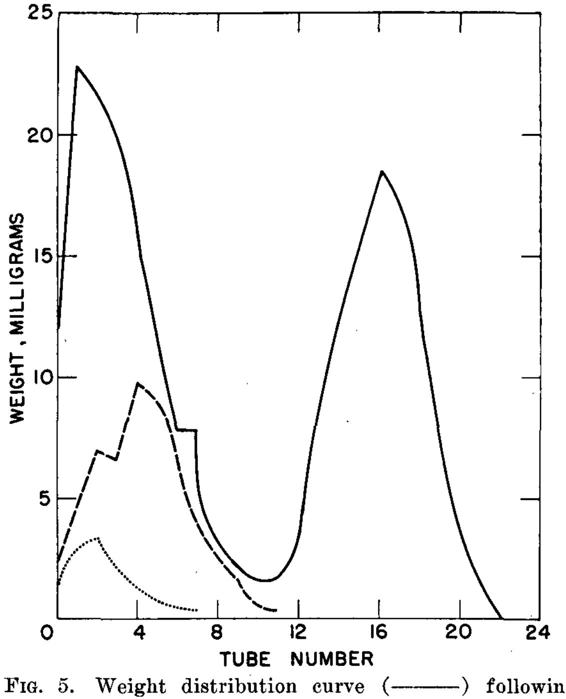

FIG. 5. Weight distribution curve (———) following countercurrent fractionation of autoxidized methyl linolenate (Experiment III). Calculated diene (– – –) and triene (....) quantities expressed as weights of methyl linolenate converted conjugated form.

model of the countercurrent distribution apparatus. The immiscible solvent pair was 90% aqueous ethanol and hexane-pentane.

Separation of oxidized material from unoxidized esters was again readily achieved as shown in Figure 5. Unoxidized esters present in tubes 11 through 23 amounted to 44.4% of the starting material. The remaining 55.6% was converted to the oxidation products found in tubes 0 through 10 and must average 2.02 moles oxygen per mole ester. By spectrophotometric analysis it was determined that the conjugated material in the first 11 tubes represented 49% of the methyl linolenate in these tubes. The ratio of diene to triene conjugation was 5.07. It is of interest to compare Figures 5 and 2. Despite the higher level and temperature of oxidation of experiment III there is a marked similarity of the two figures. The similarity is further emphasized in Table IV, where some of the important characteristics of the oxygenated fractions of experiments I, II, and III are summarized.

Summary

Methyl linolenate, oxidized to levels between 0.1 and 1.1 mol oxygen per mol ester, has been fractionated by countercurrent distribution techniques with aqueous ethanol and hexane as solvents.

The course of autoxidation of methyl linolenate differs significantly from that found for methyl oleate (reported in II of this series) and also from that reported for methyl linoleate.

Four major components are apparent from the weight distribution and ultraviolet absorption curves. They are, in order of decreasing polarity:

A. An alcohol-soluble component, approximately 40% of which is conjugated, with triene and diene conjugation present in about equal proportions.
B. An alcohol-soluble material, approximately 60% of which is diene conjugated, with triene conjugated substances being negligible.
C. A hexane-soluble oxidation product containing almost exclusively diene conjugation and occurring only at low levels of oxidation.
D. The unoxidized, hexane-soluble methyl linolenate.

It is concluded from the analytical studies that:

1. Less than half of the linolenate actually oxidized is converted to a conjugated form while more than half of the double bonds originally present are destroyed, probably through polymerization initiated by oxidative attack on the ethylenic bonds.
2. A partial separation of compounds containing conjugated triene (A) from diene (B) can be effected by countercurrent fractionation.
3. Compound C is apparently a precursor of components A and B.
4. Monomeric monohydroperoxide, if present, exists in very small quantity.
5. Dimers are formed during the oxidation process or immediately thereafter.

Acknowledgment

The authors are grateful to R. E. Childers for the Cary spectral data and to C. H. Van Etten for the semimicro hydroxyl determinations.

