Enzymatic Oxidations of Linoleic Acid and Glycerol-1-Monolinoleate in Doughs and Flour-Water Suspensions

A. GRAVELAND, Institute for Cereals, Flour and Bread TNO, Wageningen, The Netherlands

Abstract

Enzymatic oxidations of linoleic acid and glycerol-1-monolinoleate, and the products formed by these oxidations in flour-water suspensions and doughs were studied. Oxidation of linoleic acid leads through simultaneous reactions to two isomeric hydroperoxy-octadecadienoic acids and two isomeric hydroxy-epoxy-octadecenoic acids. Reduction of the former leads to hydroxy-octadecadienoic acid, while hydrolysis of the latter yields trihydroxy-octadecenoic acids. The hydroperoxy acids are formed by the enzyme lipoxygenase (E.C. 1.13.1.13), whereas the hydroxy-epoxy acids are formed by combined action of lipoxygenase and an unknown factor Y. This factor Y is localized in the gluten fraction. Oxidation of glycerol-1-monolinoleate gives a product having a hydroperoxy group and a *cis,trans* conjugated diene bond. The oxidation of glycerol-1-monolinoleate is probably a lipoxygenase reaction.

Introduction

Smith and Andrews (1) showed that much of the oxygen consumption which occurs during mixing a dough in air, is due to enzymatic oxidation of the unsaturated easily extractable lipids. This enzymatic oxidation is effected by the enzyme lipoxygenase. Lipoxygenase is specific for the oxidation of the *cis,cis*-1,4 pentadiene group in unsaturated fatty acids (2). The primary oxidation products are optically active conjugated *cis,trans*-diene hydroperoxides (3,4). These hydroperoxides oxidize the thiol-groups of protein, and affect the rheological properties of a dough (5,6).

In a previous publication (7) we have pointed out that the free linoleic and linolenic acids and their α-monoglycerides are oxidized in dough. In the present investigation, the oxidation of linoleic acid and glycerol-1-monolinoleate in flour-water suspensions and doughs were studied in more detail.

Materials

Flour

A commercial flour (not containing any bleaching or other improving agent) with a protein content (N × 5.7) of 10.1% (on 14% moisture basis) was used.

Dough

Doughs were prepared from 100 g of flour, 54 ml of distilled water, and 2 g of sodium chloride (dissolved in a part of the water). These ingredients were mixed under air in a GRL mixer (8) at a speed of 66 rpm.

Doughs used in the experiments with radioactive linoleic acid were made with a small Kenwood mixer (9). The ingredients were: 15 g of flour and 9 ml of a 3.3% sodium chloride solution.

Substrate

The substrates were: linoleic acid supplied by Fluka (Switzerland), radioactive 1-^{14}C-linoleic acid from Radiochemical Centre, Amersham, England, and glycerol-1-monolinoleate from Unilever, Vlaardingen.

Methods

Enzymatic Oxidation

In our studies concerning the enzymatic oxidations of linoleic acid in flour-water suspensions and in water extracts of flour, 6 g of flour was homogenized with an apparatus according to Potter and Elvehjem (10), 100 mg of linoleic acid was added to the mixture and incubated in a vibrating machine at 23 C. Samples were taken after 10, 30, 60, 120, 180 min and the oxidation products determined by TLC.

Extraction of Oxidation Products

The oxidation products from a flour-water suspension and those from a water extract of flour were extracted as follows. To 1 ml of the reaction mixture, 1.5 ml of a chloroform-methanol mixture (2:1) was added; this was thoroughly shaken in a Vortex vibrator, and centrifuged at 2000 × *g*. The upper layer was removed and the oxidation products determined in the bottom layer (chloroform).

The extraction of the oxidation products from a dough was carried out as follows. About 1 g of dough was homogenized with 10 ml of a chloroform-methanol mixture (2:1) with a Potter-Elvehjem homogenizer (10). The suspension obtained was centrifuged at 2000 × *g*. In the supernatant the oxidation products were determined by thin layer chromatography (TLC).

For preparative isolation of the oxidation products the dough was homogenized with a chloroform-methanol mixture (2:1) in a blender (Braun). The solvent was evaporated with a rotary evaporator and the oxidation products dissolved in chloroform. This chloroform extract containing impurities was applied directly on a large TLC plate. The recovery of the oxidation products was more than 95%.

Isolation of Lipoxygenase

Flour (6 g) was homogenized with 60 ml distilled water in a Potter-Elvehjem homogenizer (10) and the suspensions centrifuged for 20 min at 300,000 × *g* in an MSE superspeed 65 ultracentrifuge. The precipitate was discarded; the extract containing lipoxygenase was freeze-dried. This material was fractionated on a Sephadex G-100 column (Pharmacia, Uppsala, Sweden). Elution was carried out with distilled water at 4 C and the lipoxygenase-active fraction was freeze-dried.

Qualitative TLC

A coat of Silica Gel DO (Camag) 0.2 mm thick was applied to glass plates (19 × 5 cm) (7). The following solvent mixtures were used. I: benzene-diethyl ether-ethanol-acetic acid 50:40:2:0.2 [Freeman and West (11)]. II: diethyl ether-petroleum ether 40:60. III: diethyl ether-petroleum ether 60:40. The plate was developed by spraying with 50% sulfuric acid and heating it for 10 min at 200 C.

Quantitative TLC

The extracts were applied to a TLC plate in streaks (2 cm) by means of a capillary, and developed with system I. The plate was developed twice in the same direction, viz. 6 and 15 cm, then sprayed with 50% sulfuric acid and heated for 10 min at 200 C. The

intensity of the black bands on the TLC plate was measured with a densitometer (Vitatron UTD 500, filter 363 nm). The peak areas in the densitogram were measured with a planimeter. Amounts of the various components in the densitogram were determined by multiplying the area of each peak by a correction factor depending on the degree of blackening characteristic for each component.

To determine the correction factors, the oxidation products were isolated by means of preparative TLC. A mixture of equal amounts (15 mg) of linoleic acid and its four oxidation products was chromatographed. The area of the densitogram peak of linoleic acid divided by the areas of the oxidation products were: $L_1 = 1.51$, $L_2 = 1.37$, $L_3 = 1.46$ and $L_4 = 1.58$. The area of the peak of glycerol-1-monolinoleate divided by the areas of the oxidation products were: $G_1 = 1.15$ and $G_2 = 1.20$. The above factors are reliable within 0.04.

Preparative TLC

A suspension of Silica Gel DO (Camag) in ethanol (50 g/160 ml) was applied to glass plates (20 × 20 cm) with the aid of a Camag spreader, coating thickness 1 mm. The plates were first dried for 1 hr at room temperature, then for 3 hr at 120 C. After chromatography the components were made visible on the TLC plate by means of iodine vapor, scraped off and transferred into a column. The components were eluted from the silica gel with ether or with a mixture of chloroform-methanol (2:1).

Determination of Thiol Groups

Thiol groups were determined by means of amperometric titration with 0.001 M silver nitrate in a solution containing 1 M NH_4NO_3, 0.05 M NH_4OH, 0.001 M ethylenediaminotetraacetic acid and 4 M urea in a nitrogen atmosphere at 20 C (12). Doughs were dispersed in a small volume of 4.5 M NH_4NO_3; then the reagents added to the concentrations mentioned above.

Reductions—Chemical Oxidation

The component (10 mg L_1) was dissolved in 2 ml ethanol, cooled to 0 C and 20 mg $NaBH_4$ added. The solution was allowed to stand for 20 min at 0 C and for 40 min at room temperature; it was then acidified with 2 ml 4 N HCl and extracted with ether (13).

