Structure of Four Methyl Linolenate Diperoxides

P. Haverkamp Begemann, W. J. Woestenburg, and Simon Leer

Autoxidation of methyl linolenate at 37° C. yields a mixture containing not only methyl linolenate monohydroperoxide but also more polar peroxides. Four of these (isomeric) more polar compounds contain two peroxide groups each—a hydroperoxide group and a six-membered cyclic peroxide group.

In view of the fact that reversion flavors of soybean oil are mainly due to linolenic acid (Dutton *et al.*, 1953), it seemed of interest to study the primary autoxidation products of this acid.

According to the literature (Frankel *et al.*, 1961; Privett *et al.*, 1954), not only isomeric monohydroperoxylinolenates but also peroxides of a more polar character are formed. The present paper deals with the isolation and identification of four of these more polar peroxides.

METHODS

Preparation and Isolation of Peroxides. Methyl linolenate was stirred in an oxygen atmosphere at room temperature or at 37° C. until a peroxide value (PV) of ca. 400 was reached. To concentrate the peroxides formed, the mixture was distributed between 80% ethanol and light petroleum according to Frankel *et al.* (1961). The countercurrent distribution of 5 grams of substance was carried out in six separating funnels each containing 100 ml. of both phases. The lower phases were pooled and evaporated under reduced pressure until a slight turbidity was noticed. The mixture was then extracted with chloroform. Subsequently, the chloroform was removed and the concentrate separated into fractions by means of liquid-liquid partition chromatography.

Carrier, Celite (100 to 120 microns); immobile phase Carbowax-400; mobile phase isooctane ether (75 to 25) equilibrated with Carbowax.

Celite (18 grams) was loaded with Carbowax (9 ml.) and put into a column 1.5 × 20 cm. Five-milliliter fractions were collected. Elution of the peroxides was checked by spotting on paper with a solution of ferrous thiocyanate (Vioque and Holman, 1962) (0.2 gram of NH_4SCN in 15 ml. of acetone plus 10 ml. of freshly prepared 4% aqueous ferrous sulfate).

The peroxide value was determined by the ferrous thiocyanate method according to Hills and Thiel (1946) as modified by Smith (1952), using a standard curve constructed for pure methyl oleate hydroperoxide, and Lea's method (1946) using potassium iodide and acetic acid.

Molecular weights were determined in cyclohexanol according to Wilson and Heron (1941).

Reduction of Hydroperoxy Groups with Dimethyl Sulfide according to Barnard *et al.* (1961). To a solution of 3 mg. of hydroperoxide in 0.5 ml. of methanol, 50 mg. of dimethyl sulfide were added. The mixture was kept at −5° to −10° C. for 45 minutes. Subsequently the temperature was raised to 0° C. and then after 1 hour to room temperature, at which temperature the mixture was kept for another hour. Then ether was added. After the ethereal solution had been washed with water, it was dried and concentrated.

Catalytic Hydrogenation. METHOD A. With Raney Ni. About 200 mg. of Raney Ni suspended in 3 ml. of methanol were saturated with hydrogen. Subsequently ca. 30 mg. of peroxide in ca. 0.6 ml. of methanol were added. After 80 to 90% of the theoretical amount of hydrogen had been absorbed in about 10 minutes, hydrogen uptake stopped abruptly.

METHOD B. With Pd. About 30 mg. of Pd catalyst (5% Pd on $CaCO_3$), suspended in 3 ml. of methanol, were saturated with hydrogen. After addition of ca. 20 mg. of reduced peroxide in 2 ml. of methanol, hydrogenation was carried out for 5 minutes.

Periodic Acid Oxidation of Trihydroxystearates according to Marinetti *et al.* (1959). About 7 mg. of methyl trihydroxystearate were dissolved in 20 ml. of acetic acid.

The following mixtures were prepared: 4.5 ml. of trihydroxystearate solution plus 0.5 ml. of 0.02*M* sodium periodate solution; 4.5 ml. of trihydroxystearate solution plus 0.5 ml. of water; and 4.5 ml. of acetic acid plus 0.5 ml. of sodium periodate solution.

After these solutions stood for 30 minutes at room temperature, the ultraviolet absorption was measured at 300 nanometers (*A*, *B*, and *C*, respectively) using 90% acetic acid as a blank. The micromoles of diol are given by $\frac{C + B - A}{C} \times 10$.

