# Interaction of Tocored With Unsaturated Fatty Esters

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## Abstract

Tocored, an oxidation product of tocopherol and its model, 2,2,7,8-tetramethyl-5,6-chromanquinone reacted with methyl linoleate at an elevated temperature to form an adduct with isomerized linoleate. Oxidation of tocored in fatty esters produced a variety of products, depending on the degree of unsaturation of the esters. With methyl palmitate no addition product was detected but tocopurple and tocored-dimer were identified as minor products irrespective of the degree of saturation. A mechanism involving the interaction of tocored with lipid radicals to yield an adduct as the consequence of antioxidative action of tocored is postulated.

## Introduction

In an earlier paper (1) dealing with color reversion (coloration during storage) of edible soybean oil, we reported that tocored was isolated and identified as a precursor of color reversion and we assumed that the tocored in crude soybean oil is not removed by conventional refining procedures. Some of it remains in colorless form in deodorized soybean oil and is again turned into tocored, giving a red color to the oil, by atmospheric oxidation. Although the tocored-like substance recoverable from color reverted soybean oil by chromatography cannot be distinguished by means of its spectro-absorption from that synthesized from tocopherol with nitric acid oxidation, the compound is far paler than tocored and its UV absorption maximum is shifted to a somewhat shorter wave length.

Continuing research on the fate of tocopherol in autoxidizing soybean oil (2), this paper describes products from tocored and its model, 2,2,7,8-tetramethyl-5,6-chromanquinone, in purified methyl esters. The identification of several products from tocored and its model compound is also reported, together with some possible mechanisms in their formation.

## Materials and Methods

### Methyl Oleate

Methyl esters of olive oil were prepared in the usual manner and fractionally distilled, twice, in an all glass apparatus at 2 mmHg pressure.

### Methyl Palmitate

Commercial palmitic acid was esterified by refluxing for 1 hr with methanol containing 2% of concentrated sulfuric acid and fractionally distilled three times at 2 mmHg pressure. The final product shows 97% palmitate by GLC on a packed, DEGS column.

### Methyl Linoleate

Methyl linoleate was prepared by urea fractionation of safflower oil methyl esters according to the procedure of Keppler et al. (3). The final product contained more than 99% methyl octadecadienoate, as determined by GLC.

### Alkali Isomerized Methyl Linoleate

The method of Nichols et al. (4) was applied to the isomerization of methyl linoleate. Thirty grams of methyl linoleate were slowly added to 30 g of diethylene glycol and 4.5 g of sodium hydroxide, which had been heated for 15 min at 180 C. Heating at 180 C was continued for 30 min, during which time a slow stream of nitrogen was passed through the reaction mixture. Isomerized acids were isolated by dissolving the reaction mixture in two parts of water, acidifying it with 6 N sulfuric acid, then extracting it with ethyl ether. Conversion to the methyl ester was achieved by a 1 hr reflux with 100 ml of methanol containing 2 ml of concentrated sulfuric acid. Methyl esters were distilled at 2 mmHg pressure.

### Soybean Oil Fatty Acids Methyl Ester (SOFAME)

Methyl esters were prepared from molecular distilled soybean oil and distilled in vacuo.

Analytical values for these esters are summarized in Table I.

### 2,2,5,7,8-Pentamethyl-6-Chromanol (Ib, α-Tocopherol Model)

Trimethylhydroquinone was treated with isoprene under acidic conditions using fused zinc chloride as the catalyst, according to Smith et al. (5). The crude chromanol was purified by recrystallizing it three times from normal hexane.

### 2,2,7,8-Tetramethyl-5,6-Chromanquinone (IIb, Tocored Model)

Two grams of Ib were dissolved in 500 ml of ethanol and 100 ml of concentrated nitric acid added. This mixture was refluxed for 20 min. The solution was rapidly cooled, diluted with 2 liters of ice water and extracted six times with ethyl ether. The combined extracts were washed with water, dried over anhydrous sodium sulfate, then concentrated in vacuo. The residual red oil was chromatographed on a Florisil column. The deep red band which appeared on the column was eluted with ethyl ether. The red product from this elute was further purified by preparative TLC using benzene-ethyl acetate (7:3) as the developing solvent. Product IIb was obtained as red crystals. $E^{1\%}_{1\,cm}$ max in ethanol at 262, 269 and 460 mμ were, respectively, 300, 300 and 46.