For complete hydrogenation, 10 mg of the material was dissolved in 2 ml ethanol, saturated with H_2 gas, after which 5 mg PtO_2 catalyst was added and H_2 gas passed through the mixture for 5 min (13).

The oxidation was carried out according to Marinetti et al. (14). A total of 3 mg of the material was dissolved in 5 ml of acetic acid. To this, 0.5 ml 0.02 M KIO_4 solution was added; the mixture was allowed to stand at room temperature for 30 min. Then a drop of glycol in 0.2 ml water was added to remove the excess iodate.

Preparation of the 2,4-Dinitrophenylhydrazones (DNPHs)

To the above-mentioned solution, 5 ml of a 0.4% DNPH solution in 4 N HCl was added and stored at 4 C for two days. The precipitate was separated by centrifugation, washed with 0.7% DNPH solution in 2N HCl, and dissolved in chloroform (15).

IR, NMR and Mass Spectra Analyses

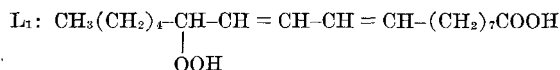
IR spectra were obtained with a Perkin Elmer unit model 237. The analyses were carried out in chloroform or KBr pellet form.

NMR spectra were obtained with a JNM-4H-100 High Resolution NMR unit. The analyses were carried out in $CDCl_3$ solution.

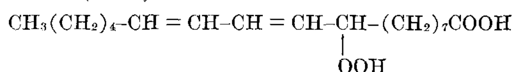
Mass spectra were obtained with an Atlas mass spectrometer, type CH 4, equipped with a TO 4 ion chamber. The ionizing current was 60 μ A and the electron beam energy 70 eV. Mass measurements were carried out with an Atlas double focussing mass spectrometer SM-1. To enable a mass spectrum to be made, the substance was first methylated with diazomethane (16) and silylated (17).

Results

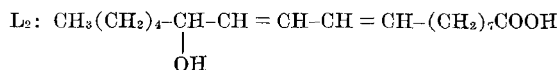
To understand the course of the experiments the structures of the oxidation products and the reaction schemes are helpful. In a dough mixed under usual conditions the oxidation of linoleic acid gives four different pairs of oxidation products (L_1–L_4). The structures of these products are:



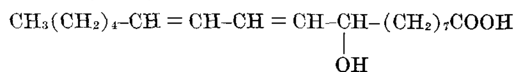
13-hydroperoxy-9(*cis*), 11(*trans*)-octadecadienoic acid (L_1-13)



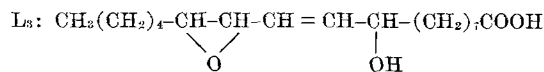
9-hydroperoxy-10(*trans*), 12(*cis*)-octadecadienoic acid (L_1-9)



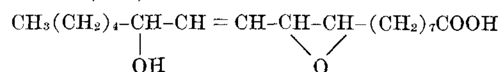
13-hydroxy-9(*cis*), 11(*trans*)-octadecadienoic acid (L_2-13)



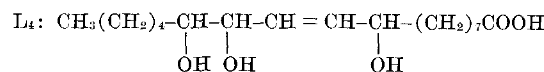
9-hydroxy-10(*trans*), 12(*cis*)-octadecadienoic acid (L_2-9)



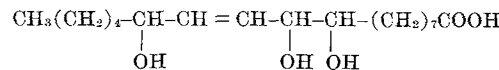
9-hydroxy-10(*trans*), 12,13(*cis*)-epoxy-octadecenoic acid (L_3-9)



9,10(cis)-epoxy-11(*trans*), 13 hydroxy-octadecenoic acid (L_3-13)



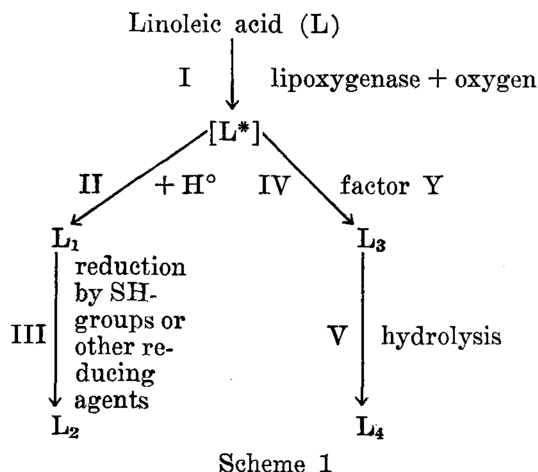
9,12,13-trihydroxy-10(*trans*)-octadecenoic acid (L_4-9)



9,10,13-trihydroxy-11(*trans*)-octadecenoic acid (L_4-13)

From the results we propose reaction scheme 1 for the oxidation of linoleic acid.

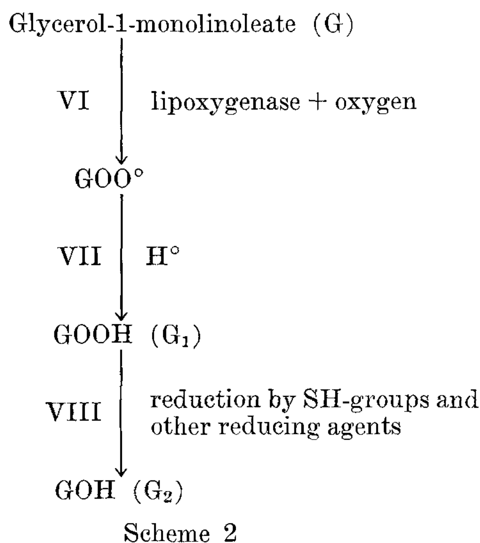
In Reaction I, a radical or a peroxy radical is formed from linoleic acid; the peroxy radical is transformed by Reaction II to the hydroperoxides (L_1); these can be reduced to the alcohols (L_2). In doughs or flour-water suspensions, thiol-groups may act as reducing agents in Reaction III. In Reaction IV, hydroxy-epoxy acids (L_3) and trihydroxy acids (L_4) are formed simultaneously. A water insoluble factor Y is involved in the production of L_3. This factor Y is partly inactivated by petroleum ether. L_3 can be transformed into L_4 through hydrolysis (Reaction V).



Scheme 1

In a dough, the oxidation of linoleic acid proceeds for about 50% by Reactions I-II-III, and for another 50% by I-IV-V. The oxidation rate of linoleic acid in a dough is 8.0×10^{-3} μmole/g flour/sec.

For the oxidation of glycerol-1-monolinoleate the reaction scheme below is suggested.



Scheme 2

From glycerol-1-monolinoleate two different oxidation products, G_1 and G_2, are formed (Fig. 1J). The component G_1 contains a hydroperoxy group and *cis,trans* conjugated diene bond. The component G_2 contains a hydroxy group and also a *cis,trans* conjugated diene bond. By Reaction VI, a peroxy radical is formed from glycerol-1-monolinoleate; the peroxy radical is transformed into a hydroperoxide G_1 (Reaction VII); G_1 is reduced to G_2 (Reaction VIII). In a dough no epoxides or trihydroxides formed from glycerol-1-monolinoleate were found. The oxidation rate is 7.6×10^{-3} μmole/g flour/sec. The results of the experiments described in this paper are summarized in Table I.

