Autoxidative Rates of Nonmethylene-Interrupted Polyenoic Fatty Acids

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Autoxidative rates of free fatty acids and methyl esters increased in the following order: oleic (c9-18:1), c5, c9-18:2, linoleic (c9,c12-18:2), c5,c9,c12-18:3, t5,c9,c12-18:3, and linolenic (c9,c12,c15-18:3). The increase of the rates due to the Δ5-olefinic bond was much lower than that due to an olefinic bond extension in a methylene-interrupted sequence. Agreement of the oxidative rates obtained from conjugated diene content with those obtained from POV showed that oxygen attack did not occur at the isolated olefinic bond, but at the methylene-interrupted diene in the oxidation of 5,9,12-18:3. The Δ5-olefinic bond may promote the oxidation of the methylene-interrupted diene intramolecularly.

There have been few papers on the autoxidation of pure polyenoic fatty acids having nonmethylene-interrupted olefinic bonds (NMIP acids). In contrast, a number of papers have been presented on the autoxidation of polyenoic fatty acids in which all olefinic bonds have a methylene-interrupted arrangement. High proportions of Δ5-olefinic NMIP fatty acids in the fatty acids from some seed lipids have been studied in our laboratory (1-3). This enabled us to prepare pure NMIP acids so as to investigate the autoxidation of Δ5-olefinic NMIP acids. The wide distribution of NMIP acids in plant and animal lipids (1-8) and the presence of the NMIP acids in partially hydrogenated oils (9,10) also have been reported.

In this study, autoxidative rates of Δ5-olefinic NMIP acids, c5,c9-18:2 acid, c5,c9,c12- and t5,c9,c12-18:3 acids, and their methyl esters were compared with those of oleic, linoleic and linolenic acids. Information on autoxidation of the NMIP fatty acids reported in this paper will be useful in further biochemical and technical investigations of these NMIP fatty acids.

MATERIALS AND METHODS

Preparation of pure fatty acids. Raw materials for preparation of pure fatty acids were olive oil for c9-18:1 acid, safflower oil for c9,c12-18:2 acid, linseed oil for c9,c12,c15-18:3 acid, ether extracts from *Taxius cuspidata* seeds for c5,c9-18:2 acid (1,2), *Pinus koraiensis* seeds for c5,c9,c12-18:3 acid (2), and *Aquilegia akitensis* seeds for t5,c9,c12-18:3 acid (3). The mixed methyl esters obtained from fatty oils by transesterification with sodium methoxide-methanol solution were fractionated by $AgNO_3$-silicic acid column chromatography (11). Each purified methyl ester was saponified, and the saponified solution was extracted repeatedly with ether to remove the unsaponifiables such as tocopherols. The solution was acidified with 1N HCl, and free fatty acids (FFA) were extracted with ether. The recovered FFA were refined just before use by silicic acid column chromatography (Kieselgel 60, 30 × 2.5 cm; Merck, Darmstadt, West Germany) with ether/hexane (1:1, v/v) for elution. Pure FFA samples