### 2,2,7-Trimethyl-6-Hydroxy-2-(4,8,12-Trimethyltridecyl)-5,8-Chromanquinone (IIIa, Tocopurple) and its Model (IIIb)

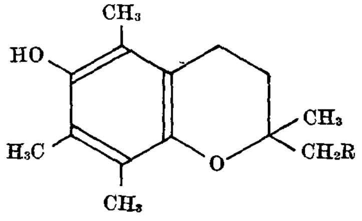
These compounds were prepared according to the procedure of Frampton et al. (6).

TABLE I

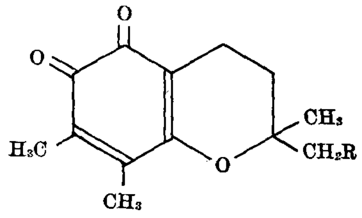
Properties of Methyl Esters

| Properties | Methyl oleate | Methyl linoleate | Methyl palmitate | SOFAME |
|---|---|---|---|---|
| Iodine value | 85.3( 85.6)[a] | 173.1(172.4) | ...................... | 138.2 |
| Saponification value | 188.2(189.2) | 190.2(190.5) | 206.9(207.5) | 189.2 |
| Specific gravity | $d^{25}_{25}$ 0.8741 | $d^{25}_{25}$ 0.8857 | $d^{50}_{4}$ 0.8438 | $d^{25}_{25}$ 0.8815 |
| Refractive index | $n^{25}_{D}$ 1.4508 | $n^{25}_{D}$ 1.4590 | $n^{30}_{D}$ 1.4357 | $n^{25}_{D}$ 1.4570 |
| GLC analysis | | | | |
| $C_{14}$ | ...... | ...... | Trace | ...... |
| $C_{16}$ | 3.0 | ...... | 97.6 | 4.2 |
| $C_{18}$ | 1.6 | ...... | 0.8 | 2.6 |
| $C_{18}$ F 1 | 88.2 | ...... | 1.6 | 24.0 |
| $C_{18}$ F 2 | 7.3 | 99.0 | ...... | 59.0 |
| $C_{18}$ F 3 | ...... | Trace | ...... | 9.7 |

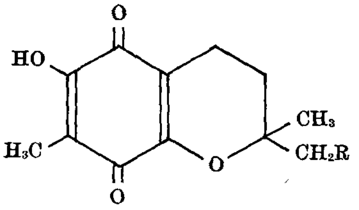
[a] Figures in parentheses show theoretical value.



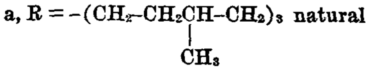
(I)



(II)



(III)



$a, R = -(CH_2\text{-}CH_2\underset{CH_3}{\underset{|}{C}}H\text{-}CH_2)_3$ natural

b, R = -H model compound

**Apparatus**

UV and IR spectra were measured with a Hitachi EPU-2 spectrophotometer and a Hitachi EPI-2 spectrophotometer, respectively. NMR spectra were measured using $CDCl_3$ solution and a Hitachi H-60 spectrometer. Chemical shifts are expressed as $\tau$ units relative to tetramethyl silane as an internal standard. TLC was carried out using Silica Gel G plates 0.3 mm (analytical) and 1 mm (preparative). Plates were activated by heating at 110 C for 2 hr and were stored in a dry cabinet until used. Gas chromatography was performed with a Perkin-Elmer 900 Gas Chromatograph equipped with a flame ionization detector. In this case, both 6 ft steel and glass column (1/8 in. i.d.) filled respectively with diethylene glycol succinate polyester and 5% SE-30 on Gas Chrom Q were used. Column temperature was 180 C with DEGS column and was programed from 150 to 220 C with the SE-30 column. Flow rate was 20–70 ml $N_2$/min. Gas chromatograms were quantified by the usual triangulation procedure.

Other materials and methods were essentially the same as described in preceding papers (1,2).

