A Dimeric Oxidation Product of γ-Tocopherol in Soybean Oil

MAMORU KOMODA and ICHIRO HARADA, Sugiyama Chemical Research Institute, Mitaka, Tokyo, Japan

Abstract

A dimeric oxidation product (5-γ-tocopheroxy-γ-tocopherol) has been isolated from soybean oil and identified. The dimer content in extracted oil was increased by elevating the moisture level in raw soybeans. With moisture increase, no change in the quantity of α-tocopherol was observed, but γ- and δ-tocopherol contents were greatly decreased and two kinds of dimer were formed from γ- and δ-tocopherols. When the moisture level in moistened beans was lowered, these dimers reverted to their corresponding original tocopherols. The same results were obtained by treating pulverized soybeans with various reducing agents. γ-Tocopherol added to autoxidizing soybean oil was oxidized more easily in the presence of oxidation products derived from tocopherols and turned into the dimeric product.

Introduction

Shone (1) isolated a new oxidation product of γ-tocopherol from tung oil. He reported that the structure of this compound is obscure but appears to contain the basic chroman ether ring structure with alkyl, hydroxyl and carbonyl substituents. McHale and Green (2) described the isolation of an analogous oxidation product from cottonseed oil deodorizer scum. He established its structure by comparing UV, IR, chromatographic behavior, nitric acid oxidation, and its maximum spectrophotometric absorption with similar properties of the authentic product obtained by the dehydrogenation of γ-tocopherol with *p*-benzoquinone. The structure proposed was 5-γ-tocopheroxy-γ-tocopherol. Takeuchi and Tatsukawa (3) recognized two components in deodorizer condensates which were positive to Emmerie-Engel reaction. He suggested that the compounds were β-, γ- and δ-tocopherol derivatives derived by substituting nonpolar radicals for the 5 or 7 hydrogen atoms.

We have isolated and identified a similar compound from soybean oil and have also studied the relationships among the moisture of soybeans, the contents of an oxidation product and individual tocopherols in extracted oil to obtain information on the interconversion among these tocopherol-related compounds. Evidence was obtained on the mutual action among γ-tocopherol, dimer and tocored from the fate of γ-tocopherol in autoxidizing soybean oil.

Materials and Methods

Chemicals

Silica gel G according to Stahl (E. Merck AG., Darmstadt, Germany) was used as adsorbent. Florisil (60–100 mesh) was purchased from Floridin Co. and α, α′-dipyridyl was a product of Wako Pure Chemical Ltd. Methanol, benzene and hexane were analytical grade reagents. Before use these solvents were tested for the presence of peroxides. Ethyl ether: 500 ml of ether was shaken with a mixture of 4 g of $AgNO_3$ in 30 ml of water and 2 g of NaOH in 50 ml of water; the ether phase was separated, dried and distilled just before use. Chloroform: contained 1% v/v of ethanol as stabilizer. Ethanol was purified by distillation in an all glass apparatus over $KMnO_4$ and KOH (1 and 2 g, respectively, per liter of ethanol). Petroleum ether: boiling range 30 C to 60 C.

Tocopherols

Purification of γ- and δ-tocopherols was as follows. The soybean oil deodorizer scum was freed from sterols by freezing a methanolic solution and the methanol-soluble oil was subjected to molecular distillation. The tocopherol concentrate obtained by molecular distillation was purified by column chromatography followed by repeated TLC on silica gel with chloroform as developing solvent. Purities of the tocopherols used in these experiments were determined by comparing their UV (4) and IR (5) spectra. $E^{1\%}_{1\,cm}$ 298 mμ in ethanol of γ- and δ-tocopherols were 82.7 (89.2%) and 87.9 (96.4%), respectively. Commercial *dl*-α-tocopherol was used without further purification, as its extinction coefficient was found to be in good agreement with that reported by Baxter et al. (4), $E^{1\%}_{1\,cm}$ 292 mμ max. = 73.20 in ethanol, for the highly purified product.

Preparation and Estimation of Tocored

Tocored was made and determined according to procedures previously reported (6).