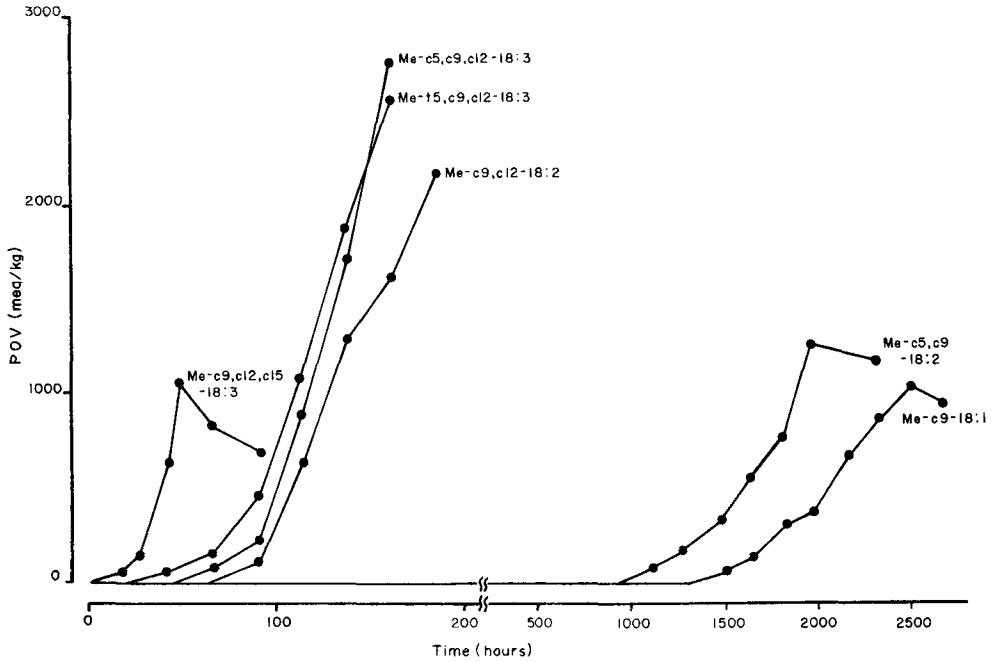

FIG. 1. Changes in POV of methyl esters during autoxidation at 50 C.

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were converted to methyl esters by heating in a sealed tube at 100 C for 10 min with 7% BF_3-methanol. Methyl esters were purified just before use by silicic acid column chromatography (Kieselgel 60, 30 × 2.5 cm, Merck, Darmstadt, West Germany) with ether hexane (5:95, v/v) for elution.

Each pure FFA and its methyl ester gave only a single spot on TLC, and showed purities of over 99% in GLC.

Oxidation of unsaturated acids and their esters. One gram of FFA or its methyl ester in a flat-bottomed glass tube (capacity 30 ml and bottom diameter 3 cm i.d.) was autoxidized by incubation in the dark at 40 or 50 C. The samples for determination of peroxide values (POV), conjugated diene contents and unoxidized substrate contents were taken from the oxidized samples at certain time intervals throughout the oxidation period. Determination of POV was carried out by the colorimetric iodine method (12) which incorporates ca. 50 mg of the sample. The conjugated diene contents were determined from the UV absorption data according to the AOCS official method (13). Samples dissolved in methanol were evaluated with a Hitachi 124 spectrophotometer (Hitachi Seisakusho Co., Tokyo, Japan). Unoxidized substrate contents in the oxidized products were analyzed by GLC. Methyl palmitate served as an internal standard. After the addition of methyl palmitate, the methyl ester samples were subjected to silicic acid column chromatography (Kieselgel 60, 10 × 2 cm, Merck, Darmstadt, West

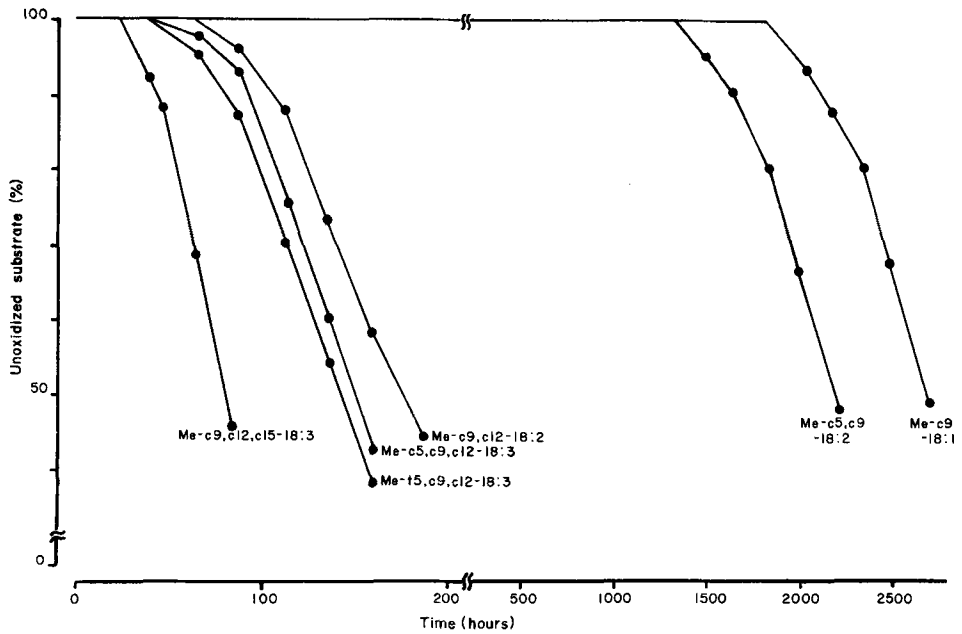

FIG. 2. Changes in the amount of unoxidized substrate during autoxidation of methyl esters at 50 C. The values are represented as the percentage of starting materials.