Flour

In a flour-water enzymatic oxidation, three pairs of products were found: hydroperoxides (L_1), alcohols (L_2) and trihydroxy acids (L_4), (Fig. 1A). The other peaks (15–26) in the densitograms shown in Figure 1 represent lipids naturally present in the flour (7). Figure 2 shows how the oxidation of linoleic acid in a suspension proceeds with time. If

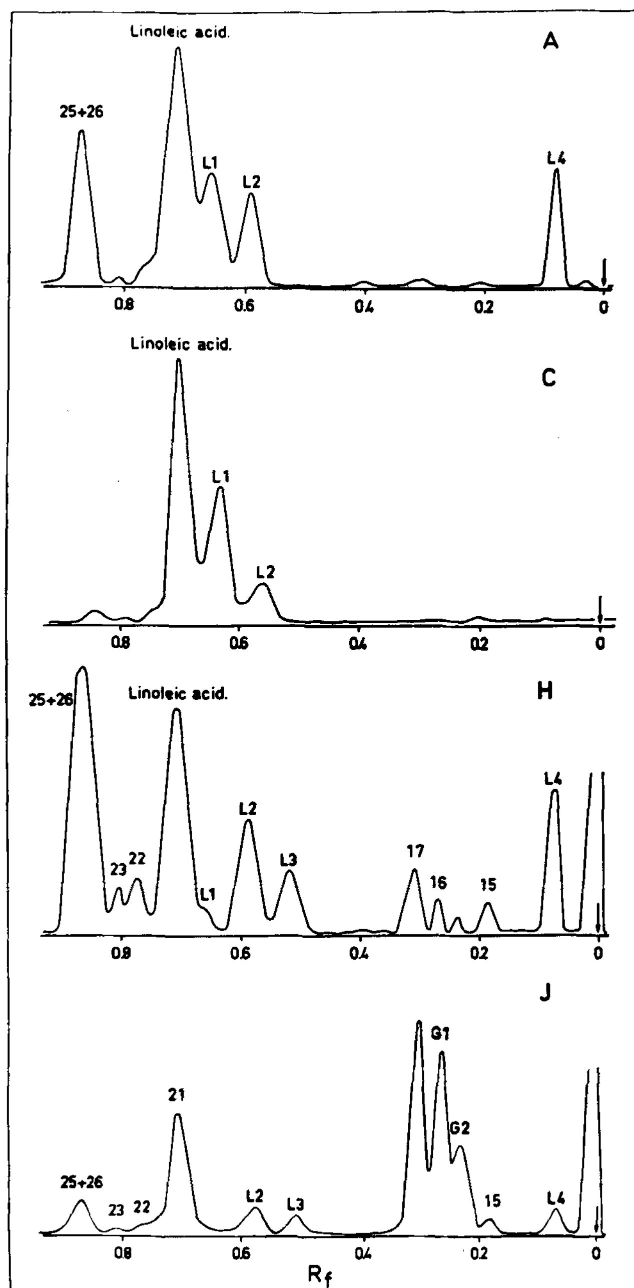


FIG. 1. Densitograms of TLC plates after charring with 50% sulfuric acid. A, Flour-water suspension incubated with linoleic acid. C, Water extract of flour incubated with linoleic acid. H, Dough mixed with linoleic acid. J, Dough of defatted flour mixed with glycerol-1-monolinoleate.

the flour is first defatted with petroleum ether less trihydroxy acids (L_4) are formed; see Table I,B. When the petroleum ether extract is recombined with the defatted flour the production of trihydroxy acids is not restored.

In a water extract of flour only two pairs of oxidation products are found; hydroperoxides (L_1) and alcohols (L_2) (Fig. 1C). After adding linoleic acid to a suspension of the water insoluble flour residue in water only small amounts of alcohols (L_2) and trihydroxy acids (L_4) could be detected. Recombining the water extract and the water insoluble residue leads to the same oxidation products as in a flour-water suspension.

The water insoluble residue was freeze-dried, and defatted with petroleum ether. Reconstitution of this defatted material and a water extract of normal flour

TABLE I
Quantities of Oxidation Products From Linoleic Acid and Glycerol-1-Monolinoleate Formed From 100 mg of Substrate

	Material	Oxidized substrate, mg	Oxidation products formed after 2 hr incubation, mg				Initial oxidation rate, μmole/g sec
			L_1	L_2	L_3	L_4	
Flour[a]							
A	Normal flour-in-water suspension	60.0	26.0	17.9		16.0	0.17
B	Defatted flour[b]-in-water suspension	58.0	32.0	16.0		10.0	0.17
C	Water solubles from normal flour	46.0	32.8	13.2			0.14
D	Water insolubles from normal flour	10.0		7.4		3.0	
E	Reconstitution of water solubles and insolubles from normal flour	58.0	25.0	18.0		14.6	0.17
F	Reconstitution of water solubles from normal flour and insolubles from defatted flour[b]	56.0	32.6	14.2		9.2	0.16
G	Water insolubles from normal flour and partially purified lipoxygenase from wheat flour	55.0	24.9	14.1		16.0	
Dough[c]			After 30 min mixing				
H	Dough from normal flour	62.0		31.6	5.0	25.0	8.0×10^{-3}
I	Dough from defatted flour[b]	60.0	7.0	35.5	3.0	15.0	7.8×10^{-3}
J	Dough from defatted flour[b]	61.0	G_1 46.0 (G_1)	G_2 15.0 (G_2)			7.6×10^{-3}

[a] A-G: substrate linoleic acid.
[b] Flour extracted with light petroleum (bp 40–60 C).
[c] H-I: substrate linoleic acid. J: substrate glycerol-1-monolinoleate.

gives also hydroperoxides (L_1) and alcohols (L_2) but a smaller quantity of trihydroxy acids (L_4).

In a suspension of water insoluble material from normal flour, to which 50 mg partially purified lipoxygenase from wheat flour was added, the same oxidation products were found as in a flour-water suspension. The quantity of 50 mg was used because under the prevailing conditions its lipoxygenase activity was equivalent to that of 6 g flour.

Linoleic acid (100 mg) added to a suspension of 1 g freeze-dried gluten in a water extract of flour gives predominantly trihydroxy acids and relatively little alcohols. Gluten was prepared from flour by hand kneading the dough in distilled water. The gluten obtained was solubilized in 0.05 N acetic acid and precipitated with 0.1 N sodium carbonate. Gluten was recovered by centrifugation at 1000 × g and freeze-dried to about 3% moisture.

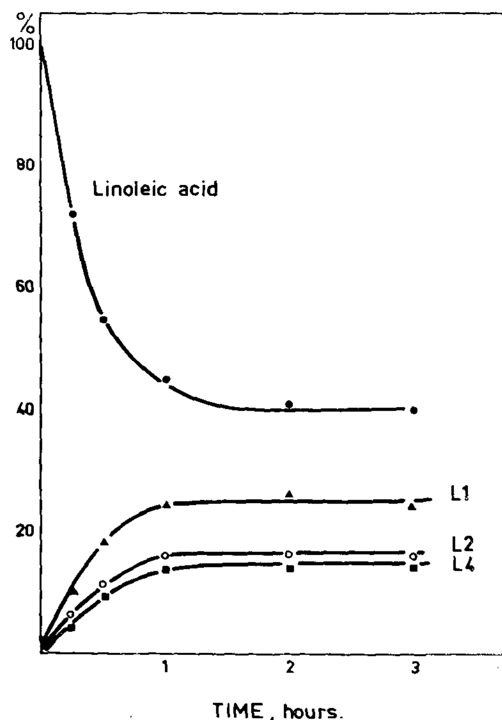


FIG. 2. Oxidation of linoleic acid in a flour-water suspension, determined by TLC. Decrease of linoleic acid and increase of oxidation products (mg) are plotted against time. Concentrations of components are expressed as percentages of total weight.

