• TECHNICAL

The Initial Stages of Autoxidation[1,2]

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Abstract

The autoxidation of polyunsaturated lipids is initiated by a discrete reaction occurring prior to the formation of *stable* hydroperoxides. The products of the initiating reaction were detected by thin-layer chromatography and by ultraviolet spectral analysis. The reaction initiating autoxidation does not necessarily involve interactions with metals, although it is catalyzed by some heavy metals when these are present in sufficient concentrations. α-Tocopherol does not inhibit the initiation reaction, and in general, the stability of preparations of esters of polyunsaturated fatty acids is related to the content of prooxygenic substances resulting from this reaction. By taking special precautions to remove these prooxygenic substances, methyl esters containing as many as six double bonds were prepared having significant induction periods at 40C during which there was no measurable uptake of oxygen.

Introduction

IN SPITE of much research on the autoxidation of fats, the significance and the mechanism of the initiation of the primary chain reaction are still matters of speculation. Generally, this reaction is written simply as the "production of free radicals R· or RO_2·" or presented as a hypothetical equation to this effect (1,2,4,6).

When the hydroperoxide theory of autoxidation was postulated (11,12,13), it was proposed that the initiation of the chain reaction occurred either by a direct abstraction of a hydrogen atom from a methylene group adjacent to a double bond or by a two-step reaction involving addition of oxygen to the double bond followed by reaction of the intermediate with hydrogen on the adjacent carbon atom (7,13). Both reactions appeared to be equally possible thermodynamically (7). Bolland (8) offered an experimental basis for the theory of a discrete initiating reaction by showing that the extrapolation of the plot of the rate of autoxidation vs. the extent of the reaction did not intersect the origin, but indicated a positive rate at zero extent of autoxidation presumably due to the "initiation reaction." In an effort to define the mechanism of this reaction, Bateman and Morris (2) carried out extensive studies on the initiating efficiencies in the autoxidation of a large number of compounds with closely related structures. While these studies were valuable contributions, they did not permit the elucidation of the chain initiating reaction.

Later Bateman, Hughes, and Morris (3) in a more detailed study of the initial stages of the autoxidation of ethyl linoleate showed that an extrapolation of the plot of the reaction rate vs. the extent of the reaction as performed by Bolland (8) was invalid because of the difference in the mode of peroxide decomposition at low and high concentrations. They proposed that in the initial stages of autoxidation, i.e., at low peroxide concentration, there was little association of the hydroperoxides, and therefore, the decomposition was a first order process. Accordingly, the curve representing the relationship between reaction rate and extent of autoxidation approached zero; the curve did not appear to pass through the origin, however. Nevertheless, Bateman et al. (1,2,3) minimized the significance of this reaction as compared to the initiation of the chain reaction by hydroperoxide decomposition, indicating that only a single molecule need be involved, and emphasizing that product analysis would yield no information concerning it.

Uri (23,24,25), in more recent studies, could not visualize the probability of molecular oxygen reacting directly with a fat molecule on the basis of the thermodynamics of the reaction and he advanced the theory with Heaton (14) that the initial attack by molecular oxygen requires traces of heavy (M) metals as catalysts in a series of electron transfer reactions of the following general type:

$$M^n + O_2 \longrightarrow M^{(n+1)^+} + O_2^-$$

Uri and Heaton also proposed that initiation through the decomposition of hydroperoxides in the initial stages of autoxidation involved heavy metal catalysts (14,23,24,25).

We show, in the present study, that if special precautions are taken to remove prooxygenic substances, methyl esters containing as many as six double bonds can be prepared having significant induction periods at 40C during which there is no measurable uptake of oxygen. These preparations, especially methyl linoleate with induction periods up to 19 hr are ideally suited for studies on the initial stages of autoxidation.

Experimental

Preparation of methyl esters. The methyl esters used in this study (Table I) were prepared by conventional methods and every possible precaution was taken to protect them from contact with atmospheric oxygen during each step.

Methyl oleate and linoleate were prepared from commercial virgin olive and safflower oils, respectively. A combination of low-temp crystallization, urea fractionation, and fractional distillation was used in their preparation (15,18,22).

Methyl linolenate was prepared from crude linseed oil by the bromination-debromination procedure (17).