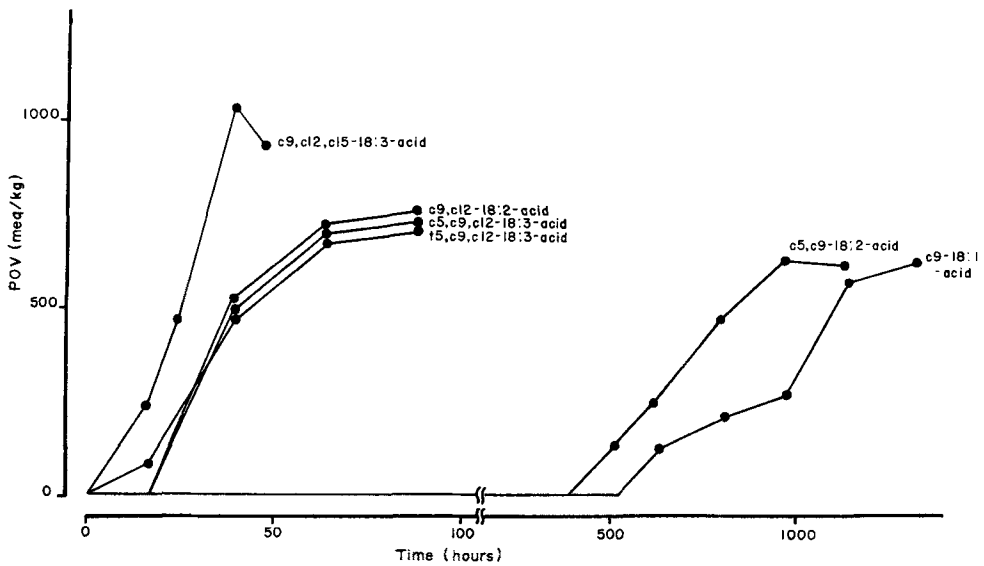

FIG. 3. Changes in POV of FFA during autoxidation at 50 C.

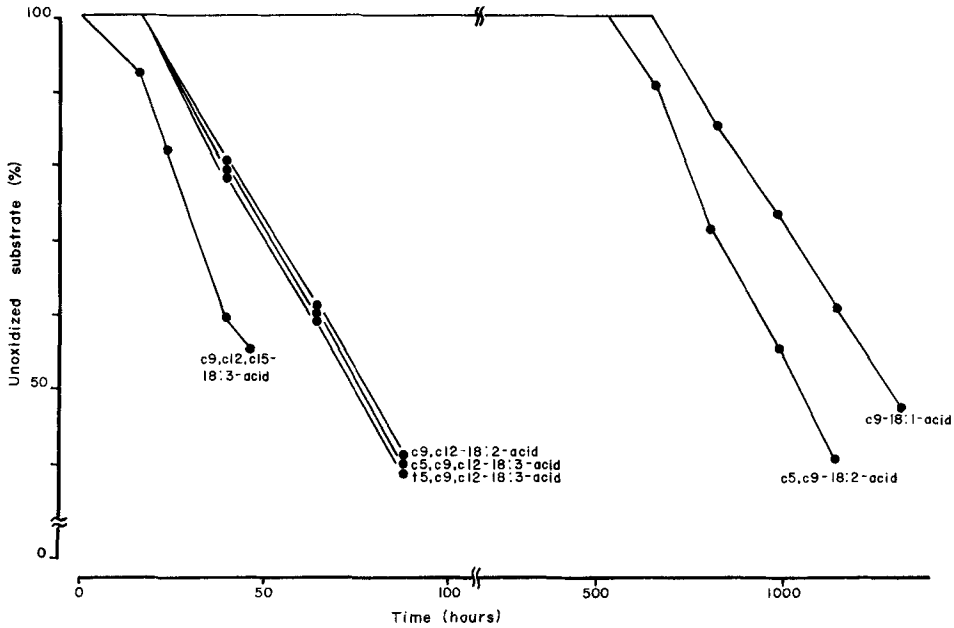

FIG. 4. Changes in the amount of unoxidized substrate during autoxidation of FFA at 50 C. The values are represented as the percentage of starting materials.