Dough

In examining the oxidation reactions in doughs, 100 g portions of flour were used, with 100 mg of linoleic acid added. Doughs were mixed in air. Samples were taken after 5, 10, 15, 20 and 25 min mixing and the oxidation products determined. In a dough made from normal flour, three pairs of oxidation products were found. Along with the alcohols (L_2) and trihydroxy acids (L_4), hydroxyepoxy acids (L_3) were formed (Fig. 1H). Figure 3 shows the course of the oxidation.

In a dough made from a flour defatted with petroleum ether, four pairs of oxidation products were formed. Along with the oxidation products named above, the hydroperoxides (L_1) could be detected. In comparison with a dough made from normal flour, less of the hydroxy-epoxy acids and of the trihydroxy

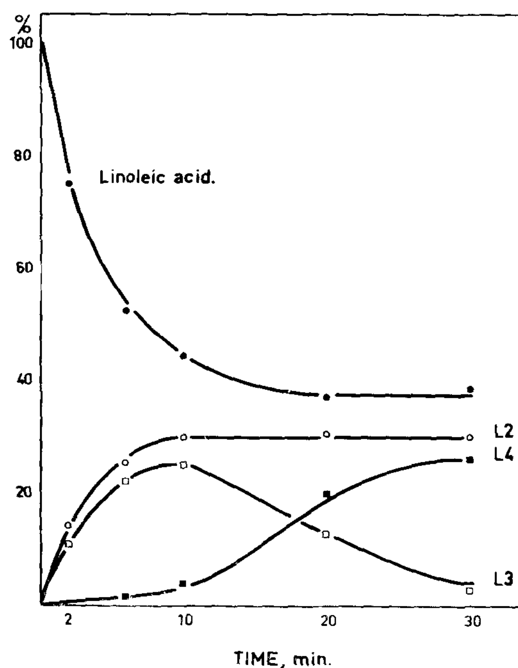


FIG. 3. Oxidation of linoleic acid in a dough mixed in a GRL mixer.

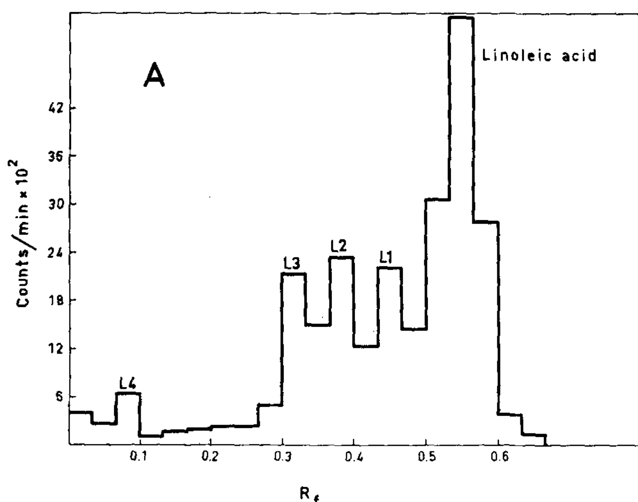


FIG. 4. The distribution of radioactivity on a TLC plate showing the products formed in a dough containing radioactive linoleic acid after 5 min mixing.

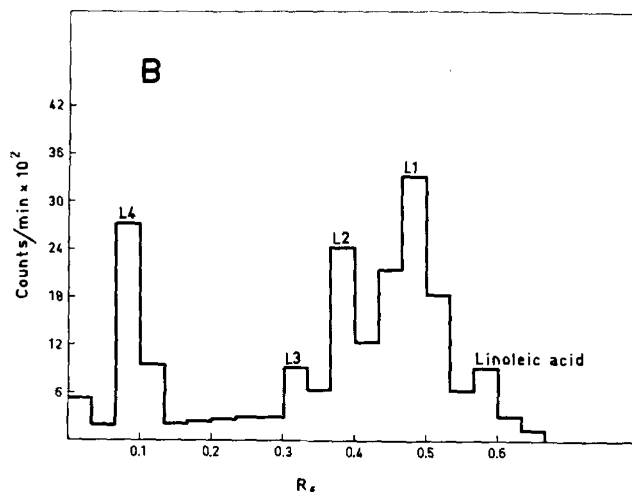


FIG. 5. The distribution of radioactivity on a TLC plate showing the products formed in a dough containing radioactive linoleic acid after 30 min mixing.

acids were formed in a dough from defatted flour (Table I, H and I).

To find out how the various oxidation products are formed from linoleic acid, a dough was mixed with radioactive linoleic acid. To a mixture of 15 g defatted flour and 9 ml 3.3% NaCl solution, 25 mg linoleic acid and 20 μC of radioactive 1-^{14}C-linoleic acid was added and mixed for 40 min. After 5, 10, 20, 30 and 40 min mixing a sample was taken and extracted with a chloroform-methanol (2:1) mixture. The extract was immediately applied to a TLC plate and developed with solvent system I. The silica gel was scraped from the plate in bands 0.5 cm wide, transferred to scintillation tubes and their activity measured with a scintillation counter. Figures 4 and 5 show that the oxidation of linoleic acid in a dough of defatted flour gives again four pairs of oxidation products L_1, L_2, L_3 and L_4. Note the high percentage of L_1 (35%). The results of this experiment agree very well with those obtained by the TLC method.

In a dough from defatted flour, with 100 mg glycerol-1-monolinoleate added, we found two oxidation products: G_1 and G_2. Figure 1J shows the composition of the reaction products after 15 min mixing. The reaction is by that time completed; G_1 and G_2 are then present in quantities of 45% and 15%, respectively.

A dough from 100 g flour and 31 mg hydroperoxides from linoleic acid (100 μmole) was mixed in nitrogen. Effect on rheological properties (elastic and viscous deformation) and the decrease of thiolgroups was about the same as when dough was mixed under air without added oxidative substances. Hydroperoxides added to a flour-water suspension cause a considerable portion of the thiol-groups to disappear within seconds.

Identification of Oxidation Products

The experiments have shown that in a dough mixing process, four oxidation products (L_1-L_4) are formed from linoleic acid, and two (G_1 and G_2) from glycerol-1-monolinoleate. Each of these products consists of two isomeric components that could be methylated with diazomethane and separated by TLC. Their quantities have been determined by densitometry. The isomers were also separated and isolated on a preparative scale, and identified with the aid of IR, UV, NMR and mass spectrometry. The results are found in the appendix.

L_1 was first reduced with $NaBH_4$, methylated and chromatographed. Solvent system II was used, the plate was developed twice. R_f values of the reduced and methylated isomers were 0.35 and 0.43, percentages 15% and 85%, respectively. The isomer having R_f 0.35 was identified to have been originally 9-hydroperoxy-10(*trans*),12(*cis*)-octadecadienoic acid; the other with R_f 0.45 13-hydroperoxy-9(*cis*),11(*trans*)-octadecadienoic acid.

L_2 was likewise separated. R_f values of the methylated isomers were 0.35 and 0.43; percentages 15% and 85%, respectively. The first isomer was identified as 9-hydroxy-10(*trans*),12(*cis*)-octadecadienoic acid; the second as 13-hydroxy-9(*cis*),11(*trans*)-octadecadienoic acid.