Methyl arachidonate, eicosapentaenoate, and docosahexaenoate were prepared by the general procedure described by Privett, Weber, and Nickell (19). The source of the arachidonate was fresh hog liver and eicosapentaenoate and docosahexaenoate commercial menhaden and tuna oils, respectively.

Just prior to the determination of the induction period, prooxygenic substances were removed by passing approx 3 g of each preparation through a column of silicic acid 3 x 25 cm. The main fractions from the column were crystallized twice as urea adducts from methanol, recovered in the usual manner, and distilled

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[2] Presented at the AOCS meeting in Chicago, 1961.

TABLE I

Induction Period[1] of Methyl Esters in Air in a Warburg Apparatus at 40 ± 0.1C

Methyl ester	I.V. (Wijs)	Absorptivity k_{233}	Purity (GLC) %	Induction period (hr)
Docosahexaenoate	421	.503	>90	0.67
Eicosapentaenoate	386	.590	>90	0.83
Arachidonate	315	.115	>99	1.0
Linolenate	259	.121	>99	1.34
Linoleate	172	.110	>99	19.0
Oleate	85	.060	>99	82.0

[1] The induction period was taken as that period during which there was no measurable uptake of oxygen.

alembically in an all-glass still under high vacuum, 10^{-2} mm Hg.

The iodine value of the final preparations were determined by the Wijs method. The ultraviolet absorption spectra were determined in a Beckman Model DU spectrophotometer, using freshly distilled and optically pure 95% ethanol as the solvent. Gas-liquid chromatographic analyses were performed with a 6′ x ¼″ packed column containing 15% ethylene glycol succinate polyester on Chromosorb W in an F & M Model 500 instrument equipped with a thermal conductivity detector. The temp of the column was 197C and helium was the carrier gas at a flow rate of 75 ml/min. Under these conditions, a standard mixture of methyl oleate-methyl stearate gave a separation factor of 1.16.

Induction period of polyunsaturated methyl esters. The induction of the methyl esters were determined on 1-g samples by means of a Warburg apparatus with 15-ml flasks at 40 ± 0.1C shaken at 120 oscillations/min through an amplitude of 2.5 cm under an atmosphere of air. Brodie's solution, sp gr 1.033, was used in the manometers. The flasks were equilibrated in the bath under an atmosphere of purified nitrogen for 30 min; then air was swept over the samples for 5 min. After a further equilibration of 10 min, the stopcocks were closed. Under these conditions, an oxygen uptake of 1.5 m.e./kg represented a differential of 1 cm on the manometers. Thus, an oxygen uptake of about 0.2 m.e./kg could be detected. The induction period was taken as that period in which there was no measurable uptake of oxygen. Table I shows the induction periods of the polyunsaturated methyl esters listed above.

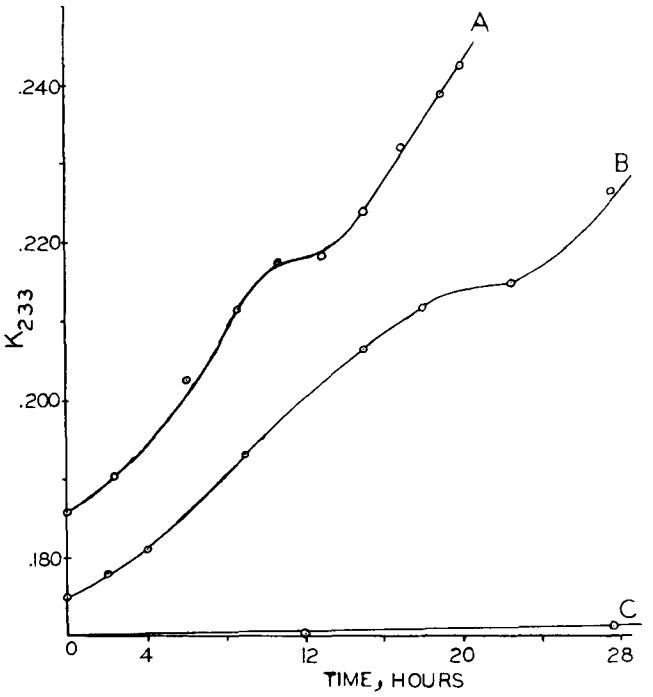

FIG. 1. UV spectral analyses. A. Methyl linoleate incubated in air in a Warburg apparatus at 40 ± 0.1C. B. Methyl linoleate (original induction period 16 hr) containing 0.01% added α-tocopherol incubated in air in a Warburg apparatus at 40 ± 0.1C. C. Methyl linoleate (original induction period 19 hr) incubated in a nitrogen atmosphere in a Warburg apparatus at 40 ± 0.1C.