Analysis of Cleavage Products. To identify the cleavage products obtained by periodic acid treatment of the trihydroxystearate, the reaction mixture (originally containing ca. 5 micromoles of trihydroxystearate) was treated in two ways.

A. After addition of 620 μg. of glycol in 0.2 ml. of water (to destroy any excess of periodic acid), 5 ml. of 0.4% 2,4-dinitrophenylhydrazine in 4*N* HCl were added. The mixture was stored in a refrigerator for 2 days, after which the precipitate was collected on a Büchner funnel, washed successively with 0.7% 2,4-dinitrophenylhydrazine in 2*N* HCl and water, and subsequently dissolved in chloroform. The solvent was removed in vacuo and the residue dissolved in 15 ml. of pure light petroleum plus 2% ether and passed over an alumina column [alumina, grade H, (Peter Spence, England) dried at 160° C., after which 8% water was added. Column 12 × 1 cm.]. After 150 ml. of light petroleum with 2% ether had been passed through the column, light petroleum–ether mixtures with increasing ether content (up to 25%) were successively passed through.

Unilever Research Laboratory, Vlaardingen, The Netherlands

The different 2,4-dinitrophenylhydrazone (DNPH) bands were examined by ultraviolet spectroscopy and by thin-layer chromatography on Carbowax plates according to Badings and Wassink (1963) and on alumina G plates. Paper chromatography according to Klein and De Jong (1956) and paper chromatography using isooctane-methanol were also applied.

B. After destroying the excess of periodate with glycol, water was added and the mixture extracted with ether. The ethereal solution was washed successively with water, sodium bicarbonate solution, and water. Subsequently, 15 ml. of 0.7% 2,4-dinitrophenylhydrazine in 2*N* HCl were added. After shaking thoroughly, the ether was carefully blown off. The mixture was stored in a refrigerator for 2 days. The precipitate was then filtered, washed with 0.7% 2,4-dinitrophenylhydrazine in 2*N* HCl, and subsequently analyzed as described above.

RESULTS

Methyl linolenate (99% pure) was autoxidized at room temperature to a PV of ca. 400 and subsequently submitted to countercurrent distribution. A thin-layer chromatogram of the concentrate is shown in Figure 1.

After it had been found that autoxidation at 37° C. yielded a peroxide concentrate similar to that obtained on autoxidation at room temperature, further experiments were carried out at 37° C.

The peroxide concentrate was separated by partition chromatography; the amount used per column was about 150 mg. In this way three peroxides were obtained. No. 1 was eluted in fractions 9 to 15, No. 2A in fractions 31 to 41, and No. 2B in fractions 48 to 52. Fractions 42 to 47 contained mixtures of 2A and 2B. The yield of pure peroxides 1, 2A, and 2B obtained from a concentrate containing 1.89 meq. of peroxide (determined according to Hills and Thiel, 1946) was 1.17, 0.30, and 0.29 meq., respectively.

Some characteristic values of these three peroxides are given in Table I.

On hydrogenation, peroxide 1 yields a mixture of monohydroxystearates. In view of its molecular weight it must be a mixture of isomeric monohydroperoxylinolenates with a theoretical PV of 6170, which value was found by the Hills and Thiel method. From this it follows that the hydroperoxy group in a polyunsaturated compound reacts to the same extent with ferrous thiocyanate as the hydroperoxy group in methyl oleate hydroperoxide, which was used as a standard in the determination.

Table I. Characteristic Values of Three Peroxides Obtained by Autoxidation of Methyl Linolenate