## Results and Discussion

**Color Changes of Tocored in Methyl Esters**

In order to get some clue as to the mechanism in the reaction of tocored, 20 ml of esters containing 0.03–0.05% of tocored and 0.05% of tocopherols were put into a test tube and heated in an oil bath at 140 C. Changes in absorption at 460 m$\mu$ corresponding to the maximum of tocored were determined periodically. In the case of methyl oleate or SOFAME, peroxide value was measured at suitable intervals.

When tocored alone was added to a saturated compound, such as methyl palmitate or liquid paraffin, no changes in absorbance of tocored were observed for 60 min. The absorbance gradually decreased in methyl oleate after 10 min and some peroxides were formed simultaneously. When tocored coexisted with $\alpha$-tocopherol in methyl palmitate or oleate, the absorbance decreased with the duration of heating. However, when tocored coexisted with $\gamma$-tocopherol, the absorbance did not change in methyl palmitate and increased in methyl oleate. When SOFAME was treated in the same manner, absorbance at 460 m$\mu$ decreased rapidly in the initial stage and again increased on further heating. Evidently coloration owing to tocored occurred even in esters without $\gamma$-tocopherol, just as in the ester containing tocored and $\gamma$-tocopherol.

From these results, it is evident that the color change of tocored occurs only in the case of unsaturated fatty esters.

**Reaction of Tocored Model (IIb) With Methyl Linoleate**

Two grams of IIb were added to methyl linoleate and heated in a vacuum at 160 C for 5 min. The red color of the solution became yellow-orange. The linoleate was dissolved in 500 ml of petroleum ether and placed on a column of 200 g of Florisil. The column was then washed with an additional 1 liter of petroleum ether to remove the linoleate (Fraction 1). It was then eluted with 700 ml of ether-methanol (1:1) to remove chroman substances. Evaporation of the solvent from this elute gave 50 g of a deep red oil (Fraction 2), insoluble in petroleum ether. TLC of Fraction 2 showed the presence of several separate colored products in addition to a considerable amount of IIb when a mixture of benzene-ethyl acetate (7:3) was used as the developing solvent (Fig. 1). During the elution procedure, a purple colored zone developed at the top of the Florisil column. Elution of this zone with 500 ml of methanol gave a purple solid (Fraction 3).

The deep red oil (Fraction 2) was dissolved in a mixture of petroleum ether-ethyl ether (4:1). This mixture was applied to a column packed with silica gel, which was developed with increasing proportions of ethyl ether in petroleum ether. One hundred and fifty fractions of 10 ml each were collected. A yellow oil (compound A) which was eluted at first was less polar than any other *o*-quinoid fraction, as judged by TLC. IR and UV spectra indicated that the chroman ring did not exist intact in this product, but no further investigation was attempted.

Elution with 600 ml of petroleum ether-ethyl ether (4:1) gave a deep red oil (Fraction 4). TLC of Fraction 4 showed two separate red spots when developed with a mixture of benzene-ethyl acetate (7:3), the $R_f$ being 0.53 (Compound B) and 0.43 (Compound C), respectively. The next fraction eluted with 500 ml of petroleum ether-ethyl ether (1:1) produced a red solid (Fraction 5). TLC (benzene-ethyl acetate 7:3) showed one major spot, $R_f$ 0.18 (Compound E), and traces of Compounds B and C.

**Isolation and Identification of Compound B**

The mixture of oxidized compounds obtained in Fraction 4 from the silica gel column was separated by TLC. A sample of deep red oil was dissolved in benzene and applied to silica gel plates. Plates were developed with a mixture of benzene-ethyl acetate