There were no absorption maxima in the ultraviolet spectrum of these compounds, but since a maximum eventually developed at about 233 mμ with methyl linoleate on the formation of diene conjugated hydroperoxides, the measurements were made at this wavelength.

Before a standard method was developed for the removal of prooxygenic substances, there was considerable variation in the induction period from one preparation of the same ester to another. This was found to be related, in general, to the absorption of ultraviolet light and techniques which reduced the absorption of ultraviolet light effected an extension of the induction period.

UV absorption during and after the induction period. Since most of the fundamental studies on autoxidation have been carried out on methyl linoleate preparations of this ester were used in these studies.

During the induction period there was a general increase in the UV absorption as indicated by measurements at 233 mμ (Figure 1, curve A). The general shape of this curve was readily reproducible, but was displaced to higher or lower levels, depending on the UV absorption of the original sample. The plateau always occurred just prior to the end of the induction period.

Figure 1, curve B shows that α-tocopherol had no effect on the increase in UV absorption during a period corresponding to the induction period of the original sample. There was no measurable uptake of oxygen during this period, but soon thereafter, a continuous slow absorption of oxygen was observed in this sample.

Figure 1, curve C shows that in the absence of oxygen there was no increase in the UV absorption of the sample, indicating that a slow rate of oxidation occurred during the induction period in the presence of oxygen, although no uptake of oxygen could be measured manometrically.

At the end of the induction period, the UV absorption of the sample increased sharply with the formation of *stable* (conjugated diene) hydroperoxides. The increase in the extinction coefficient at 233 mμ was related directly to the increase in oxygen absorption when the measurements were made, beginning with the end of the induction period (Figure 2, curve A). This relationship had been observed previously by Lundberg and Chipault (16) and by Bolland and Koch (5). When the increase in UV absorption, taken from the beginning of the induction period, was calculated in terms of the extinction coefficient of the hydroperoxides, curve B (Figure 2) was obtained. Figure 3 shows the results obtained by calculating the UV absorption of the sample in terms of hydroperoxides with a molecular extinction of 29,000 compared to the experimental values calculated from oxygen uptake, i.e., curves A and B, respectively. These results showed that the extent of the UV absorption of autoxidized methyl linoleate was not simply due to the formation of hydroperoxides, but was the resultant of the entire autoxidation process.

Evidence for the formation of nonhydroperoxidic

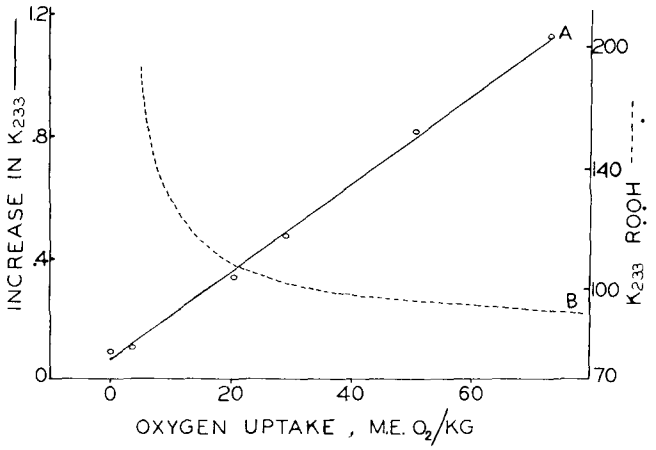

FIG. 2. Autoxidation of methyl linoleate. A. Relationship between oxygen uptake and increase in diene conjugation from the end of the induction period. B. Oxygen uptake expressed as monomeric conjugated diene hydroperoxides with a molecular extinction coefficient of 29,000.

compounds during the induction period. The foregoing results (Figures 1, 2, and 3) indicate that nonhydroperoxidic substances exhibiting UV absorption are formed during the induction period. Further evidence for the formation of such substances was obtained by thin-layer chromatographic analyses (TLC) of samples of "autoxidizing" methyl linoleate taken at intervals during and after the induction period.