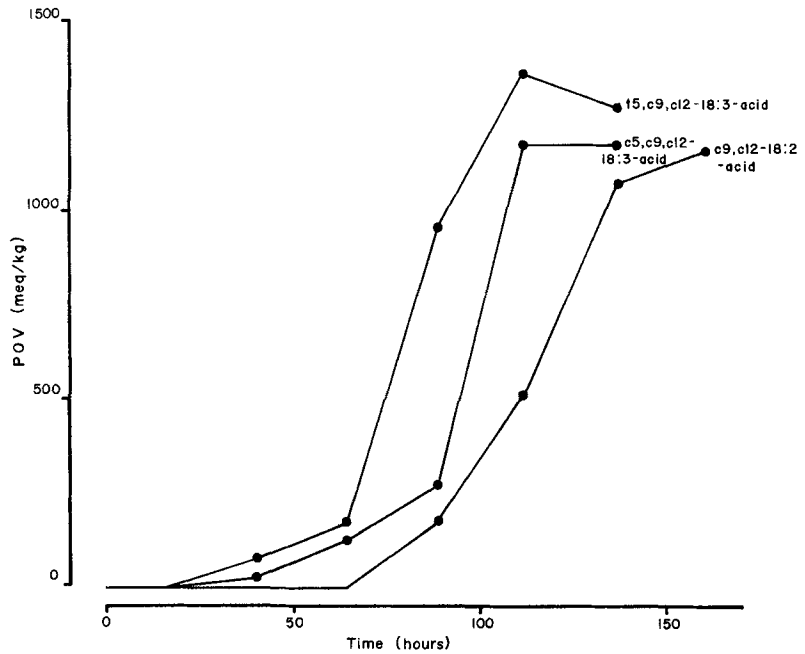

FIG. 5. Changes in POV of c9,c12-18:2, c5,c9,c12-18:3 and t5,c9,c12-18:3 acids during autoxidation at 40 C.

Germany) with ether/hexane eluents to remove the oxidized products. The unoxidized ester samples that were eluted with hexane/ether (5:95, v/v) were analyzed by GLC. The unoxidized substrate contents were calculated from the peak area ratios of the substrate methyl ester to the internal standard. The FFA samples were converted to methyl esters with 7% BF_3-methanol after addition of methyl palmitate, and then subjected to silicic acid column chromatography and to GLC. GLC was carried out with a Shimadzu GC-6AM instrument equipped with flame ionization detectors (FID) and an integrator Shimadzu E1A (Shimadzu Seisakusho Co., Kyoto, Japan) by using a 1.5-m × 3-mm i.d. glass column packed with 10% DEGS on 80/100 mesh Chromosorb W at column temperature 170 C. The detector and injector were held at 230 C. The flow rate of the nitrogen carrier gas was 30 ml/min.

Oxidation of mixtures of FFA or methyl esters. A mixture of 100 mg each of c9-18:1, c5,c9-18:2, c9,c12-18:2, c5,c9,c12-18:3, t5,c9,c12-18:3, and c9,c12,c15-18:3 acids or their methyl esters was autoxi-