Quantitative Determination of Individual Tocopherols

Soybean oil was saponified in the presence of pyrogallol with KOH solution, and unsaponifiable matter was extracted with ethyl ether according to the system of the Analytical Methods Committee (7). The unsaponifiable matter was dissolved in 0.4 ml of benzene. 30–40 μl (100–200 μg) of benzene solution was spotted as a narrow band across the starting line of a large silica gel plate (20 × 20 cm), which had been activated in an oven at 110 C for 2 hr and cooled. Fifteen μl portions of the same test solution were spotted on both edges of the plate as a guide for finding the tocopherol bands for analysis. The plate was then developed with chloroform to a distance of 15 cm. After removal and drying, Emmerie-Engel reagent was sprayed on the test spots to determine the tocopherol positions. The appropriate zone was removed using a 0.5 cm blade held in a vertical position. The loosened material was transfered to a small glass filter and the tocopherols were extracted with a minimum quantity of ethyl ether. The solvent was removed in vacuo, and the residue was dissolved in 9 ml of ethanol. The tocopherol and dimer contents of the solutions were determined by the method of Emmerie-Engel. The solution from the blank portions, treated in the same way, was used in the reference cell. Standard graphs were prepared for each of the three tocopherols and dimer showing the correlation between the optical density of the color complex and the quantity of tocopherol taken.

Preparation of 5-γ-Tocopheroxy-γ-Tocopherol

Quantities of 0.5 g of γ-tocopherol and 1 g of *p*-benzoquinone were dissolved in 50 ml of benzene and refluxed for 30 min. The benzene was evaporated. The purple oily residue was dissolved in petroleum

ether and *p*-benzoquinone crystals were removed by filtration. The petroleum ether solution was run through a short Florisil column (1.5 × 5 cm) to remove any remaining *p*-benzoquinone. The refined petroleum ether solution was concentrated and chromatographed on a Florisil column (2 × 20 cm). The first 100 ml fraction was discarded. Elution with 200 ml of 5% ethyl ether in petroleum ether yielded a yellow oil (fraction 2). TLC showed one major spot (Rf 0.78–0.86) and one minor spot (Rf 0.34–0.44) of unreacted γ-tocopherol, when developed with chloroform. The mixture obtained as fraction 2 from the Florisil column was purified by TLC. A sample of yellow oil dissolved in benzene was applied to silica gel TLC-plates (20 × 20 cm) and developed with chloroform. The front band was scratched off and extracted with ethyl ether. The ether extract was then filtered and the solvent was removed. The slight yellow residual oil was dissolved in benzene and further purified by repeating TLC until a pale viscous oil, soluble in ethanol, $E^{1\%}_{1\,cm}$ 293 mμ max. = 80.6 was obtained. The reducing power in the Emmerie-Engel reaction was 45.5% that of γ-tocopherol. δ-Tocopherol was oxidized under conditions similar to those used for the preparation of γ-tocopheroloxide. Chromatography of δ-tocopherol-oxide on TLC showed only a very poorly defined spot in the dimer position and also the presence of various other products.

A mixture of 80 mg of dimer obtained from γ-tocopherol and 1 g of $LiAlH_4$ in 30 ml of ethyl ether was allowed to stand overnight at room temperature. The excess reagent was decomposed with ethanol and water and the ether layer filtered and concentrated in vacuo to give a yellow brown oil (80 mg). The product was further purified by TLC employing chloroform as the developing solvent. The IR and TLC behaviors of this reduced compound were identical to those of γ-tocopherol.

Soybeans and Soybean Oil

Raw soybeans used in these experiments were from a 1966 crop of Illinois soybeans and had 12% moisture. From these, soybeans with varying moisture contents were prepared as follows: (a) Moistened soybeans; 3.5 kg of raw soybeans and distilled water were put into a glass bottle and the bottle was shaken vigorously. The bottle was kept for seven days at room temperature, during which period it was shaken several times per day. The soybeans thus prepared proved to be moistened homogeneously. (b) Dried soybeans; three samples, each containing 3.5 kg of raw soybeans were dried in a vacuum dryer below 50 C until their moisture contents were reduced to 6.7%, 7.8% and 9.2%, respectively.

Three kilograms of each soybean sample were ground and passed through a 700 μ sieve, then extracted with normal hexane at room temperature.

Molecular Distillation

A five-inch centrifugal molecular still CMS-5 (Consolidated Electrodynamics Corporation, Rochester Division) was used.