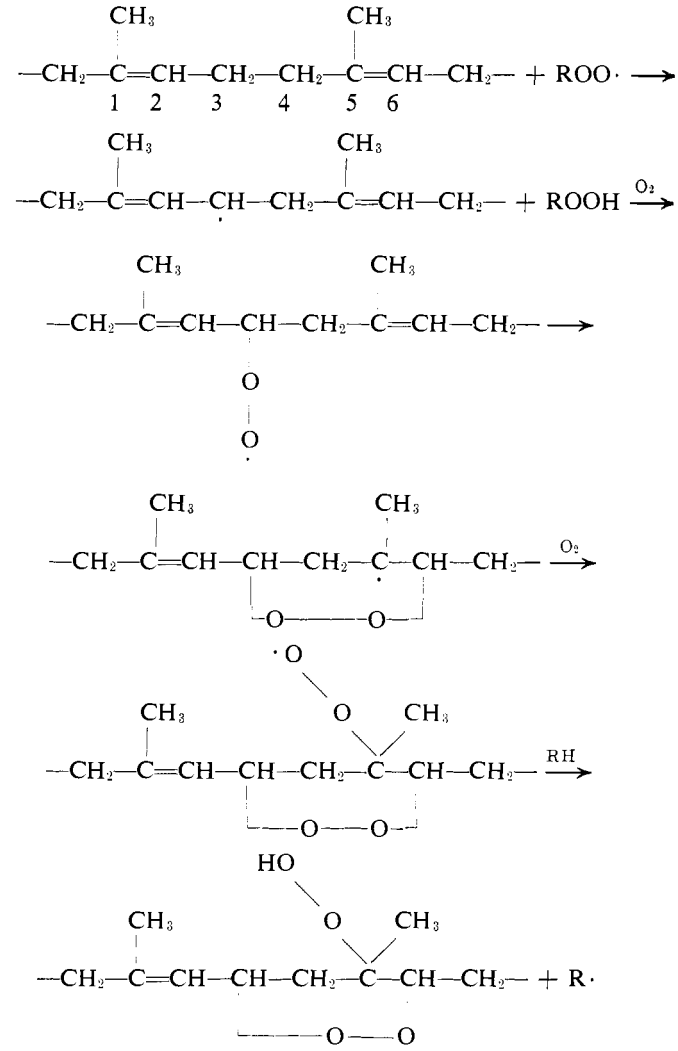
Peroxide	Peroxide Value: By ferrous thiocyanate method	Peroxide Value: By Potassium Iodide Method, 15 min.	Peroxide Value: By Potassium Iodide Method, 60 min.	No. of Conjugated Double Bonds[a] per Peroxide Group, Determined with Ferrous Thiocyanate	Mol. Wt.
1	6170	6020	5690	0.79	284
2A	5580	8790	7820	0.97	347
2B	5820	8700	9520	1.03	324

[a] Derived from extinction at 234 nm. of a methanolic solution, assuming ϵ to be 29,000 (Privett and Blank, 1962).

In view of the results obtained with the Lea method and the PV determined by the ferrous thiocyanate method, it seemed highly probable that 2A and 2B contain two peroxy groups, one of which is a hydroperoxy group.

This hypothesis was confirmed by an oxygen determination (Vieregge, 1965). 2A contains 27.05% oxygen and 2B contains 26.95% oxygen. For $C_{19}H_{32}O_6$, 26.93% oxygen can be calculated. The theoretical PV according to the ferrous thiocyanate method is 5618.

It occurred to the authors that 2A and 2B might have a structure (as regards peroxy groups) similar to the squalene peroxide identified by Bolland and Hughes (1949). According to them, the peroxy radical at the 3rd C atom in the 1–5 diene system adds to the double bond between the 5th and 6th C atom to form a cyclic peroxide bearing a radical group. This new radical yields a new peroxy radical, which is converted into a hydroperoxide.



On hydrogenation with Raney nickel catalyst, this squalene peroxide is converted into a compound with three hydroxy groups, two of which are vicinal.

If the 2A and 2B peroxides contain peroxy groups similar to squalene peroxide, they may be expected to give tri-

hydroxystearate on hydrogenation. This compound was indeed formed.

Hydrogenation of 2A with Raney nickel catalyst yielded a mixture as shown in the thin-layer chromatogram in Figure 2. The hydrogenation product of 2B gave a similar thin-layer chromatogram.

The main components of these two hydrogenation mixtures, designated $2A_1$, $2A_2$, $2B_1$, and $2B_2$, were isolated by scraping them from a set of thin-layer chromatoplates and again chromatographed (Figure 2). According to mass spectroscopy, these four fractions were all trihydroxystearates. The mass spectra (AEI mass spectrometer Models MS9 and MS12) of $2A_1$ and $2B_1$ were identical with that of the model substance methyl 9,10,12-trihydroxystearate prepared according to Kass and Radlove (1942). $2B_2$ was shown to be 13,15,16-trihydroxystearate, and $2A_2$ appeared to be a mixture of 13,15,16-trihydroxy- and 9,10,-12-trihydroxystearate.