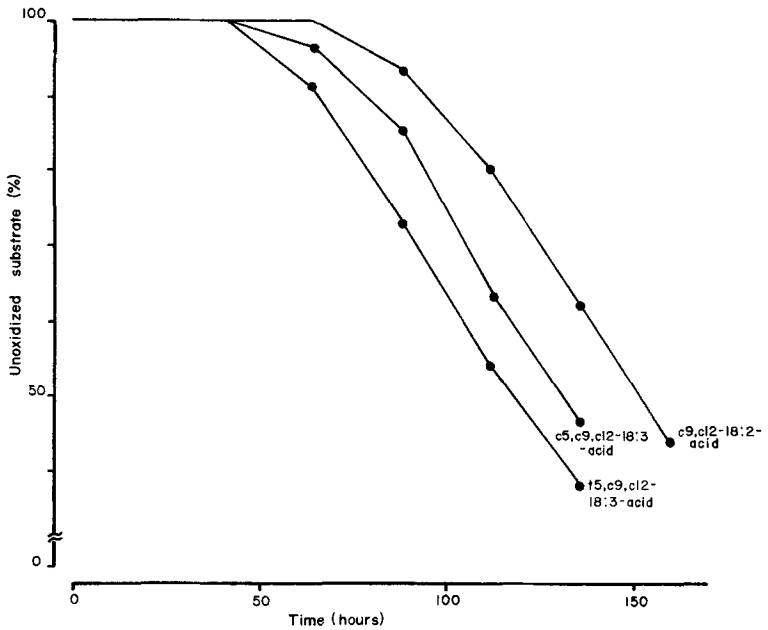

FIG. 6. Changes in the amount of unoxidized substrate during autoxidation of c9,c12-18:2, c5,c9,c12-18:3 and t5,c9,c12-18:3 acids at 40 C. The values are represented as the percentage of starting materials.

TABLE 1

Comparison of Oxidative Rates of FFA and Their Methyl Esters During Autoxidation at 50 C

	Iodimetric analysis[a] (hr)		Unoxidized substrate contents determination[b] (hr)	
	FFA	Methyl esters	FFA	Methyl esters
c9-18:1	610	1570	700	1860
c5,c9-18:2	480	1200	560	1440
c9,c12-18:2	21	83	22	91
c5,c9,c12-18:3	21	71	22	77
t5,c9,c12-18:3	17	55	22	64
c9,c12,c15-18:3	7	21	10	34

[a]Time to gain 100 meq/kg of POV.
[b]Time for unoxidized substrate to decrease by 5%.

dized at 40 or 50 C in the flat-bottomed glass tubes. The autoxidative rate of each FFA or its methyl ester was estimated by calculating the decrease of the unoxidized substrate content, which was determined by using GLC. The pretreatment of oxidized samples for GLC was carried out in the same way as described above. The unoxidized substrate content was calculated from the peak ratio of each methyl ester to an internal standard. GLC was carried out with a Shimadzu GC-7A equipped with dual FID detectors by using a wall-coated open-tubular (wcot) column coated with SP-2340 (Supelco Inc., Bellefonte, Pennsylvania) (50 m × 0.3 mm i.d.). The flow rate of the hydrogen carrier gas was 0.5 ml/min. The splitting ratio was 1/100. The column temperature was 170 C. The detector and injector temperatures were held at 230 C.

RESULTS AND DISCUSSION

Oxidative rates of each single substrate. The changes in POV and unoxidized substrate contents of FFA and their methyl esters during autoxidation at 50 C are shown in Figures 1-4. The oxidative rates of the methyl esters increased at the early stage in the following order: c9-18:1; c5,c9-18:2; c9,c12-18:2; c5,c9,c12-18:3; t5,c9,c12-18:3, and c9,c12,c15-18:3, as shown in Figures 1 and 2. Those of the FFA increased almost in the same order, but differences in rates among c9,c12-18:2 acid, c5,c9,c12-18:3 acid, and t5,c9,c12-18:3 acid were much smaller in the oxidation at 50 C, as shown in Figures 3 and 4. To clarify the order, oxidative rates of these FFA were compared at a lower temperature (Figs. 5 and 6). We suggest that the differences in oxidative rates of

these three FFA could be made clear by changing the incubation temperature, because low temperature slows down the progress of oxidation. The autoxidative rates of three fatty acids at 40 C increased in the following order: c9,c12-18:2, c5,c9,c12- and t5,c9,c12-18:3 acids. This was the same order as that described in the oxidation of the methyl esters at 50 C (Figs. 1 and 2). Tables 1 and 2 show the oxidative rates at both temperatures which are indicated as the time in hr to gain 100 meq/kg POV and 5% decrease of unoxidized substrate contents. The oxidative rate of the FFA was greater than that of its corresponding methyl esters in all six cases. These results are in agreement with those reported in a previous paper (14). The data in Tables 1 and 2 also show that the rates generally increased with increasing numbers of olefinic bonds, and that an increase in the rates due to an isolated Δ5-olefinic bond was much lower than that due to an olefinic bond extension in a methylene-interrupted sequence. Additionally, it is noteworthy that t5,c9,c12-18:3 was oxidized more quickly than its *cis* isomer, c5,c9,c12-18:3, both as the methyl esters and as FFA.