TABLE I

Properties of Compound I, II and 5-γ-Tocopheroxy-γ-Tocopherol

	Compound I	Compound II	5-γ-Tocopheroxy-γ-tocopherol
Appearance	Yellow viscous oil	Yellow viscous oil	Yellow oil
Rf[a]	0.83	0.70	0.86
$E^{1\%}_{1\,cm}$ max. (EtOH)	292mμ = 66.9	290mμ = 65.0	293mμ = 80.6
Tocopherol[b] equivalence (%)	48.0	23.0	45.5
Diazotised[c] o-dianisidine coupling reaction	(±)	(±)	(−)
$LiAlH_4$ reduction	γ-Tocopherol A trace of δ-Tocopherol	δ-Tocopherol	γ-Tocopherol

[a] Silica gel TLC, chloroform, 10 cm migration of front.
[b] The response in Emmerie-Engel assay procedure for γ-tocopherol.
[c] Spray reagent: 5% K_2CO_3 followed by diazotized *o*-dianisidine. Tocopherols may be identified by the resultant coupling colors: γ-tocopherol; blue-green changing to indigo, δ-tocopherol; reddish-brown changing to purple (8).

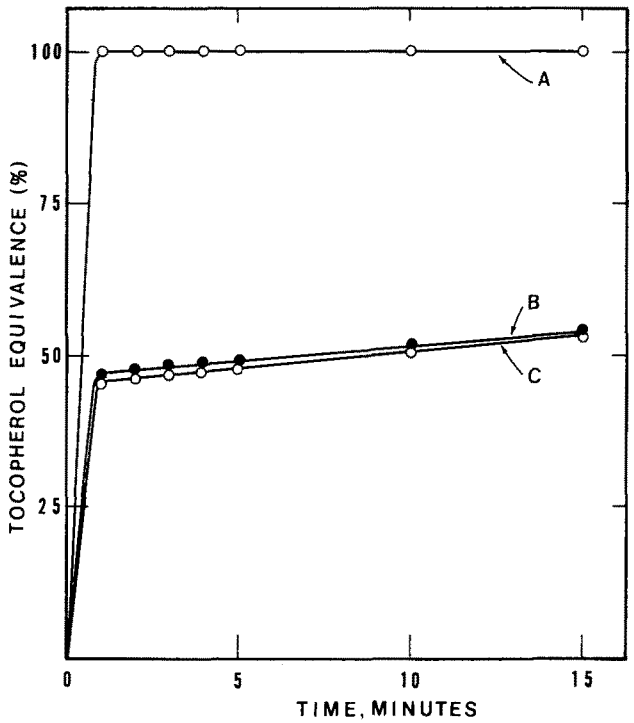

FIG. 1. Oxidation curves of (A) γ-tocopherol; (B) compound I; and (C) 5-γ-tocopheroxy-γ-tocopherol in Emmerie-Engel assay procedure.

Results and Discussion

Identification of 5-γ-Tocopheroxy-γ-Tocopherol

The extracted crude oil from moisture controlled soybeans (moisture 18.3%) was degummed and subjected to molecular distillation. The unsaponifiable matter from the molecularly distilled oil (205–210 C, 8–10 μ) was freed from sterols by cooling a methanolic solution and subjecting the methanol soluble oil to chromatography on Florisil. The oxidation products and tocopherols were eluted together with 5% ethyl ether in petroleum ether and a yellow

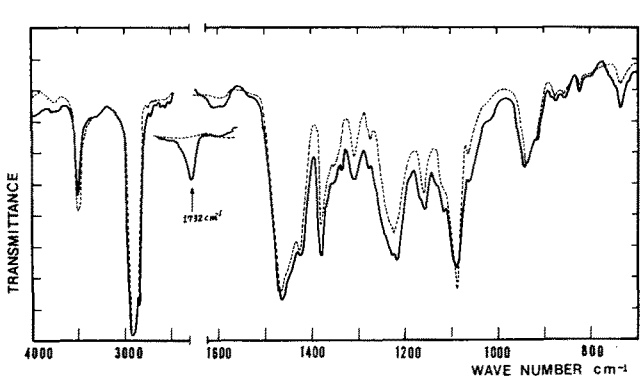

FIG. 2. IR spectrum (thin film) of the compound I (——) identified as 5-γ-tocopheroxy-γ-tocopherol (- - - - -).

oil was obtained. This yellow oil was rechromatographed in chloroform on a 1 mm thick silica gel plate and was separated into five fractions which gave intense red spots with the Emmerie-Engel reagent. Rechromatography in chloroform of the two fastest moving substances on similar thick plates showed one major spot, Rf 0.83 (I) and two minor spots, Rf 0.70 (II), 0.25, respectively. Table I shows the properties of compounds I, II and 5-γ-tocopheroxy-γ-tocopherol prepared from γ-tocopherol.