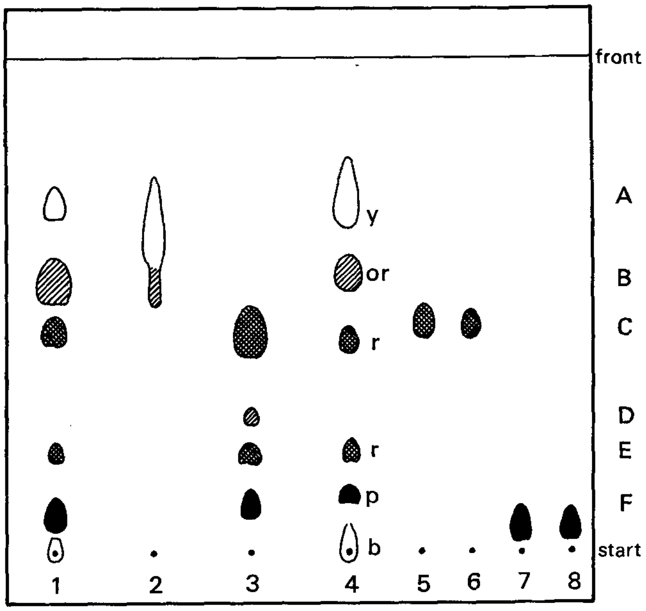


FIG. 1. Composite thin layer chromatograms of products derived from oxidation of tocored model in methyl esters. Nonpolar eluate, unsaturated fatty ester adduct, tocored, its isomer and dimer and tocopurple donated A, B, C, D, E and F, respectively. 1, methyl linoleate; 2, peroxidizing methyl linoleate; 3, methyl palmitate; 4, tocored in SOFAME; 5, 6, 7 and 8, standards: tocored (II a and b), tocopurple (III a and b), respectively. y, yellow; or, orange-red; r, red; p, purple; b, brown. Developed with benzene-ethyl acetate (7:3).

(7:3). The two major spots separated. The front band containing silica gel was scratched off the plate and extracted with ethyl ether. The solvent was removed in vacuo, and the residual red oil (Compound B) was redissolved in benzene and further purified by repeated TLC to eliminate any trace of compound C. After purification by TLC using chloroform-ethyl ether (9:1) as the developing solvent, Compound B was obtained as a red viscous oil. IR (Fig. 2) and UV absorption spectra showed no changes in additional chromatographs. $E^{1\%}_{1\,\mathrm{cm}}$ max in ethanol were 264, 272 and 460 mμ = 158, 171 and 13, respectively. The IR spectrum showed no hydroxyl band. Five bands were present in the carbonyl area. The first corresponded to ester carbonyl and the others to unconjugated and conjugated carbonyl groups. In addition a peak at 985 cm$^{-1}$, which corresponded to conjugated double bonds of the linoleate, was observed. The NMR spectrum of compound B (Fig. 3) was significantly different from that of IIb. The area for the aromatic methyl group showed only one peak at 8.18. Additional signals due to methyl linoleate were observed.

Methyl linoleate has a signal at 7.25 due to the methylene group between its two double bonds. With the alkali isomerized linoleate this signal is, of course, not clear. Furthermore, NMR spectra of compound B and the alkali isomerized linoleate have additional signals at 4.20, which indicates the presence of a conjugated double bond group. If compound B is a mixture of IIb and isomerized linoleate, these components should be separable by gas chromatography. Figure 4 is a typical gas chromatogram of Compound B. No methyl linoleate was resolved. Figure 5 also shows chromatograms of the alkali isomerized linoleate containing 10% of IIb after heating at 160 C for 10 min under reduced pressure. The content of isomerized methyl linoleate (c,d) did not change but the all-*cis* linoleate (b) decreased about 8%. A chromatogram of this sample revealed three small peaks (a, g and i), corresponding to the peaks of Compound B. In this case, we concluded that the isomerization of methyl linoleate proceeds via catalytic action of the quinone (6), then produces the adduct at an elevated temperature.

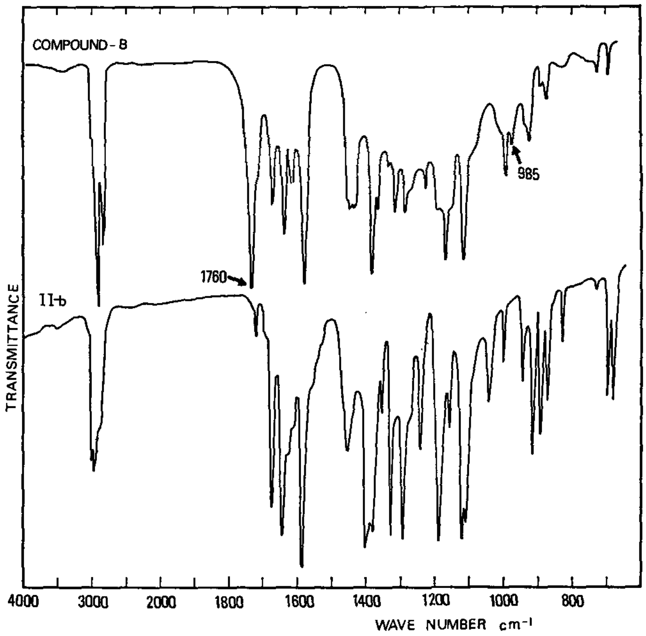


FIG. 2. IR spectra of compound B and the tocored model (IIb).