Oxidative rates of each substrate as mixtures. It is possible that the individual FFA and their methyl esters that were used for oxidative rate determinations contained antioxidants or oxidation promoters as minor components. To compare the oxidative rates under completely uniform conditions, FFA or their methyl esters were mixed in nearly equal ratios. The mixtures were oxidized and analyzed by GLC for unoxidized substrate contents of each component by using the procedures described above. The results are shown in Figures 7 and 8. The orders obtained agreed with those described in the separate oxidation of each single acid. FFA and methyl esters of c9,c12,c15-18:3, t5,c9,c12-18:3, c5,c9,c12-18:3, and c9,c12-18:2 oxidized more slowly as mixtures than as a single substrate. On the contrary, FFA or methyl esters of c5,c9-18:2 and c9-18:1 oxidized more quickly as the mixtures than as a single substrate. Retardation in the oxidation of the polyenoic components as the mixtures is attributed to the dilution effect with c9-18:1 and c5,c9-18:2, which are less susceptible to autoxidation. On the other hand, acceleration in the oxidation of c9-18:1 and c5,c9-18:2

TABLE 2

Comparison of Oxidative Rates of FFA of c9,c12-18:2; c5,c9,c12-18:3 and t5,c9,c12-18:3 During Autoxidation at 40 C

	Iodimetric analysis[a] (hr)	Unoxidized substrate contents determination[b] (hr)
c9,c12-18:2	78	81
c5,c9,c12-18:3	64	67
t5,c9,c12-18:3	47	54

[a]Time to gain 100 meq/kg of POV.
[b]Time for unoxidized substrate to decrease by 5%.

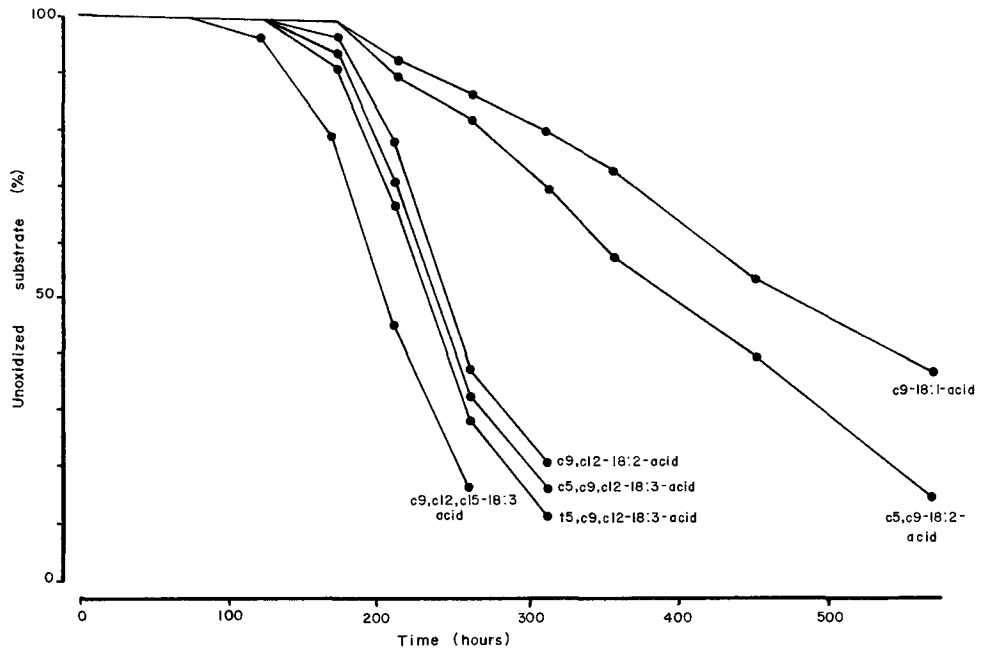

FIG. 7. Changes in the amount of unoxidized substrate during autoxidation of FFA mixtures at 40 C.