As shown in Table I, the yellow viscous oils, I and II, recovered from the fastest moving spots showed absorption maxima at 290–292 mμ in ethanol, which is typical of a 5,7-disubstituted tocopherol (9). The response in the Emmerie-Engel assay procedure for compound I was approximately equivalent to one-half of γ-tocopherol (Fig. 1), which was consistent with the presence of an oxidizable phenol group. The IR of I (Fig. 2) is similar to that of γ-tocopherol. The hydroxyl band is sharper than that in either of the tocopherols and the intensity of the aromatic ether band (1070 cm^{-1}) is enhanced. McHale and Green (2) reported a similar interpretation of a substance which they isolated from cottonseed oil deodorizer scum.

The spectrum of the authentic preparation differs from those of materials from soybean oil that have a weak band at 1732 cm^{-1}. The UV spectra of I and II indicated the absence of an α,β-unsaturated ketone function. The occurrence of an absorption band for the carboxyl group at 1732 cm^{-1} resulted from a trace amount of remaining fatty acids.

Compound I was refluxed with $LiAlH_4$ for 3 hr in ethyl ether. Alcohol was added to destroy the catalyst and the compounds were extracted with ethyl ether. The TLC showed several spots. The major component was purified by repeated silica gel TLC and was proven to be γ-tocopherol by its IR and TLC

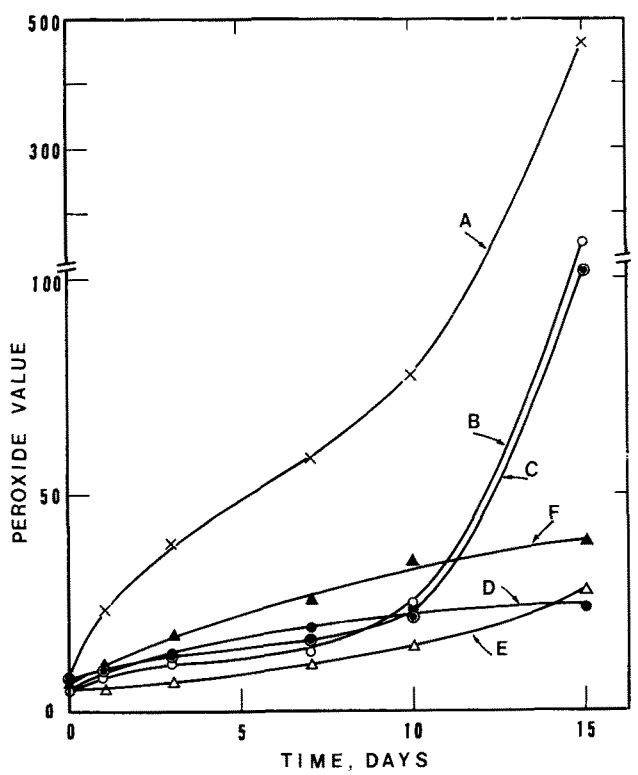

FIG. 3. Increase of peroxide value in soybean oil during storage at 40 C. A, no addition; B, 0.015% dimer; C, 0.02% tocored; D, 0.1% γ-tocopherol; E, 0.015% dimer + 0.1% γ-tocopherol; F, 0.01% tocored + 0.1% γ-tocopherol.

TABLE II
Contents of Individual Tocopherol in Soybean Oil

Moisture of soybeans (%)	Total tocopherol (mg/g of oil)	POV	Tocopherols found in oil[a] % of total tocopherol			
			α	γ	δ	dimer
6.7	1.49	4.1	5.9	60.0	27.5	6.6
7.8	1.49	4.3	6.7	60.1	27.7	5.6
9.2	1.57	4.2	6.6	59.4	28.8	5.3
11.6	1.06	3.9	7.4	57.5	30.0	5.2
14.0	1.25	4.7	8.8	39.5	28.3	23.4
16.0	0.44	7.5	7.6	32.1	19.4	24.8
18.0	0.46	11.7	12.0	19.5	13.2	55.3
4.3[b]	1.02	4.6	3.8	59.0	19.4	17.8

[a] Figures are averages of three samples.
[b] Soybeans with 18% moisture were dried.

behaviors. When compound II was treated by the same procedures as compound I, δ-tocopherol was obtained as its main constituent.