These results were confirmed by periodic acid oxidation: Fractions $2A_1$ and $2B_1$ and the model substance methyl 9,10,12-trihydroxystearate consumed comparable amounts of periodic acid—namely, about 70% of the theoretical amount.

For the model substance, the ultraviolet spectrum of a chloroform solution of the DNPH obtained by method A showed a maximum at 376 nm. after elution from the alumina column with light petroleum–5% ether. It occupied the $7\frac{1}{2}$ position on a thin-layer chromatoplate (according to Badings and Wassink, 1963) and the $8\frac{1}{2}$ position on paper (Klein and De Jong, 1956). Also on an alumina plate its R_f value was identical with that of 2-nonenal DNPH. Thus the carbonyl formed was 2-nonenal.

By method B not only 2-nonenal DNPH was obtained but also a DNPH was eluted from the alumina column with light petroleum–25% ether which occupied the $3\frac{1}{2}$ position on paper chromatography (isooctane-methanol) and the $3\frac{3}{4}$ position on the Badings plate. On an alumina plate, however, its R_f value was much lower than might be expected for an aldehyde DNPH. In fact, its R_f value was identical with that of the model substance, the DNPH of methyl 9-aldononanoate (which on paper occupied the $3\frac{1}{2}$ position and on a Badings plate the $3\frac{3}{4}$ position). The ultraviolet spectrum of a chloroform solution showed a maximum at 358 nm.

The same results were obtained with the DNPH's from $2A_1$ and $2B_1$.

The aldehydes to be expected on periodic acid cleavage of methyl 9,10,12-trihydroxystearate are 3-hydroxynonanal and methyl 9-aldononanoate. The DNPH of 2-nonenal is obtained instead of the 3-hydroxynonanal derivative because of the elimination of water from the hydroxy group at C atom 3 and a hydrogen atom at C atom 2 (Scanlan and Swern, 1940).

Fraction $2B_2$ was also submitted to periodic acid cleavage. It consumed 73% of the amount of periodic acid expected for a vicinal diol. The carbonyl compounds formed were converted into their DNPH's in two ways (Methods A and B). The two DNPH's obtained with Method A were the same as those obtained with Method B. The ultraviolet spectra of the chloroform solutions showed maxima at 358 and 372 nm.

The first DNPH was run, next to model DNPH's, on a Badings plate and on an alumina plate. It appeared to be propanal DNPH. The second DNPH occupied the $7\frac{3}{4}$ position on a Badings plate and is thus comparable with that of 2-nonenal DNPH. However, on an alumina plate its R_f value was much lower than that of 2-nonenal DNPH. Consequently, it was supposed to be an aldo-ester DNPH (2–3 unsaturated in view of its ultraviolet spectrum). On Badings plates aldo-ester DNPH's occupy a position 6 C atoms lower than the aldehyde DNPH of the same chain length. α-β Unsaturation lowers the position on a Badings plate by $1\frac{1}{4}$ C atoms. Therefore, the second DNPH was supposed to be methyl 15-aldo-13-pentadecenoate DNPH. This was confirmed by mass spectroscopy which showed a parent peak of mass 448. On the basis of the cleavage products obtained, $2B_2$ must be methyl 13,15,16-trihydroxystearate.

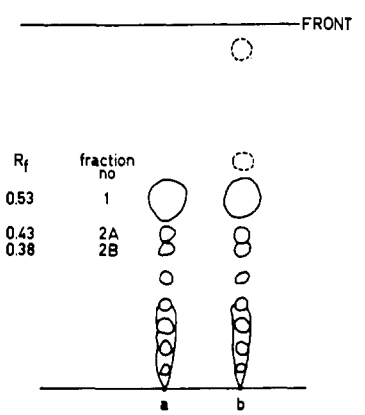


Figure 1. Thin layer chromatogram of peroxide concentrate from autoxidized methyl linolenate

a. Sprayed with KI (Lea, 1946) (1 ml. of saturated aqueous solution in 30 ml. of ethanol–acetic acid–chloroform 5:3:2 v./v.)
b. Sprayed with molybdophosphoric acid and heated at 200° C. for 5 minutes. Layer thickness, 0.25 mm., absorbent SiO_2
Eluent, light petroleum–diethyl ether–chloroform (50:40:10 v./v.)