It appears from these data that the conjugated linoleate adduct, in which either carbon chain adjacent to the conjugated double bonds in the linoleate combined with the tocored moiety, was formed from IIb upon heating.

### Isolation of Compound C

Silica gel containing the more slowly moving band was scratched off the plate and extracted with ethyl ether. The ether extract was concentrated in vacuo. The residual red crystal was submitted to repeated TLC by the techniques described above. The red crystal agreed in melting point, IR, UV, NMR spectra and TLC behavior with an authentic original sample of IIb.

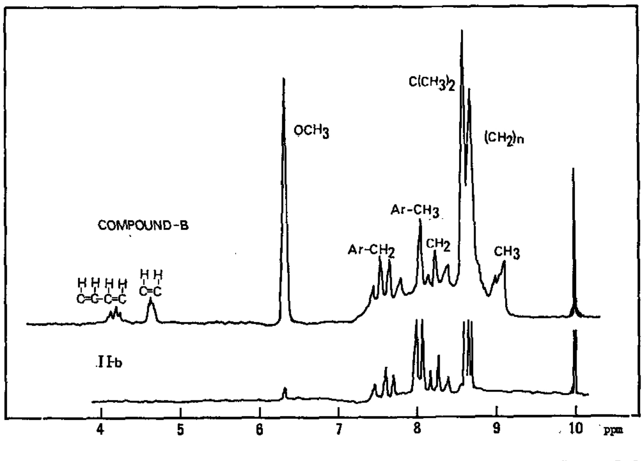


FIG. 3. NMR spectra of compound B and the tocored model (IIb).

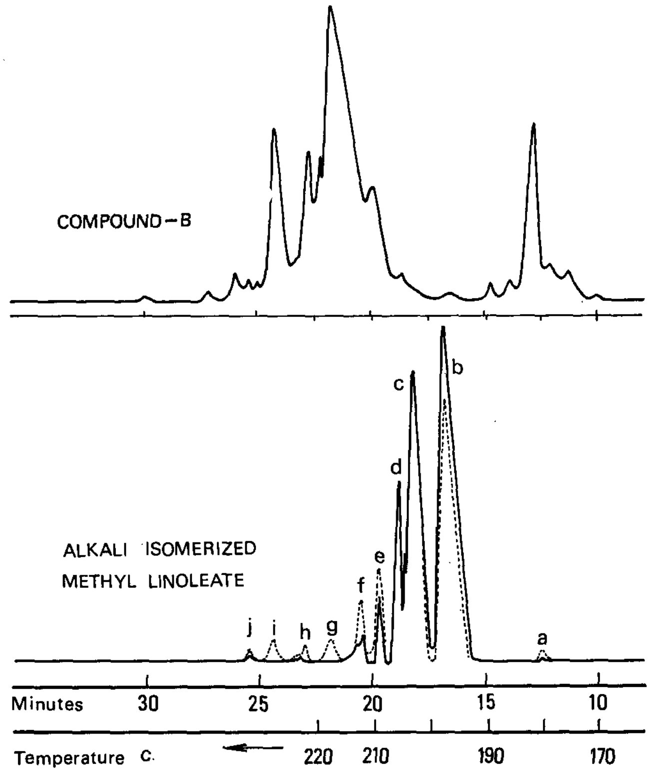


FIG. 4. Gas chromatograms of compound B and alkali isomerized methyl linoleate, dotted line; 10% IIb was added, showing the presence of compound B. Both linoleates were heated at 160 C for 10 min in a vacuum.