The yield of compound II was very poor. This difference may be ascribed to the δ-tocopherol content of the soybean oil which was about half that of the γ-tocopherol. In addition, when the δ-tocopherol was dehydrogenated with *p*-benzoquinone, the reaction product showed only poorly defined properties of a dimer. This may be due to a weakness in the C-5 and 7 positions of δ-tocopherol owing to the absence of a methyl group.

When compounds I and II were purified by TLC, a red spot (Rf 0.25) was obtained but sufficient quantities could not be purified for structural studies. The TLC behavior of the red spot was very similar to that of the tocored previously reported (6).

The evidence thus obtained supports the suggestion that compounds I and II were probably derived by oxidation from γ- and δ-tocopherols during the storage of soybeans or during the oil extraction process.

Relationship of Moisture in Soybeans to the Quantity of Individual Tocopherols and of Compounds I and II

Analytical results are shown in Table II for oils extracted from soybeans at 8 different moisture levels.

As shown in Table II, tocopherol contents in oil from ordinary soybeans (moisture 11.6%) were in agreement with earlier determinations (10,11). The α-tocopherol content in soybeans was not changed by changing the moisture level of the beans, but a rapid decrease in γ- and δ-tocopherols appeared as the moisture level was increased from 14% to 18%. Consequently, as the moisture content was raised the total amount of I and II (dimer) was remarkably increased.

The dimer in soybeans with high moisture (18.0%) was approximately five times that of ordinary soybeans. Since the response of dimers in the Emmerie-Engel assay procedure was approximately equivalent to one-half that of γ-tocopherol, these dimeric compounds may correspond to a tenfold reduction in the quantity of γ- and δ-tocopherol.

It has previously been reported (6,12) that lipid content varies with the moisture content of soybeans

TABLE III
Total Tocopherol Contents After Treatment With Various Reducing Agents

Reducing agents	Total tocopherol	
	(mg/g of oil)	Restoration (%)
Raw soybeans (moisture 11.6%)	1.06	.....
Moistened soybeans (moisture 18.0%)	0.46	.....
Ascorbic acid	1.02	96.2
$LiAlH_4$	0.84	79.2
$NaBH_4$	0.90	84.9
Pyrogallol	1.07	100.9

(moisture range 7.8–17.6%), but that variations in lipid content are less than a few per cent on a dry basis. These observations suggest that lipid content does not greatly affect the variation in tocopherol content. In addition, the tocopherol content in crude soybean oil, obtained by mechanical pressing at room temperature, was also decreased by an elevated moisture level in raw soybeans (6).

It seems from the above results that γ- and δ-tocopherols were converted into dimer in seeds by moistening and that this conversion may be related to the peroxidation of unsaturated fatty acids.

Soybeans with 18% moisture were dried in a vacuum dryer below 50 C until the moisture was reduced to about 4%, then individual tocopherols and dimer in the crude oil from these dried soybeans were determined. As shown in Table II, the dimer decreased about 70% from its highest content. However, γ- and δ-tocopherol were regenerated, the regeneration ratio by drying being, respectively, 99% and 62% of the quantity of these tocopherols in the original soybeans (moisture 11.6%). These phenomena were also observed when raw soybeans were dried at low temperatures or in the sun (12). IR and TLC behavior of γ-tocopherol isolated from dried soybeans were identical to those of an original γ-tocopherol with a strong band at 3450 cm^{-1}.

Conversion of Dimer to Tocopherol

To convert dimer to tocopherol, the ethyl ether suspension containing 5 g of pulverized soybeans (moisture 18%) was mixed with 0.5 g of various reducing agents. The suspension was stirred for 30 min at room temperature, filtered and washed with ethyl ether. The ether extract was washed and evaporated. The residue, to which a little absolute ethanol and benzene were added, was dried again. The total tocopherol contents of these extracts are shown in Table III.