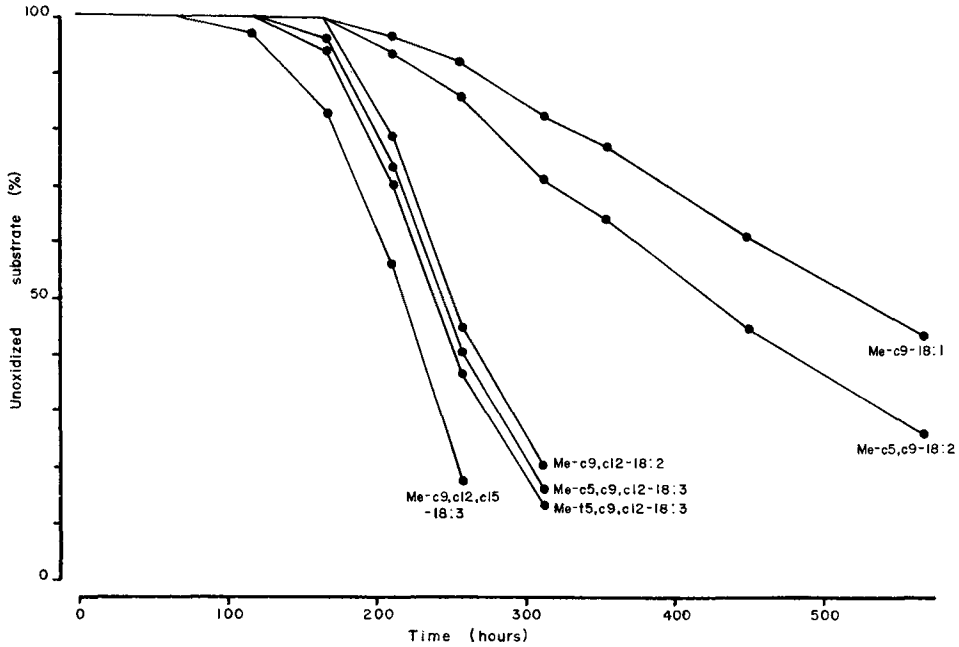

FIG. 8. Changes in the amount of unoxidized substrate during autoxidation of methyl ester mixtures at 50 C.

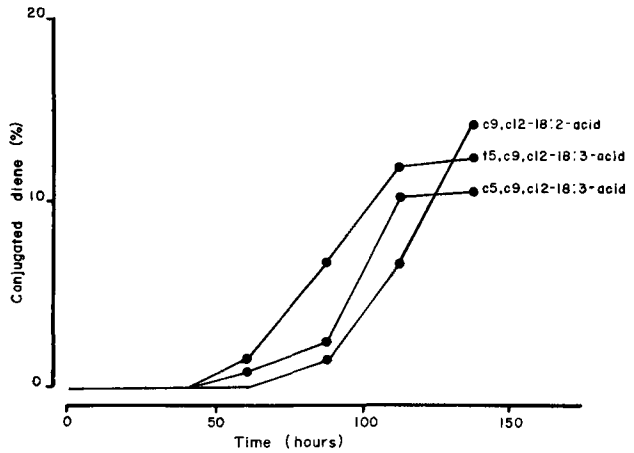

FIG. 9. Changes in the content of conjugated diene during autoxidation of c9,c12-18:2, c5,c9,c12-18:3 and t5,c9,c12-18:3 acids at 40 C. The values are represented as the percentage of starting materials.

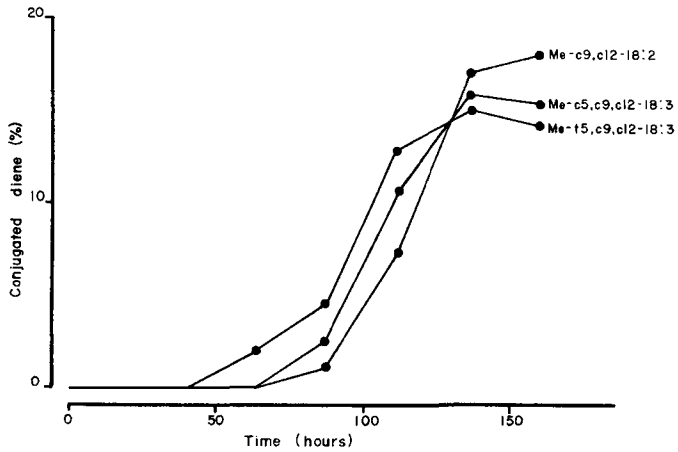

FIG. 10. Changes in the content of conjugated diene during autoxidation of Me-c9,c12-18:2, Me-c5,c9,c12-18:3 and Me-t5,c9,c12-18:3 at 50 C.

acids and methyl esters as mixtures probably is due to the oxidation promotive effect of free radicals formed in the autoxidation of the other substrates which are more susceptible to autoxidation. At any rate, the order of the oxidative rates among the FFA or methyl esters of the substrates was confirmed by the oxidation of the mixtures.