L_3 was also methylated and chromatographed. When solvent system III was used, R_f values were 0.23 and 0.30 with 15% and 85% respectively. The first isomer was identified as 9,10(*cis*)-epoxy-11(*trans*),13-hydroxy-octadecenoic acid; the second as 9-hydroxy-10(*trans*),12,13(*cis*)-epoxy-octadecenoic acid.

We did not succeed in separating the L_4 component into isomers. Our experiments have shown that L_4 originates from L_3. There is every likelihood that L_4 does also consist of two isomers in portions of 15% and 85%. This assumption is confirmed with the DNPHs that were obtained after breakdown. The two isomers of L_4 were identified as: 9,10,13-trihydroxy-11(*trans*)-octadecenoic acid and 9,12,13-trihydroxy-10(*trans*)-octadecenoic acid.

Discussion

The experiments in which linoleic acid was incubated with flour-water suspensions and with water extracts from flour showed that in the suspensions two isomeric hydroperoxides (L_1) are formed by the water-soluble enzyme lipoxygenase. These hydroperoxides may be reduced to the corresponding hydroxy acids (L_2). Along with the hydroperoxy acids and hydroxy acids, also trihydroxy acids (L_4) are formed. These are not formed in a water extract from flour. Therefore a water-insoluble factor Y must be responsible for the step leading to the component L_4. Also, a water-soluble factor is required for the reaction leading to L_4. This is true because after addition of linoleic acid to a suspension of

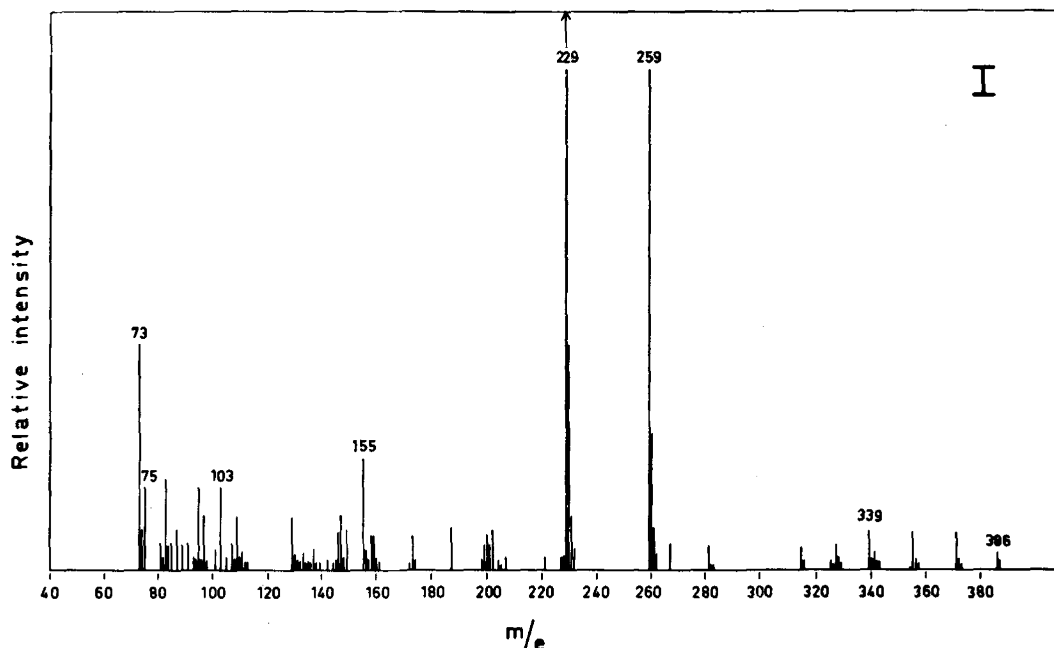


FIG. 6. Mass spectrum of the component L_2-9 after methylation, hydrogenation and silylation.

insoluble material of flour in water only small amounts of hydroxy acids and trihydroxy acids were detected. Recombining flour residue and water extract restored the production of L_4 to the same level as produced by the original flour. The soluble factor required for the formation of L_4 is the enzyme lipoxygenase because incubating linoleic acid with a mixture of partly pure lipoxygenase from wheat flour and water-insoluble material from flour forms trihydroxy acids.

By fractionating the flour into gluten, starch and water-solubles it was found that the water-insoluble factor Y is localized in the gluten. The formation of trihydroxy acids is partially reduced by extracting the water-insoluble flour residue with petroleum ether. Reconstituting the residue with the petroleum ether extract does, however, not lead to identical results. The water-insoluble factor Y is partly inactivated by petroleum-ether.

If hydroperoxy acids are added to a water extract from flour, or to a flour-water suspension, or to a dough, they are transformed only into hydroxy acids. The transformation proceeds more rapidly in the first two conditions than in dough. The reaction is accomplished by the reducing substances (Reaction III). Thiol groups are very effective in this respect. There are no trihydroxy acids formed.

In a dough made from normal flour, another extra component was found, viz. a pair of hydroxy-epoxy acids (L_3). According to the course of the reactions suggested in Figure 3, L_3 is a primary reaction product, while L_4 is a secondary product. If L_3 is added to a water extract from flour or to a flour-water suspension, it is hydrolyzed (Reaction V) to L_4; if added to a dough, the transformation proceeds more slowly.

Defatting the flour with petroleum ether leads to an increase of the production of L_1 and L_2, while the production of L_3 and L_4 is decreased (Table I, H and I). Defatting does not affect the lipoxygenase activity but inactivates the unknown factor Y. The degree of inactivation is related to the intensity of the petroleum ether extraction. Reconstituting the flour with the petroleum ether extract does not restore the original reaction course.

The oxidation of linoleic acid proceeds 20 times faster in a flour-water suspension than it does in a dough and the reactivity of factor Y controls the formation of the oxidation products.

Since it has been shown that lipoxygenase is required for the formation of L_3, and that L_3 does not originate from L_1, it is logical to assume that it is derived from an intermediate product formed by lipoxygenase from linoleic acid (Reaction I). Scheme 1 is based on the supposition that Reactions II and IV, leading to L_1 and L_3 respectively, start from the same intermediate product L*. These two simultaneous reactions leading to L_1 and L_3 might also start from linoleic acid.

The experiment with radioactive linoleic acid showed that the total activity during the entire mixing process remained constant. Apparently, no volatile oxidation products were formed, which could escape during mixing. A very small part (1%) of the total activity was found to stay at the place of application (Fig. 4 and 5). This shows that very few polymeric products form from the linoleic acid which stay at the starting point and, consequently, are not found by the TLC method. When compared with dough kneaded in a GRL mixer, there is a considerable amount of L_1 (35%). This probably results from the relatively more linoleic acid added and the different mixer used.

The oxidation of glycerol-1-monolinoleate is very likely a lipoxygenase reaction because a normal hydroperoxide is formed. No other oxidation products were found along with the hydroperoxide and the alcohol derived from glycerol-1-monolinoleate. Evidently, the factor Y does not react with this substrate.

Appendix

L_1: The IR spectrum of the methylated product points to the presence of a methyl ester (1730 and 1173 cm^{-1}), a *cis,trans* conjugated double bond (3040, 982 and 948 cm^{-1}) and a hydroperoxy group (3550 cm^{-1}).