Since the substances formed during the induction period were present in extremely small concentration, the solvents used in TLC had to be purified and freshly distilled. The absorbent, Silica Gel G, also had to be exhaustively extracted, first with $CHCl_3$, then with diethyl ether to remove traces of organic material.

Figure 4 shows thin-layer chromatoplates of methyl linoleate that had been exposed to air for various periods of time in a Warburg apparatus as described above. These analyses were carried out on 6-mg samples, using Silica Gel G as the absorbent and 10% diethyl ether in petroleum ether (b.p. 30–60C) as the solvent system. The spots were visualized by charring the organic material after spraying the plates with 50% aqueous sulfuric acid. The first sample, "zero time," was taken at the beginning of the induction period. The second sample, "11 hr," was taken in the middle of the induction period. The last sample, "33 hr," had a peroxide value of 6 m.e./kg. The hydroperoxides were readily detected in the latter sample as the spot having an R_f value of about 0.15 (Figure 4).

A small amount of highly polar material was detected in the 11-hr sample as well as in the 33-hr sample as a spot in the base line. This material was recovered from the plates and found to exhibit a broad absorption band in the conjugated diene absorbing region of the UV spectrum. It had a strong prooxidant action on being added back to fresh methyl linoleate.

In order to determine whether this polar material could account for all the increase in UV absorption observed during the induction period, another series of experiments was performed. Samples of up to 150 mg of methyl linoleate that had been exposed to air were applied in a row of spots on 20 x 20 cm plates and chromatographed in the same solvent system (10% diethyl ether in petroleum ether, 30–60C). Two bands of absorbent layer corresponding to A and B,

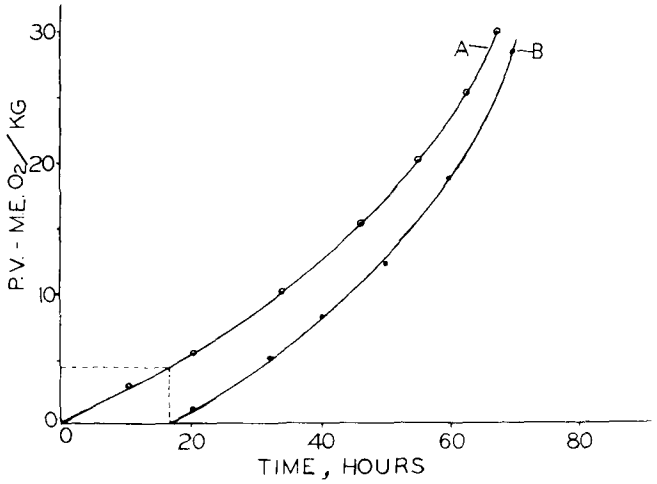

FIG. 3. Autoxidation of methyl linoleate. A. Hydroperoxides calculated on basis of increase in UV absorption. B. Experimentally determined values.

Figure 4 were scraped off the plates still wet with solvent and extracted with freshly distilled diethyl ether. The recovered materials were analyzed by TLC and UV absorption.

These analyses showed that in addition to the polar material on the base line of the chromatoplate, UV absorbing material was present in the trailing edge of the methyl ester spot (Zone A, Figure 4). TLC of this fraction (Figure 5) showed that it contained substances with greater polarity than the original methyl ester. The spot designated as "compound A" in Figure 5 represents the UV absorbing material in the trailing edge of the methyl ester spot. The other spots obviously arose from the breakdown of this material. A distinguishing feature between the substances in zone A and *stable* hydroperoxides is that *stable* diene conjugated hydroperoxides isolated from autoxidized methyl linoleate can be rechromatographed without any apparent decomposition.

Hydroperoxides could not be detected in the zero- and 11-hr samples of methyl linoleate by recovering the material from zone B and analyzing it in a similar manner. Thus, it was estimated that if any hydroperoxides were present in these samples, they were in concentrations of less than 0.001%.