REFERENCES

1. Fugger, J., Zilch, K. T., Cannon, J. A., and Dutton, H. J., to be published, J. Am. Chem. Soc.,*73* (5), (1951).
2. Franke, W., and Jerchel, D., Ann., *533*, 46 (1937).
3. Franke, W., and Monch, J., Ann., *566*, 200 (1944).
4. Bergström, S., Ark. f. Kemi, Min. och Geol., *21A*, No. 14, 1-18 (1945).
5. Bolland, J. L., and Gee, G., Trans Faraday Soc., *42*, 236 (1946).
6. Lundberg, W. O., Chipault, J. R., and Hendrickson, M. J., J. Am. Oil Chem. Soc., *26*, 109 (1949).
7. Farmer, E. H., Trans. Faraday Soc., *42*, 288 (1946).
8. Goldschmidt, S., and Freudenberg, K., Ber., *67*, 1589 (1934).
9. Farmer, E. H., Koch, H. P., and Sutton, D. A., J. Chem. Soc. *(1943) 541.*
10. Ogg, C. L., and Cooper, F. J., Anal. Chem., *21*, 1400 (1949).
11. Zilch, K. T., and Dutton, H. J., to be published, Anal. Chem., *23* (1951).
12. Craig, L. C., and Craig, D., Chap. IV, pp. 205-292, "Techniques in Organic Chemistry," Vol. III, Ed., A. Weissberger, Interscience, 1950.
13. Melvin, E. H. Private communication.
14. Rusoff, I. I., Holman, R. T., and Burr, G. O., Oil and Soap, *22*, 290 (1945).
15. Prater, A. N., and Haagen-Smit, A. J., Ind. Eng. Chem., Anal. Ed., *12*, 705 (1940).
16. Schmidt, E., Ber., *52*, 409 (1919).
17. Lea, C. H., Proc. Royal Soc., *108B*, 175 (1931).
18. Lundberg, W. O., and Chipault, J. R., J. Am. Chem. Soc., *69*, 833 (1947).
19. Elving, P. J., and Warshowsky, B., Anal. Chem., *19*, 1006 (1947).
20. Van Etten, C. H., of this laboratory, unpublished procedure.
21. Clark, E. P., Ind. Eng. Chem., Anal. Ed., *13*, 820 (1941).
22. Erdmann, E., Bedford, F., and Raspe, F., Ber., *42*, 1334 (1909).
23. Craig, L. C., Golumbic, C., Mighton, H., and Titus, E., J. Biol. Chem., *161*, 329-330 (1945).

[Received November 8, 1950]

Report of the Referee Board, 1950-1951

FOR the year ending May 31, 1951 33 Referee Chemists were appointed. All except two were renewals. Two were appointed for the first time. Twenty-four were given certificates on cottonseed oil cake and meal and fatty oils. Nine held restricted certificates either from choice of application or from discretion of the Board.

The chemists represent 11 states, 21 cities, and 25 different laboratories. To date no chemist has qualified for certification in the North Central States. While the Board feels that this is unfortunate, we also feel that our requirements are reasonable and a sustained and planned effort on the part of chemists in these areas to meet these requirements would result in certification.

The Referee Board was the official sponsor of a special set of four samples of refined cottonseed oil and four samples of refined soybean oil for collaborative tests by the new spectrophotometric color method. These samples were planned primarily as practice samples for the benefit of the Referee Chemists and prospective applicants for referee certification, but they were offered to and requested by nearly all collaborators on crude vegetable oils. This was essentially a service to the Referee Chemists and did not imply or constitute responsibility of the Board for this or any other specific A.O.C.S. method.

Considerable progress was made by A. S. Richardson in his final period as chairman of the Board to clarify and establish the policies and requirements of the Board.

The Board has strongly adhered to the policy of requiring that new applicants meet certain formal educational requiremens.

Many inquiries were received during the year relative to certification and handled in an expeditious manner.

The Board strongly urges any prospective applicants to participate in the Smalley check sample work even though an application for certification is not contemplated in the immediate future.

Applications for certification for 1951-52 already have been given some consideration, and they will be handled before May 31, 1951.

R. R. KING
J. P. HARRIS
J. R. MAYS, JR.
A. S. RICHARDSON
R. W. BATES, chairman