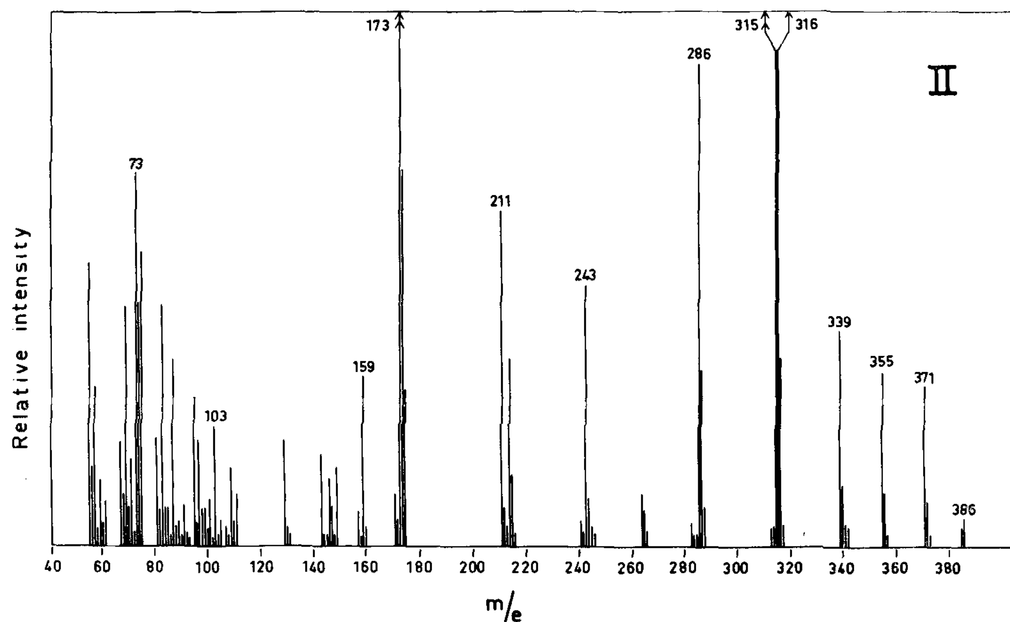


FIG. 7. Mass spectrum of the component L_2-13 after methylation, hydrogenation and silylation.

L_2: The IR spectrum of the methylated product points to the presence of a methyl ester (1730 and 1173 cm^{-1}), a hydroxy group (3620 cm^{-1}) and a *cis,trans* conjugated double bond (3040, 982 and 948 cm^{-1}). The two isomers of L_2 were isolated by preparative TLC with system II as solvent. A mass spectrum was recorded for both isomers. All the mass spectra mentioned below display a number of very characteristic peaks, viz.: 73 ($Si(CH_3)_3^+$); 75 ($HO^+ = Si(CH_3)_2$); 103 ($CH_2 = O^+Si\ (CH_3)_2$) and 147 ($(CH_3)_2Si = O^+–Si(CH_3)_3$). Furthermore, all spectra contain the peaks with m/e values of p(arent)-15 and p-31; attributable to a loss of CH_3 and $CH_3 + CH_4$ the TMS group (18). Peaks with m/e values of p-90 and p-180 are indicative of the loss of one or two trimethyl silanol groups (18). The molecule breaks next to a C atom bearing a TMS group, and preferably between two adjacent C atoms bearing a TMS group (19).

The mass spectrum of L_2-9 (methylated, hydrogenated and silylated) (Spectrum I) shows a small parent peak (m/e 386). The values 371 (p-15) and 355 (p-31) are scarcely visible. The molecule has broken on either side of the C atom where the TMS group is attached; this produces two large peaks, viz. 229 ($CH_3(CH_2)_8$–CH–OTMS) and 259 (TMSO–CH–$(CH_2)_7$–$COOCH_3$). Also, there are peaks 339 (p–CH_3–MeOH) and 155 (O = CH–$(CH_2)_7$–C = O). From these data it appears that L_2-9 is 9-hydroxy-10 (*trans*),12(*cis*)-octadecadienoic acid.

The mass spectrum of L_2-13 (methylated, hydrogenated, silylated) (Spectrum II) shows a parent peak (m/e 386), and 371 (p-15), 355 (p-31) and 339 (p-15-MeOH). There are also two large peaks 315 (TMSO–CH–$(CH_2)_{11}COOCH_3$) and 173 (CH_3–$(CH_2)_4$–CH–OTMS) derived from the rupture on either side of the C atom to which the TMS group is attached. Other peaks are 286 ($(CH_2)_{11}$–C(OCH_3)–OTMS) and 243 (O = CH$(CH_2)_{11}$–C(OH)OCH_3) and 211 (O = C–$(CH_2)_{11}$–C = O). From these data it appears that L_2-13 is 13-hydroxy-9(*cis*),11(*trans*)-octadecadienoic acid.

L_1-9 and L_1-13 reduced with $NaBH_4$ give L_2-9 and L_2-13. Therefore, the structure of L_1-9 is 9-hydroperoxy-10(*trans*),12(*cis*)-octadecadienoic acid, and that of L_1-13 is 13-hydroperoxy-9(*cis*),11(*trans*)-octadecadienoic acid.

L_3: The IR spectrum of the methylated compound points to the presence of a methyl ester (1730 and 1173 cm^{-1}), a hydroxy group (3620 cm^{-1}), a *trans* double bond (3040 and 965 cm^{-1}) and an epoxy group (weak absorption at 830 and 845 cm^{-1} (21). Furthermore, this spectrum resembles very much that of L_2.

The UV spectrum shows two very weak absorption bands at 277 and 316 nm. From these data it can be concluded that L_3 contains no conjugated double bonds and only one *trans* double bond.

The NMR spectrum of the methylated compound is made up as follows. A triplet at 9.1 τ(3H; CH_3–CH_2–), a broad peak with top at 8.7 τ(20H; –(CH_2)–), multiplets between 7.6 and 8.0 τ(2H; CH_2–$COOCH_3$), two broad peaks with tops at 6.6 and 7.1 τ(2H; H–C–C–H), a singlet at 6.4 τ(1H; OH)

and a singlet at 6.3 τ(3H; OCH_3), a broad peak between 5.8 and 6.1 τ(1H; H–C–OH) and a multiplet with top at 4.3 τ(2H; HC = CH). From this spectrum it appears clearly that L_3 comprises a hydroxy group and a *cis*, epoxy group, viz. a broad peak at 7.1 τ(20). All the above-mentioned spectra of L_3 were made of mixtures of the two isomers.

The methylated product L_3 was hydrogenated with H_2/PtO_2. The R_f value of the product formed was 0.25 (system I); this is between 0.14 (L_4 methylated) and 0.43 (L_3 methylated). The epoxy ring passes over into a hydroxy group, forming a diol. The IR spectrum showed clearly that there was more than one hydroxy group present. The weak absorption bands of the epoxy group (830 and 845 cm^{-1}) had disappeared. The epoxy group was converted into two hydroxy groups by boiling for 2 hr with acetic acid, followed by hydrolysis and esterification with diazomethane (22). The resulting product had the same R_f value as L_4, viz. 0.14 (system I). The IR spectrum was identical to that of L_4.