Effect of hydroperoxides and heavy metals on the induction period of methyl linoleate. For studies on the effect of hydroperoxides, a concentrate was isolated by countercurrent extraction according to the method of Privett, Lundberg, and Nickell (20) from methyl linoleate autoxidized at 0C to a peroxide value

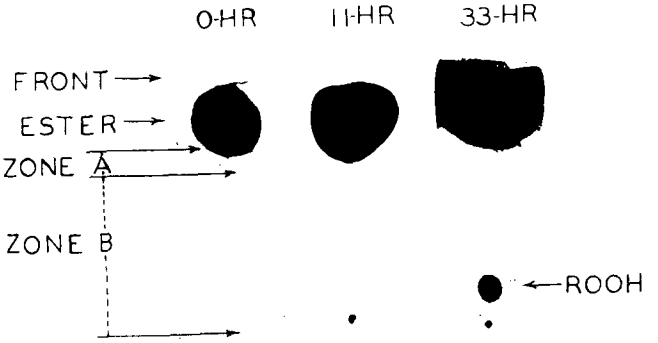

FIG. 4. TLC analysis of samples of methyl linoleate incubated in air in a Warburg apparatus at 40 ± 0.1C. A. Sample taken at the beginning of the induction period. B. Sample taken about the middle of the induction period, 11 hr. C. Sample taken beyond the end of the induction period, 33 hr exposure to air.

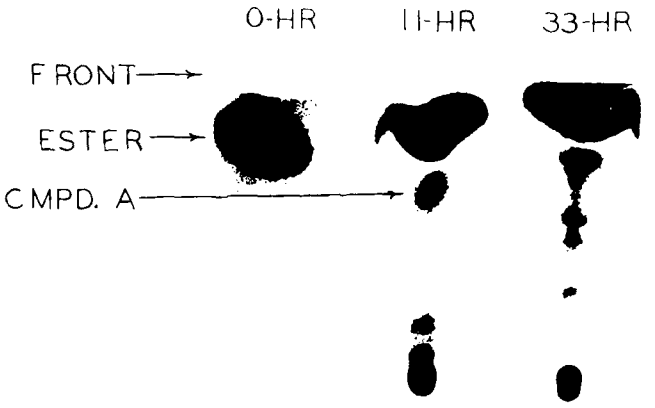

FIG. 5. TLC of the material recovered from the band designated as Zone A in Samples A, B, and C in Fig. 4.

of 600 m.e./kg. The product was about 90% pure conjugated diene methyl octadecadienoate hydroperoxide (21).

In these experiments, the hydroperoxide concentrate was dissolved in diethyl ether and added to the empty Warburg flasks first. Then the ether was evaporated in a stream of nitrogen, the sample of methyl linoleate added and equilibrated as described above. The effect of the hydroperoxide concentration on the rate of the initiation of autoxidation, which is expressed as the reciprocal of the induction period, is shown in Figure 6.

Similar experiments were carried out on the effect of metals on the induction period of methyl linoleate. Cuprous, cupric, ferric, ferrous, manganous, and cobaltous chlorides, dissolved in methanol, were added to the Warburg flasks. The solvent was removed in a stream of nitrogen, methyl linoleate was added, and the system equilibrated.

Cuprous, cupric, ferrous, and ferric chlorides in concentrations below 1 ppm had little effect on the initiation of autoxidation in samples of methyl linoleate having long induction periods (19 hr). Manganous chloride was much more active than these salts and cobaltous chloride was the most active. However, in concentrations below 25 p.p.b., cobalt chloride also had no effect on the induction period of methyl linoleate (Figure 7). Above this concentration, its activity was directly proportional to its concentration, as might be expected if its function were purely catalytic (9,10).

The concentrations of the metals used in these experiments were well above those present in the purified preparations of methyl linoleate. This was indicated by the determination of the cobalt and copper content of purified preparations of methyl linoleate reported by Uri (23,25) as well as by semiquantitative neutron activation analysis performed on samples of methyl linoleate used in this study. No iron or cobalt was detected in these samples and the presence of copper was debatable; a weak response was obtained for manganese. Since these analyses were carried out on 10 g of material, it was estimated on the basis of the sensitivity of the analysis for the individual metals that cobalt, if present, and manganese were in concentrations of less than 1 p.p.b. Since the test for the presence of copper was borderline, it also appeared to be present in concentrations of the order of only p.p.b. No iron could be detected in these preparations, but since the limit of detection for this metal is 25 μg, it would have to be present in a concentration of about 2.5 ppm to be found by activation analysis.