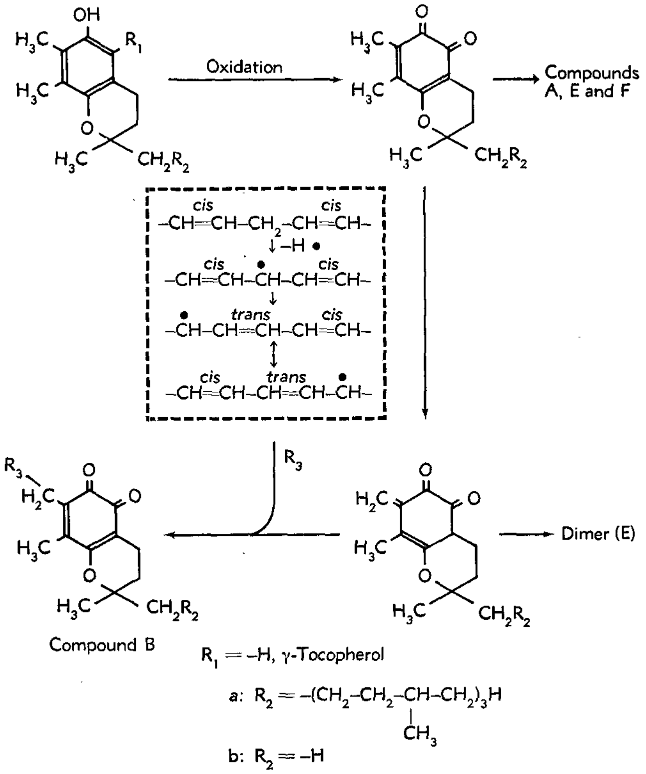


FIG. 5. Mechanism of formation of the unsaturated fatty ester adduct and other products from tocored oxidation.

### Isolation of Compound E

Fraction 5 from the column chromatography was submitted to repeated TLC as described above. Upon removal of the solvent in vacuo, a brilliant reddish purple crystal, insoluble in ethyl ether but soluble in alcohol, with melting point higher than 190 C, was obtained. UV and IR spectra were very similar to those of IIb, showing an absence of ester carbonyl and the presence of the carbonyl group and chroman. Chromatographic behavior of this compound indicated a molecule of high polarity. The chloroform solution of Compound E deposits an amorphous solid. Therefore, tocored dimer seemed to be formed.

### Isolation of Compound F

A purple solid obtained from Florisil column chromatography was dissolved in ethyl ether and filtered. The solvent was removed in vacuo, and the orange residual oil, Compound F, was redissolved in benzene and purified by repeated TLC using benzene-ethyl acetate (1:1), chloroform-ethyl ether (9:1) as the developing solvents. The orange gum showed indicator properties, turning yellow in acid and purple in basic media. It was proven to be tocopurple model (IIIb) by its UV, IR spectra and TLC behavior. The UV absorption maximum in ethanol was at 295 mμ; Frampton (6) reported a maximum at 296 mμ. The IR spectrum ($CS_2$): 3350 $cm^{-1}$ (OH), 1650, 1610 (C = O, C = C), 1380, 1350 ($CH_3$), 1290 (=C–O–C), 1155 (=C–OH).

### Reaction of IIb in Peroxidizing Methyl Linoleate

Fifty grams of methyl linoleate were put into each of four Petri dishes (14 cm). These dishes were placed on a horizontal rotating disc installed in a cabinet. The linoleate was irradiated by UV light for 10 hr at 24–40 C and the peroxide value was measured intermittently. At the end of the irradiation period (POV 218 meq/kg), the linoleate was combined and 2 g of IIb was added. This solution was heated at 170 C for 15 min in vacuo. The procedure used for the isolation of oxidized products of IIb was essentially the same as that applied to methyl linoleate.

The initially isolated products from the Florisil column were brown oils which showed only very poorly defined spots, corresponding to Compounds B and C. This also indicated a high yield of Compound A and the formation of other extraneous products. Chromatographing the crude products on a silica gel column and elution with petroleum ether-ethyl ether mixtures of increasing polarity, produced a considerable amount of yellow oil in addition to several different products. The eluted substances from all fractions were too small to carry further investigations. While some of these may have been derived from IIb, it seems that the major portion of this material must have originated from fragmentation of the oxidizing methyl linoleate. The main oxidized product (Compound A) was less polar than Compound B.