From these results it appears that L_3 comprises a hydroxy and an epoxy group and a *trans* double

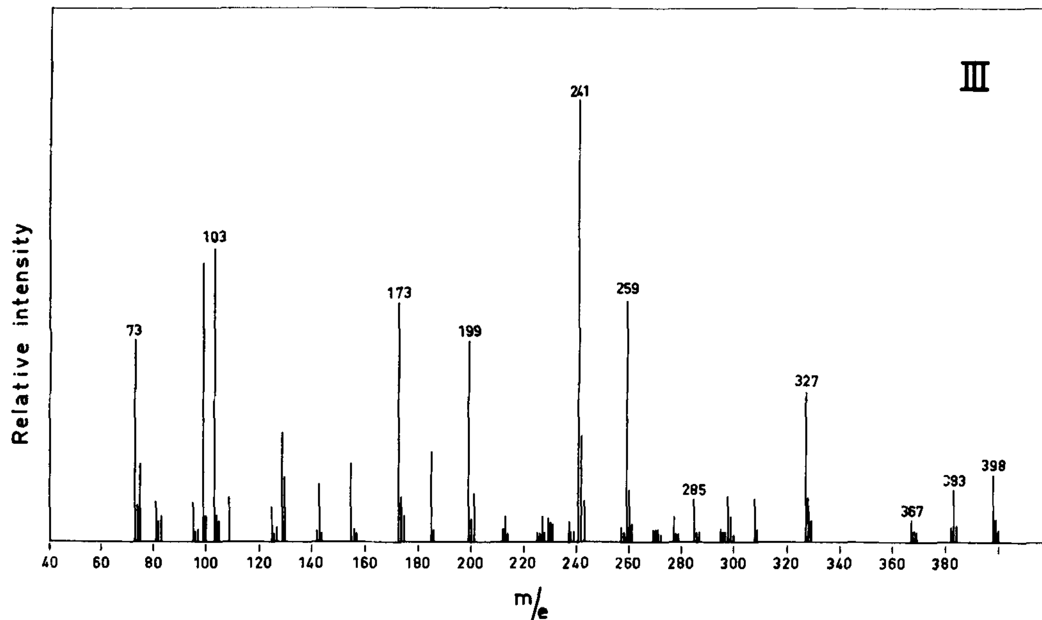
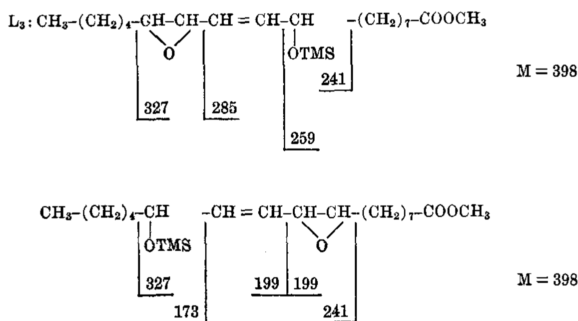


FIG. 8. Mass spectrum of a mixture of two isomers L_3, after methylation and silylation.

bond. The locations of these functional groups can be determined by reference to the mass spectrum. The mass spectrum (III) of a mixture of two isomers L_3 shows a clear parent peak (m/e 398). Peaks 383 (p-15) and 367 (p-31) are also present. Peaks 327, 259, 241, 199 and 173 correspond with the fragments represented in the formulae below.



L_4: The IR spectrum of the methylated compound points to a polyhydroxycarboxylic acid which moreover contains an unsaturated (*trans* 968 cm^{-1}) double bond. There are no absorption bands in the UV spectrum. The NMR spectrum of the methylated compound is made up as follows. A triplet at 9.1 τ(3H; CH_3–CH_2–), a broad peak with top at 8.7 τ(20H; –$(CH_2)_n$–), a multiplet between 7.6 and 8.0 τ(2H; CH_2–$COOCH_3$) a broad peak between 5.8 and 6.3 τ(2H; H–C–OH) and a broad peak between 6.4–6.8 τ(1H; H–COH), a singlet at 6.4 τ(3H; HO–C) and a singlet at 6.3 τ(3H; OCH_3), a multiplet with top at 4.3 τ(2H; HC = CH). This spectrum was photographed in $CDCl_3$ at 25 C. Of this substance also an NMR spectrum was made in $CDCl_3$ at 45 C. The only change observed was a shift of the OH peak from 6.4 τ to 7.2 τ. These two spectra show clearly that L_4 comprises three OH groups and a double bond.

With the oxidative decomposition it was demon-

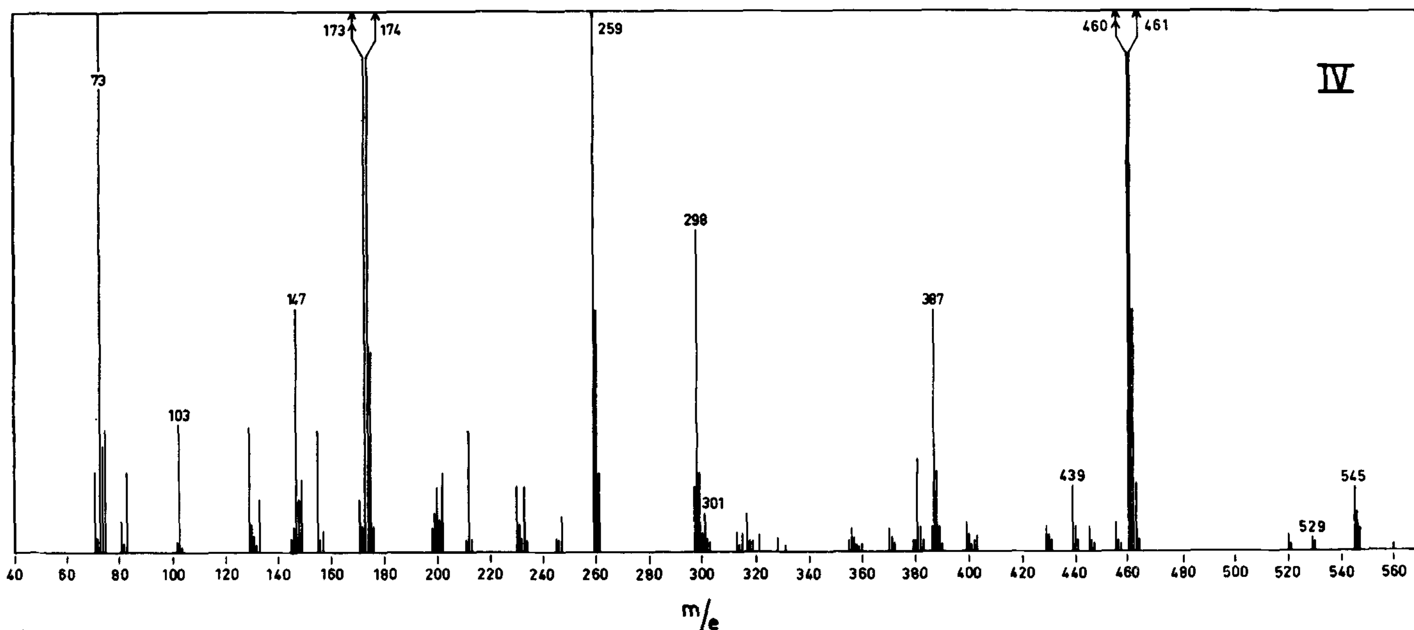


FIG. 9. Mass spectrum of a mixture of two isomers L_4, after methylation and silylation.