Since highly purified methyl linoleate is devoid of hydroperoxides, iron has little catalytic activity in these preparations because it functions as a catalyst for autoxidation primarily through the decomposition of hydroperoxides. In contrast, the catalytic action of cobalt does not appear to involve its direct reaction with hydroperoxides. The relative effect of cobalt and iron on the decomposition of hydroperoxides was demonstrated by an experiment in which these metals, in the form of their chlorides, were allowed to react with methyl linoleate hydroperoxide in air-free diethyl ether at room temp for 20 hr. The concentration of the methyl linoleate hydroperoxide was 1% and the metals 0.5%. In spite of the high ratio of metal to hydroperoxide, only the iron reacted with the hydroperoxides as shown by TLC analysis (Figure 8).

The detection of compounds prior to *stable* hydroperoxide formation in the autoxidation of methyl linoleate is highly significant because it requires a complete reevaluation of the general concept of the mechanism of the initiation of autoxidation. At present, the nature of these compounds is unknown, but studies on their structures and properties are in progress with the view of defining the mechanism of their formation and their role in the initiation of autoxidation.

These studies show that even if one assumes that immeasurably small amounts of *stable* hydroperoxides may be present in the purified preparations of methyl linoleate, their effect on the initiation of autoxidation would be insignificant.

Since it was necessary to add much larger amounts of iron, copper, and cobalt than were present in these preparations of methyl linoleate to obtain a significant shortening of the induction period, it was evident that metals were not required for the initiation of autoxidation. This conclusion was supported further by the observation that the activity of cobalt was related to the concentration of the substances formed prior to *stable* hydroperoxides. For example, the addition of 1 ppm of cobalt completely eliminated the induction period in samples of methyl linoleate which had an induction period of less than 9 hr. One may speculate that cobalt, like metals, may function primarily as catalysts for autoxidation through their relationship with the compounds formed prior to *stable* hydroperoxides just as iron functions primarily through its role in peroxide decomposition. Certainly

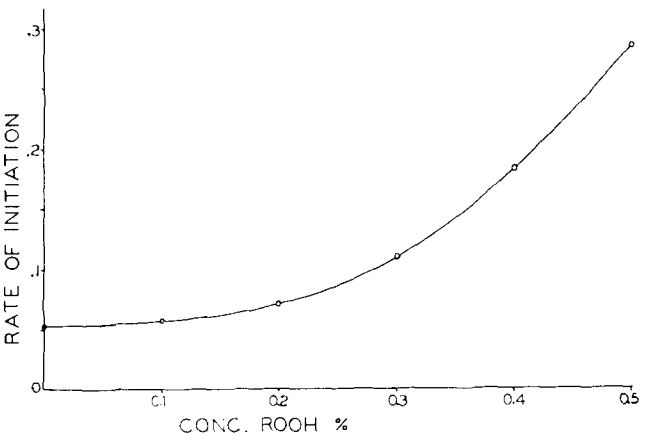

FIG. 6. Effect of added hydroperoxide on the rate of initiation of autoxidation of methyl linoleate with an induction period of 19 hr. The rates of initiation are expressed as the reciprocal of the induction periods which were determined in a Warburg apparatus at 40 ± 0.1C.

there is an inherent difference between the activity of these metals in catalyzing autoxidation and peroxide decomposition.

Although the findings reported here on the nature of the initiation reaction are directly opposed to the theories of Heaton and Uri (14), their studies actually pertain largely to the initiation of autoxidation through the decomposition of hydroperoxides. These investigators studied the effects on "observed rates" of autoxidation, whereas our studies involved observations taken during the induction period in which there is no measurable uptake of oxygen.

Although the initiation of autoxidation has been considered mostly of academic interest since the reactions detected here occur prior to the detection of hydroperoxides, are not inhibited by *a-tocopherol*, and result in the formation of extremely unstable compounds, they may have more than a little practical significance. For example, flavor reversion of soybean oil and development of incipient off-flavors in egg and milk powders are not inhibited by *a*-tocopherol or phenolic antioxidants in general, and are detected at the threshold levels of organoleptic tests; that is, at oxidative changes of the order of parts per billion.