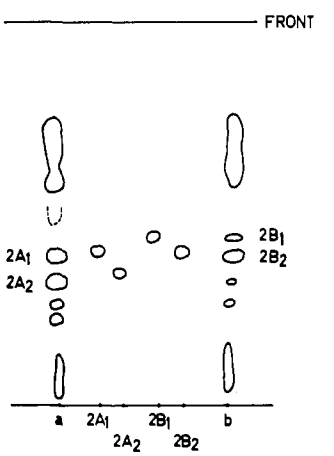


Figure 2. Thin layer chromatograms of hydrogenation products of cyclic linolenate peroxides

a. Hydrogenation mixture of 2A *b.* Hydrogenation mixture of 2B (see Figure 1).
Layer thickness 0.25 mm., absorbent SiO_2
Eluent, ethyl acetate–diethyl ether (25 to 75 v./v.)
Detection, spraying with molybdophosphoric acid and heating at 200° C. for 5 minutes

The results of periodic acid cleavage of the four hydrogenation products are listed in Table II.

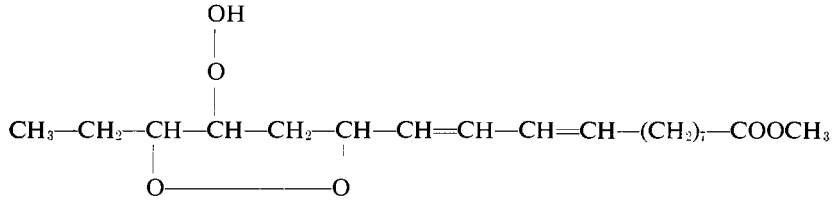
In the analysis of cleavage products Method A gives the higher yields of aldehyde DNPH whereas Method B gives the higher yields of aldo-ester DNPH.

The yields of aldehydes mentioned in Table II, column 4, are based on the results of Method A and the yields of aldo-esters on the results of Method B.

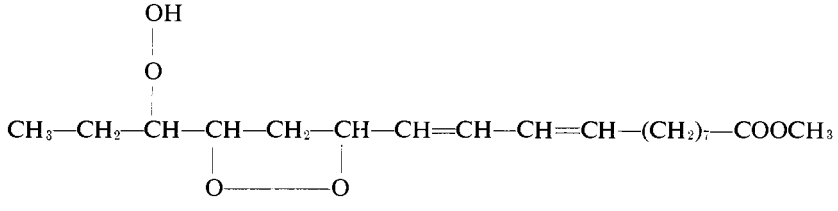
Those parts of 2A and 2B which yield $2A_1$ and $2B_1$ on hydrogenation are assumed to be formed according to the reactions on page 683.

Both isomers I and II should give methyl 9,10,12-trihydroxystearate on hydrogenation. Analogously, that part of 2B which forms $2B_2$ on hydrogenation is thought to be formed by initial attack at the 11th C atom instead of at the 14th C atom.

The structure of the final peroxide is assumed to be as follows:



or



Apparently, $2A_2$ is derived from a mixture of such peroxides having the conjugated diene structure partly on the methyl side of the chain and partly on the ester side of the chain.

Obviously the structure of the trihydroxystearates obtained by hydrogenation of the cyclic peroxides gives no clue as to the presence of a six-membered peroxide ring system (a 1,2-dioxahexane group) or of a five-membered ring (1,2-dioxapentane).