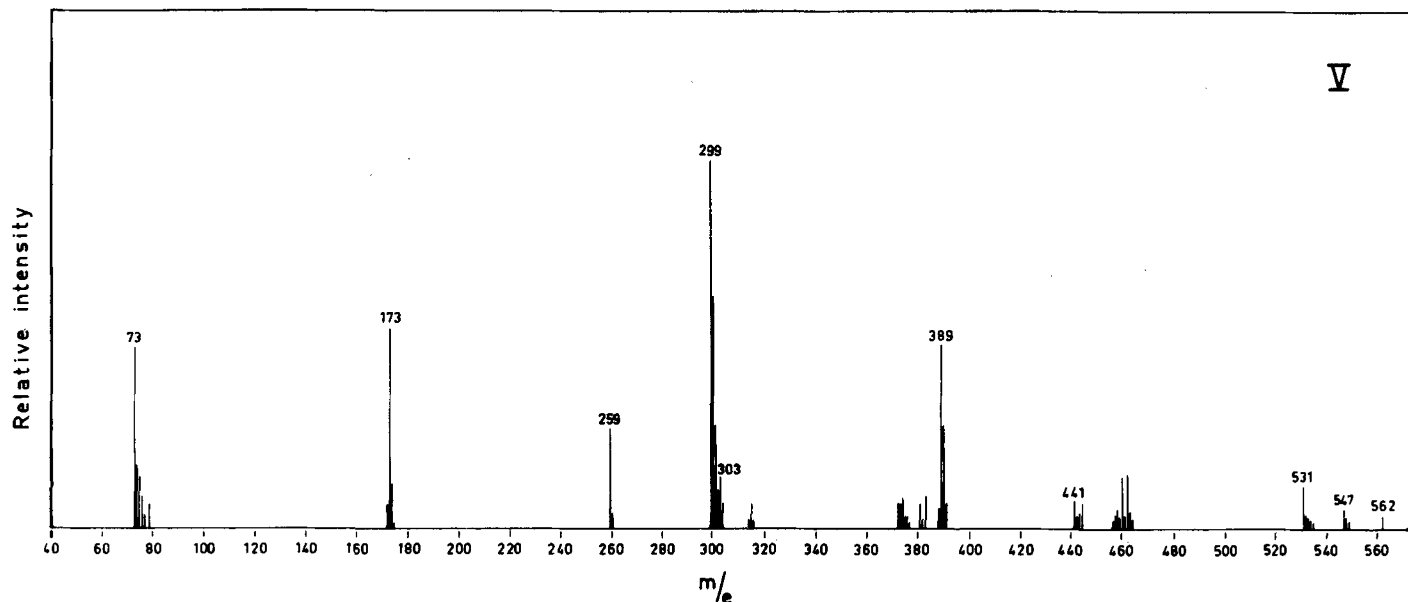


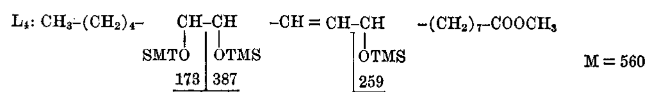
FIG. 10. Mass spectrum of a mixture of two isomers L_4, after methylation, hydrogenation and silylation.

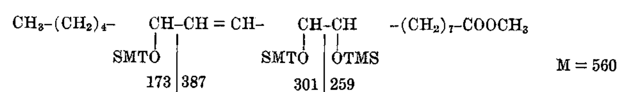
strated that L_4 contains two vicinal hydroxy groups and also consists of two isomers. After the periodate oxidation of L_4 (methylated and hydrogenated) the products formed were converted to DNPHs. The latter were separated on TLC with benzene as solvent. A separation into four components with R_f values 0.13, 0.26, 0.33 and 0.54 was obtained.

With the aid of the system of Badings and Wassink (23) and with the DNPH of hexanal as reference substance the component with R_f 0.54 was proved to be hexanal. Of this component an IR spectrum was also made; it appeared to be identical to the spectrum of hexanal. With a silica gel plate and a mixture of ethyl acetate and ether as solvent (24), as well as with the DNPH of 4-hydroxy-nonanal the component with R_f 0.26 was proved to be 4-hydroxy-nonanal. The IR spectra of the component and the reference substance were identical. The IR spectrum of the component with R_f 0.13 indicates the presence of a methyl ester (1730 and 1174 cm^{-1}) and a hydroxy group (3620 cm^{-1}). In all probability, this substance is methyl-9-hydroxy, 12-aldododecanoate. The IR spectrum of the component with R_f 0.33 indicates the presence of a methyl ester (1730 and 1174 cm^{-1}) but no hydroxy group. It is very probable that the substance is methyl-9-aldononanoate.

It was determined gravimetrically and spectrophotometrically that the hexanal to 4-hydroxy-nonanal ratio is roughly 85:15. From these results it appears that L_4 consists of two isomers and that each isomer contains three hydroxy groups, of which two are vicinal. The locations of these hydroxy groups were determined by mass spectrometry.

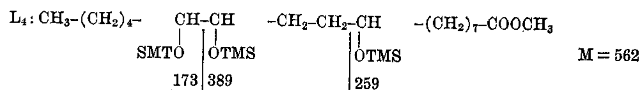
In the mass spectrum (IV) of a mixture of two isomers L_4 (methylated, silylated) the parent peak 560 (elem. form. $C_{28}H_{60}C_5Si_3$) is very small. The characteristic TMS peaks 73, 75, 103 and 147 are present. Further striking features are 545 (p-15); 529 (p-31); 439 (529–$(CH_3)_3$ SiOH); 297 (387–$(CH_3)_3$ SiOH) and 460 (p-100: $C_{22}H_{48}O_4Si_3$ = p-hexenol (19)). This large peak is indicative of a TMS group on the 13 position. Peaks 173, 387, 259 and 301 correspond with the fragments represented in the formulae below.

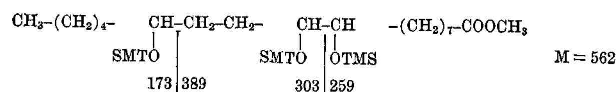




If we may conclude from the publication by Capella and Zorzut (19) that in this case, too, the molecule will break preferably between two adjoining C atoms bearing a TMS, then the two structures can be derived from this spectrum.

A mass spectrum (V) was also made of L_4 (methylated, silylated, hydrogenated). Again, there was a very small peak at 562 (elem. form. $C_{28}H_{62}O_5Si_3$). Peaks 73 and 75 are present, but not peaks 103 and 147. Also present are 547 (p-15) and 531 (p-31) again. Peak 462 (p-100) and 441 (531–$(CH_3)_3SiOH$) and 299 (389–$(CH_3)_3SiOH$) are visible in the spectrum. The small peak at 460 shows that a small amount of nonhydrogenated L_4 is present. Other important peaks are: 389, 303, 259 and 173. These peaks can be derived from the formulae below.





G_1 and G_2: G_1 reduced with $NaBH_4$ is transformed into G_2. The UV spectrum shows strong absorption at 234 nm of both compounds, i.e., a conjugated diene bond. After staining the TLC plate with a 5% KI solution in water and then with a 4% starch solution, band G_1 turned blue, which points to the presence of a hydroperoxy group. The IR spectrum of G_1 indicates the presence of an ester bond, a *cis,trans* conjugated diene bond (3030, 982 and 950 cm^{-1}) and a hydroperoxy group (3540 cm^{-1}).

The IR spectrum of G_2 also suggests the presence of an ester bond, a *cis,trans* conjugated diene bond (3030, 982 and 950 cm^{-1}) and a free hydroxy group (3600 cm^{-1}).

It is very probable that G_1 is derived from glycerol-1-monolinoleate by peroxidation, resulting in a hydroperoxy group in position 9 or 13, and G_2 the same compound as G_1, with a hydroxy group instead of a hydroperoxy group.

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