The induction periods of hydroperoxide-free polyunsaturated fatty acid esters appear to be related largely to their content of prooxygenic substances, specifically the types of compounds formed prior to the detection of hydroperoxides. Since these compounds are unstable and form a variety of secondary products which can catalyze autoxidation, no single treatment is completely effective in removing all of them. Treatment with silicic acid removes the more polar compounds as well as hydroperoxides, but is obviously ineffective in removing the parent substances which have R_f values on TLC almost the same as that of the methyl ester. Since the application of both urea fractionation and distillation increases the stability of polyunsaturated methyl esters, they are particularly effective in removing these compounds. However, even the purest preparation often contains traces of highly polar substances, indicating that it is extremely difficult to eliminate these substances completely. As the technique for the removal of the prooxygenic substances formed prior to the detection of hydroperoxides is improved, longer induction periods than those reported here will undoubtedly be observed. Nevertheless, preparations of polyunsaturated fatty acids of high stability may be obtained by conventional procedures by using the following guides:

1. The original oil or fat should be as fresh as possible.
2. All operations should be carried out so as to avoid contact of the product with atmospheric oxygen at all times.
3. The entire operation should be carried out as quickly as possible.
4. Steps should be incorporated toward the end of the procedure, as described herein, to remove in so far as possible prooxygenic substances.

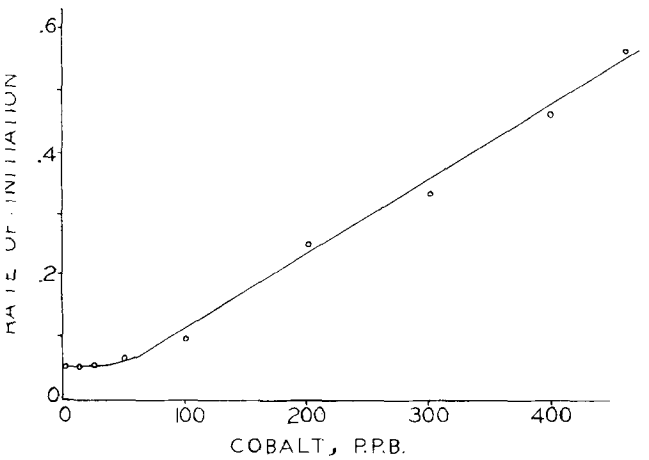

FIG. 7. Effect of added cobalt (as cobaltous chloride) on the rate of initiation of the autoxidation of methyl linoleate with an induction period of 19 hr. The rates of initiation are expressed as the reciprocal of the induction periods which were determined in a Warburg apparatus at 40 ± 0.1C in air.

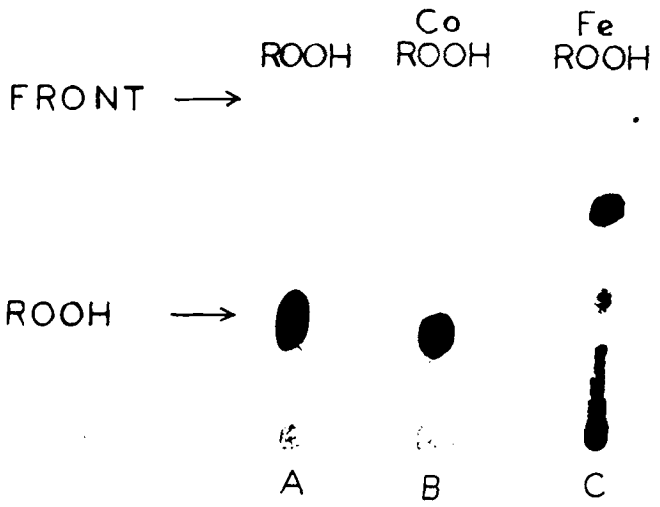

FIG. 8. TLC analysis of the products of the reaction of methyl linoleate hydroperoxides (1% concentration) with cobalt and iron chloride (0.5% concentration) in diethyl ether. A, Hydroperoxide; B, Hydroperoxide plus cobalt chloride; C, Hydroperoxide plus ferric chloride.

Acknowledgments

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