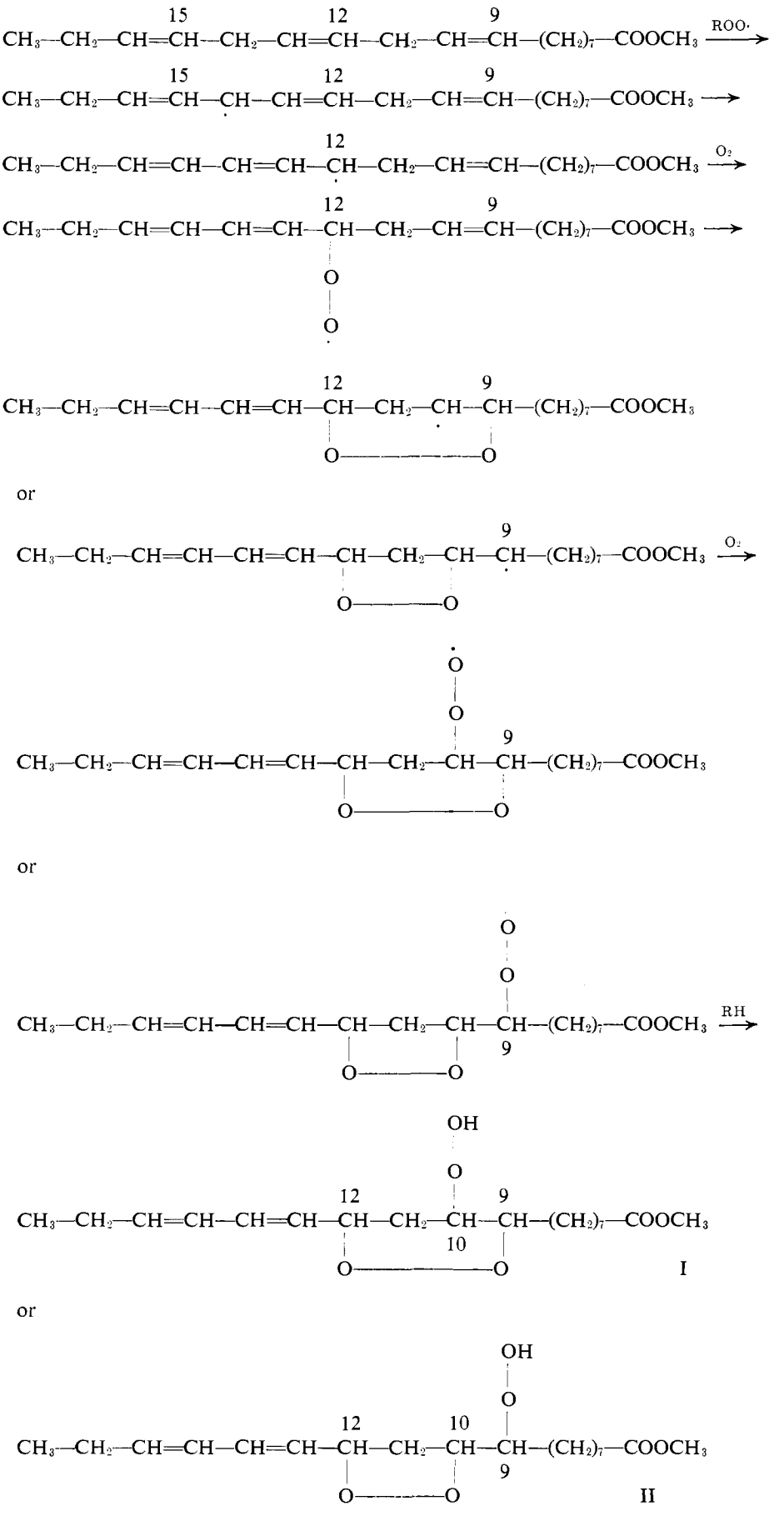
The following method was used to identify two of the four cyclic peroxides: The hydroperoxy group was converted into the hydroxyl group by treatment with dimethyl sulfide. 2A and 2B each gave two hydroxy compounds $2A^a$, $2A^b$, $2B^a$, and $2B^b$—for example, Figure 3—which were separated by TLC. Each of these four cyclic peroxides, carrying a hydroxyl group was then hydrogenated with palladium as catalyst. The hydrogenation products, when submitted to TLC, showed, in addition to trihydroxystearate, ca. 15% of a compound with a higher R_f and carrying a carbonyl group as revealed by spraying with dinitrophenylhydrazine reagent (Figure 3, *b*).