### Oxidation of IIb in Methyl Palmitate

The method described before was used with the exceptions that methyl linoleate was substituted for methyl palmitate and the reaction time was doubled (10 min). On TLC (benzene-ethyl acetate, 7:3) the crude oxidized products showed three red spots and a purple spot. The largest spot, $R_f$ 0.45 (Compound C) was due to starting tocored IIb. One of these spots, $R_f$ 0.18 (red), corresponded to Compound E and the other (purple, $R_f$ 0.12) to Compound F. The middle red spot, $R_f$ 0.30 (Compound D) was eluted with ethyl ether. Upon removal of the solvent in vacuo, an orange oil was obtained, which, even after repeated purifications by TLC, gave an orange oil quite impossible to crystallize. Its absorption spectrum was identical with that of IIb. Judging from its chromatographic behavior, its color, and UV, IR absorption spectra, this orange oil seems to be a paraquinone analogous to a product obtained by Frampton (7) upon the isomerization of o-quinone.

### Oxidation of Tocored in Soybean Oil

Two grams of tocored (IIa) were dissolved in 150 g of soybean oil from which unsaponifiable matter had been removed by molecular distillation. The solution was heated at 200 C for 5 min under reduced pressure, then was dissolved in 1 liter of petroleum ether. This solution was placed on a column of 200 g of Florisil. TLC of the crude oxidized products showed the presence of compounds corresponding to A, B, E and F, in addition to unchanged tocored. The crude products were purified by chromatography according to procedures described in the isolation of model compounds. UV and IR spectra of isolated compounds were identical to those of model samples.

Figure 4 shows the spectra of methyl linoleate containing the tocored model (IIb) before and after heating. As most of the IIb had been changed by the heat treatment, the spectrum of B revealed less absorption than that of ester A characterized by the *o*-quinoid structure with a maximum at 460 mμ. Attempts to isolate the discolored products derived from tocored and its model in the presence of methyl linoleate by chromatography failed, as oxidation and dimerization were predominant. A number of colored products were produced and these were separable by TLC.

Discoloration of tocored occurred only in the case of unsaturated esters with the progress of peroxide formation. This coincides with the mutual relationship between autoxidation of esters and formation of Compound B.

Data presented in this paper show the following probable relationships among the oxidation products of tocopherol and methyl linoleate (Fig. 5).

The red color faded to pale yellow when a mixture of tocored or its model and a reducing agent, such as $LiAlH_4$ or ascorbic acid, was stirred at room temperature. Purification of the products was not possible but their IR spectra showed a strong hydroxyl band and weak carbonyl bands with a maximum at 291 mμ in the UV spectra indicating possible conversion to 5,6-dihydroxy chroman. The reduction product, however, is oxidized in air extremely rapidly—so rapidly that the compound reoxidizes and its oxidation product turns red as soon as the reducing agent is removed. Smith et al. (8) reported a similar result in the reaction of tocored with aqueous sodium bisulfite.

A hydrogen atom is known to be abstracted in the earlier stage of autoxidation of methyl linoleate due to enhanced reactivity of the doubly activated methylene group at $C_{11}$ and the linoleate becomes a free radical. Discoloration of tocored in unsaturated fatty esters may be partly due to reduction by the hydrogen atom which is furnished from peroxidized linoleate.

We presumed that a quinone methide-like substance, the structure of which was proposed as an intermediate in various reactions (9,10), was formed from the tocored upon heat treatment in unsaturated esters. This quinone methide then combined with the fatty free radical to form the adduct (B) just as a complex formation occurred between the antioxidant and free radical (11,12). As described in a previous paper (2), this appears to be the strongest proof that the tocored shows antioxidative potency to soybean oil and that it plays an important role as the chain breaking agent. As the free radical is a resonance hybrid, it appears that the adduct is mainly a mixture of diene isomeric adducts. This can actually be seen in the gas chromatogram (Fig. 4) of Compound B.

A number of other reactions seems to be possible. Two tocored molecules may react by a Diels-Alder type reaction to give dimer, similar to the proposal by Harley-Mason and Laird (13) for the dimer of *o*-benzoquinone. It may also be possible that the quinone methide reacts with the tocored or with another molecule of quinone methide to give dimers. These dimers may correspond to Compound E.

Recently Porter et al. (14) have reported for the formation of an addition compound of oxidized $\alpha$-tocopherol and linoleic acid.

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