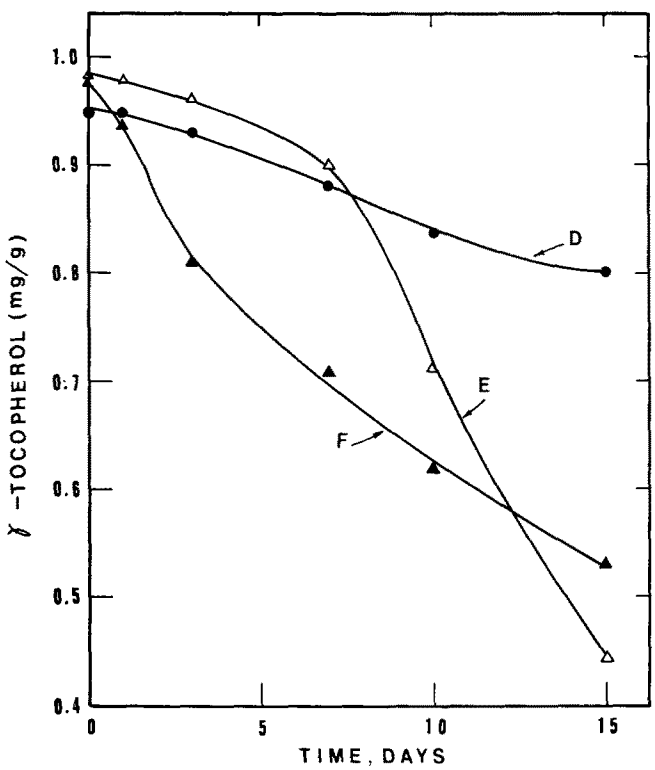

FIG. 4. Oxidation of γ-tocopherol in soybean oil at 40 C. Symbols are the same as in Fig. 3.

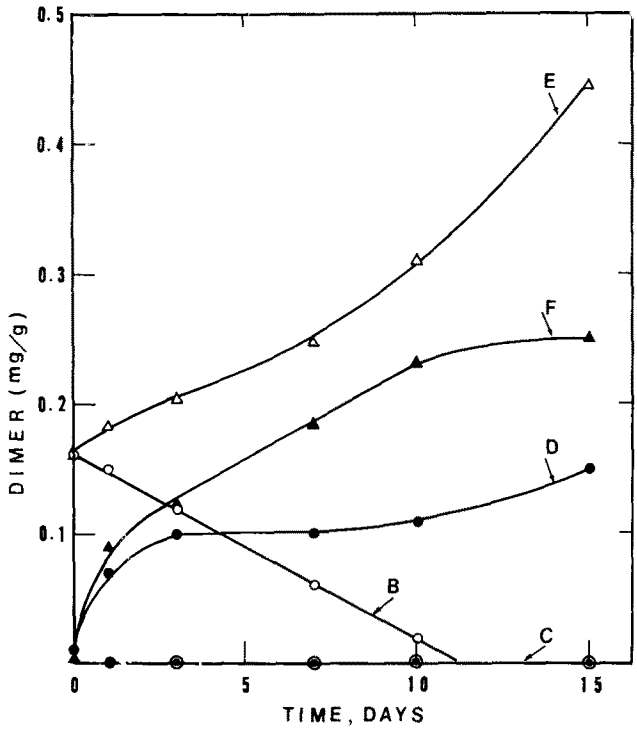

FIG. 5. Changes of dimer content during the storage of soybean oil at 40 C. Symbols are the same as in Fig. 3.

In the case of ascorbic acid or pyrogallol, the oxidation product was almost completely converted to tocopherol, but in the case of a strong reducing agent, such as $LiAlH_4$ or $NaBH_4$, the conversion rate to tocopherol was about 90%. Reduction by the latter agents probably proceeds by a different mechanism from that in case of ascorbic acid.

The results shown in Table II and III and in previous reports (6,12) suggest that a certain oxidation-reduction system in soybean cells may be activated in response to changes of moisture level in soybeans and that oxidation of tocopherols by moistening, or reduction of dimer to the original tocopherols by drying, may proceed in the cells. It is thought that some enzymes may be involved in this redox system, but no evidence for this supposition has yet been obtained.