Mass spectroscopy showed them to be ketodihydroxystearates. The position of the different substituents was determined by mass spectroscopy of their di-trimethylsilyl ethers. Thus the ketonic hydrogenation product, designated $2A^a_{Pd}$ was identified as methyl 9-keto,10,12-

Table II. Results of Periodic Acid Cleavage of the Four Hydrogenation Products of Peroxides 2A and 2B

Compound	Consumption of Periodic Acid, % of Theory	Main[a] Carbonyl Compounds Formed	Yield, μMoles, Starting from 5 μMoles of Trihydroxyester	Identity
Model 9,10,12-trihydroxystearate	69	2-Nonenal	4.35	
		Methyl 9-aldononanoate	3.64	
$2A_1$	86	2-Nonenal	3.25	9,10,12-Trihydroxystearate
		Methyl 9-aldononanoate	4.0	
$2A_2$	65	Methyl 15-aldo-13-pentadecenoate	0.95	13,15,16-Trihydroxystearate
		Propanal	0.67	+
		2-Nonenal	0.18	9,10,12-Trihydroxystearate
		Methyl 9-aldononanoate	0.38	
$2B_1$	97	2-Nonenal	2.4	9,10,12-Trihydroxystearate
		Methyl 9-aldononanoate	2.7	
$2B_2$	73	Propanal	1.24	13,15,16-Trihydroxystearate
		Methyl 15-aldo-13-pentadecenoate	0.76	

[a] Methanal, ethanal, and acetone originating from solvents disregarded.



or

or

or

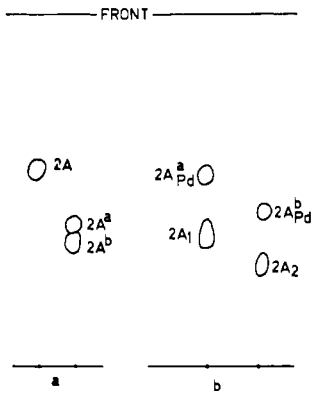


Figure 3. Thin layer chromatography on silica gel G

a. Peroxide 2A and its dimethyl sulfide-reduction products. Eluent, diethyl ether–light petroleum–chloroform (50:40:10 v./v.)

b. Hydrogenation products of 2A^{a} and 2A^{b} using palladium as catalyst
Eluent, ether–ethyl acetate (75 to 25 v./v.)
Detection: see Figure 2

dihydroxystearate, $2A^{b}_{Pd}$ as methyl 13-keto,15,16-dihydroxystearate, $2B^{a}_{Pd}$ as methyl 9-keto,10,12-dihydroxystearate, and $2B^{b}_{Pd}$ as methyl 13-keto,15,16-dihydroxystearate.

As the keto group can have its origin only in the cyclic peroxy group (the hydroperoxy group being previously converted into the hydroxyl group), 2A^{a} and 2B^{a} carry the 9,12-dioxahexane group with a hydroxyl group at the 10 position.

The fact that the cyclic peroxy groups in 2A^{b} and 2B^{b} split in such a way that the keto group occurs only at the 13 position makes identification of these two compounds impossible. By analogy, it is assumed that these latter two compounds also contain the 1,2-dioxahexane ring.

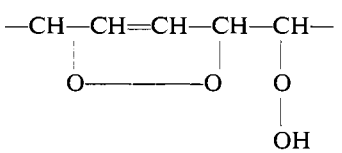
We may conclude that two of the four cyclic peroxides are methyl 9,12-epidioxy-10-hydroperoxy-13,15-octadecadienoate. The difference in chromatographic properties of the peroxides, and of the trihydroxystearates derived from them, must be due to stereoisomerism.

The remaining two cyclic peroxides are tentatively identified as methyl 13,16-epidioxy-15-hydroperoxy-9,11-octadecadienoates.

The possibility that by autoxidation of linolenate five-membered ring peroxides are formed in addition to six-membered ring peroxides cannot be excluded. Although only 9-keto,10,12-dihydroxystearate has been detected in the hydrogenation products of 2A^{a} and 2B^{a}, small amounts of 10-keto,9,12-dihydroxystearate may have escaped us.