Specificity during oxidation. Figures 9 and 10 show an increase of the conjugated diene contents during autoxidation of t5,c9,c12-18:3, c5,c9,c12-18:3, and c9,c12-18:2 as FFA at 40 C and as methyl esters at 50 C. The order in the increase of the conjugated diene formation agrees with that of the increase of POV in the oxidation of c and t5,c9,c12-18:3 and c9,c12-18:2. The oxidative rates obtained by the iodimetric analysis of the hydroperoxide contents and by the UV analysis of the conjugated diene contents are shown comparatively in Table 3. The rates from the conjugated diene contents were obtained with the assumption that isomerization of a methylene-interrupted diene to conjugated diene leads simultaneously to formation of a hydroperoxy group. The agreement of the oxidative rates from the conjugated diene contents with those from the hydroperoxide contents indicated that oxidation of the NMIP fatty acids and their methyl esters essentially occurred only on the methylene-interrupted diene. The oxidative rates of t5,c9,c12-18:3 and c5,c9,c12-18:3 were higher than that of c9,c12-18:2 both in FFA and methyl esters. This is explainable by the promotive effect of the Δ5-olefinic bond to oxidation of the methylene-interrupted diene.

It is well known that the *cis*-olefinic bond oxidizes more quickly than the *trans*-olefinic bond in the oxidation of 9-18:1 (15,16). A similar relationship was expected in the oxidation of t5,c9,c12-18:3 and

TABLE 3

Comparison of Oxidative Rates of FFA and Their Methyl Esters Obtained by POV and Conjugated Diene Contents

	Iodimetric analysis[a] (hr)		Conjugated diene content[b] (hr)	
	FFA	Methyl esters	FFA	Methyl esters
c9,c12-18:2	98	98	102	103
c5,c9,c12-18:3	89	92	95	96
t5,c9,c12-18:3	67	80	73	87

[a]Time for unoxidized substrate contents calculated from POV to decrease by 5%.
[b]Time for unoxidized substrate contents calculated from conjugated diene contents to decrease by 5%.

c5,c9,c12-18:3, but t5,c9,c12-18:3 oxidized more quickly than c5,c9,c12-18:3. This also showed that oxidation of the Δ5-olefinic bond itself will not have an influence directly on the oxidative rates of 5,9,12-18:3, and that the oxidation promotive effect of a *trans*-5-olefinic bond will be higher than that of a *cis*-5-olefinic bond. The oxidation promotive effect of the Δ5-olefinic bond to the methylene-interrupted diene may be correlated with one or both of the following factors: (i) Acceleration of radical chain reactions by the molecular assisted homolysis (17) of a hydroperoxy group on the 9 or 12 carbon by the Δ5-olefinic bond, or (ii) stimulation of the hydrogen abstraction on the 11 carbon by a peroxy radical or oxygen with the influence of the Δ5-olefinic bond. Walkup reported the π-π interaction of the Δ5-olefinic bond with the Δ9-olefinic in the long chain 5,9-dienoic fatty acid (8). The hydrogen abstraction on the 11 carbon may be promoted by this interaction.

The NMIP acids are included as the acyl components in the polar lipids, in the neutral lipids, and in the biomembranes of some plants and animals such as gymnospermae (1,2), aquilegia (3), sea urchins (6) and clams (7). The fluidity of the membrane increased with an increasing degree of unsaturation of FFA in the membrane, but an increase in the number of double bonds from 3 to 4, 5 or 6 did not evoke a further increase in fluidity (18). Therefore, the NMIP acids containing three double bonds such as c5,c9,c12-18:3 and t5,c9,c12-18:3 improve the fluidity of the membrane by bending configuration similar to 20:4 (n-6) and 20:5 (n-3) acids. On the other hand, our present study showed that the oxidative rates of c5,c9,c12-18:3 and t5,c9,c12-18:3 were much lower than that of c9,c12,c15-18:3. Since the oxidative rates of lipids accelerated markedly with increases in their unsaturation (19), c9,c12,c15-18:3 and t5,c9,c12-18:3 will make the membrane more stable to autoxidation than 20:4 (n-6) and 20:5 (n-3) acids, while the accelerated effects of these NMIP acids on the membrane fluidity are the same as those of 20:4 (n-6) and 20:5 (n-3).

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