Oxidation of γ-Tocopherol in Autoxidizing Soybean Oil

The oxidation was carried out under atmospheric conditions using 0.1% γ-tocopherol, 0.015% dimer and 0.01–0.02% tocored dissolved in soybean oil, from which unsaponifiable matter had been removed by molecular distillation. All preparations were held at 40 C in the dark and changes in the contents of γ-tocopherol and its oxidation products during storage were determined periodically. The results are shown in Figures 3–6. Figure 3 shows the comparative antioxygenic potencies of γ-tocopherol and its oxidation products for soybean oil.

γ-Tocopherol (Fig. 4,D) decreased at a steady rate during storage. After 15 days of storage, the unsaponifiables of D contained about 85% residual γ-tocopherol plus additional dimer. The decrease of γ-tocopherol in E and F, which contained dimer or tocored in addition to γ-tocopherol, was twice as much as the decrease in D and there was no evidence of the possibility of restoration of γ-tocopherol from dimer or tocored.

When dimer or tocored alone was added to soybean oil (Fig. 5,B; 6,C), they rapidly decreased. But when

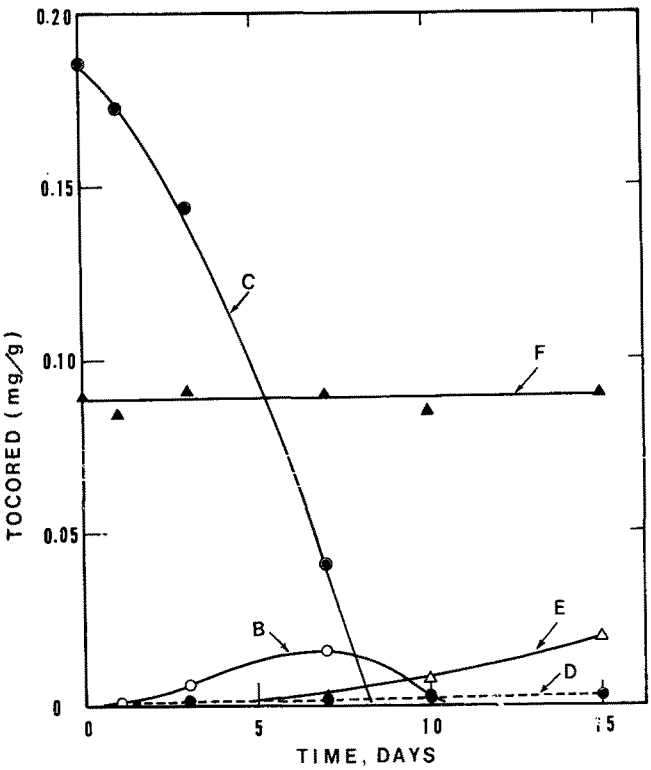

FIG. 6. Changes of tocored content during the storage at 40 C. Symbols are the same as in Fig. 3.

γ-tocopherol coexisted with them the presence of a new reducing substance was indicated by the TLC data of the unsaponifiables of D, E and F (Emmerie-Engel reaction positive). The size of this one spot, (Rf 0.83) on TLC developed with chloroform, became larger with the duration of storage. The IR of this compound was in good agreement with that of 5-γ-tocopheroxy-γ-tocopherol.

Interesting examples of the sparing effect of γ-tocopherol on tocored and dimer are shown in Figure 6. After eight days of storage, the tocored in C disappeared completely. But in case of F, tocored was extremely stable in coexistence with γ-tocopherol. Tocored was not produced from γ-tocopherol directly (D) but from dimer, as the TLC data and the UV spectra of B and E show the appearance of tocored during storage. Comparing E with B, the delay in appearance of tocored in E may involve the protective effect of γ-tocopherol on dimer.

The findings presented here lend some support to the tentative relationship (shown in Fig. 7) between γ-tocopherol and its isolated oxidation products from autoxidizing soybean oil.