DISCUSSION

In the past 15 years, several structures of cyclic monomeric peroxides have been proposed—e.g.,



which was assumed to be formed by 1,4-addition of oxygen to the conjugated diene group in linoleic acid hydroperoxide (Bergström *et al.*, 1950; Cannon *et al.*, 1952; Lundberg *et al.*, 1949; Privett and Nickell, 1956). These structures do not contain a conjugated diene group as our peroxides and are not expected to form trihydroxystearates with the —CH—CH—CH$_2$—CH— grouping. Dahle *et al.* (1962) suggested addition of the —CH—O—O· radical to the remaining isolated double bond during autoxidation of linolenate and a cyclic five-membered ring peroxide—not containing the adjacent hydroperoxy group—was proposed.

It is generally assumed that the oxygen ring structure is stable under conditions of iodometric determinations (Cannon *et al.*, 1952). When determining the PV value of their squalene peroxide according to Dastur and Lea (1941), Bolland and Hughes (1949) found one peroxy group (the hydroperoxy group) per molecule.

However, Criegee and Müller (1956) stated that 1,2-dioxane "reacted incompletely" with the usual peroxide reagents. We also found that the cyclic peroxy group reacts to a certain extent with KI in glacial acetic acid.

ACKNOWLEDGMENT

Mass spectra were recorded and interpreted by J. de-Bruijn. Work was carried out under supervision of B. G. Tarladgis.

LITERATURE CITED

Badings, H. J., Wassink, J. G., *Neth. Milk Dairy J.* **17,** 132 (1963).
Barnard, D., Bateman, L., Cunneen, J. L., "Organic Sulfur Compounds," Vol. I, N. Kharash, Ed., Chap. 21, Pergamon Press, New York, 1961.
Bergström, S., Blomstrand, R., Laurell, S., *Acta Chem. Scand.* **4,** 245 (1950).
Bolland, J. L., Hughes, H., *J. Chem. Soc.* **1949,** p. 492.
Cannon, J. A., Zilch, K. T., Burket, S. C., Dutton, H. J., *J. Am. Oil Chemists' Soc.* **29,** 447 (1952).
Criegee, R., Müller, G., *Ber.* **89,** 238 (1956).
Dahle, L. K., Hill, E. G., Holman, R. T., *Arch. Biochem. Biophys.* **98,** 253 (1962).
Dastur, N. N., Lea, C. H., *Analyst* **66,** 90 (1941).
Dutton, H. J., Evans, C. D., Cowan, J. C., *Trans. Am. Assoc. Cereal Chem.* **11,** 116 (1953).
Frankel, E. N., Evans, C. D., McConnell, D. G., Selke, E., Dutton, H. J., *J. Org. Chem.* **26,** 4663 (1961).
Hills, G. L., Thiel, C. C., *J. Dairy Res.* **14,** 340 (1946).
Kass, J. P., Radlove, S. B., *J. Am. Chem. Soc.* **64,** 2253 (1942).
Klein, F., De Jong, K., *Rec. Trav. Chim.* **75,** 1285 (1956).
Lea, C. H., *J. Soc. Chem. Ind.* **65,** 286 (1946).
Lundberg, W. O., Chipault, J. R., Hendrickson, M. J., *J. Am. Oil Chemists' Soc.* **26,** 109 (1949).
Marinetti, G. V., Erbland, J., Stotz, E., *J. Am. Chem. Soc.* **81,** 861 (1959).
Privett, O. S., Blank, M. L., *J. Am. Oil Chemists' Soc.* **39,** 564 (1962).
Privett, O. S., Nickell, C., *J. Am. Oil Chemists' Soc.* **33,** 156 (1956).
Privett, O. S., Nickell, C., Tolberg, W. E., Paschke, R. F., Wheeler, D. H., Lundberg, W. O., *J. Am. Oil Chemists' Soc.* **31,** 23 (1954).
Scanlan, J. T., Swern, D., *J. Am. Chem. Soc.* **62,** 2309 (1940).
Smith, G. H., *J. Sci. Food Agr.* **3,** 26 (1952).
Vieregge, H., private communication, University of Groningen, The Netherlands, 1965.
Vioque, E., Holman, R. T., *Arch. Biochem. Biophys.* **99,** 522 (1962).
Wilson, H. N., Heron, A. E., *J. Soc. Chem. Ind.* **60,** 168 (1941).

Received for review December 6, 1967. Accepted March 25, 1968. Division of Agricultural and Food Chemistry, 154th Meeting, ACS, Chicago, Ill., September 1967.