There is much information (13–15) available on the reactions between tocopherol and free radicals. In 1955, Inglett and Mattill (13) reported that products were isolated after reacting α-tocopherol and its model 6-hydroxy-2,2,5,7,8-pentamethylchroman with the relatively stable benzoyloxy radical. Both the chromanoxy radical and the isomeric 5-benzylic radical were of importance in the induction of free radical reactions with tocopherol and its model 6-hydroxychroman, followed by dimerization and further oxidation. They also reported that γ-tocopherol reacted with benzoyl peroxide gave the tocored. This

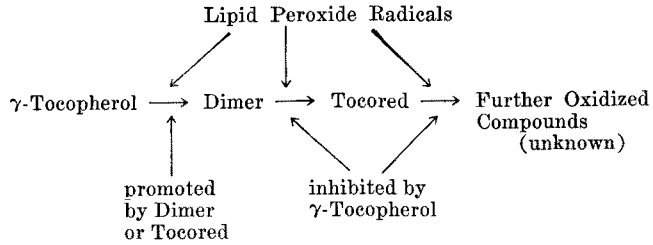

FIG. 7. Possible course of oxidation of γ-tocopherol in autoxidizing soybean oil.

supplementary evidence indicates that γ-tocopherol is the precursor of red quinone in autoxidizing cottonseed oil (16) and further illustrates the vulnerability of the C-5 position of γ-tocopherol molecule. McHale and Green (2) reported that nitric acid oxidation of 5-γ-tocopheroxy-γ-tocopherol gave two molecules of the *o*-quinone of γ-tocopherol.

The generally accepted mechanism of the inhibition of lipid peroxidation posits that the nature of the stable products from phenolic inhibitor radicals mainly depends on reaction conditions. For example, in the presence of higher lipid-peroxide radical concentrations in the system, the inactive inhibitor radical will mainly combine with the active peroxide radical while dimers of inhibitor molecules are mainly formed at low concentrations (17).

In our experiments, formation of the dimeric product of tocopherols was proven at the initial stage of autoxidation of soybean oil.

As shown in Figures 4–7, there are mutual effects among γ-tocopherol, dimer and tocored and apparently a reasonable explanation may be obtained by comparing the curves in Figure 3. Namely, these compounds show different antioxidative potencies to soybean oil.

Although the present study is not completed, it seems likely that the hypothesis in Figure 7 may offer a suggestion as to the antioxigenesis of tocopherol in unsaturated lipids.

ACKNOWLEDGMENT

This research has been financed in part by a grant made by the U.S. Department of Agriculture, Agricultural Research Service, under P.L. 480.

REFERENCES

1. Shone, G., Chem. Ind. (London) 335–336 (1963).
2. McHale, D., and J. Green, Ibid. 982–983 (1963).
3. Takeuchi, T., and T. Tatsukawa, J. Japan Oil Chem. Soc. *16*, 185–193 (1967).
4. Baxter, J. G., C. D. Robson, J. D. Tayler and R. W. Leaman, J. Am. Chem. Soc. *65*, 918–924 (1943).
5. Morris, W. W., Jr., and E. O. Haenni, J. Assoc. Offic. Agr. Chemists *45*, 92–98 (1962).
6. Komoda, M., N. Onuki and I. Harada, Agr. Biol. Chem. *31*, 461–469 (1967).
7. Analytical Methods Committee 1959, report prepared by the vitamin E panel. Analyst. *84*, 356–372 (1959).
8. Weisler, L., C. D. Robson and J. G. Baxter, Anal. Chem. *19*, 906–909 (1947).
9. McHale, D., P. Mamalis, J. Green and S. Marcinkiewicz, J. Chem. Soc. 1600–1603 (1958).
10. Whittle, K. J., and J. F. Pennock, Analyst. *92*, 423–430 (1967).
11. Govid, M. K., S. V. Rao and K. T. Achaya, J. Soc. Food Agr. *16*, 121–124 (1965).
12. Komoda, M., N. Onuki and I. Harada, Agr. Biol. Chem. *30*, 906–912 (1966).
13. Inglett, G. E., and H. A. Mattill, J. Am. Chem. Soc. *77*, 6552–6554 (1955).
14. Skinner, W. A., and R. M. Parthurst, J. Org. Chem. *31*, 1248–1251 (1966).
15. Goodhue, C. T., and H. A. Risley, Biochem. Biophy. Res. Comm. *17*, 549–554 (1964).
16. Swift, C E., G. E. Mann and G. S. Fisher, Oil & Soap *21*, 317–321 (1944).
17. Moore, R. F., and W. A. Waters, J. Chem. Soc. 243 (1954).

[Received May 30, 1968]