The Antioxygenic Synergism of Various Acids with α-Tocopherol[1]

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The synergistic action of certain inorganic and polyhydroxy organic acids with phenolic inhibitors in the stabilization of fat substrates has long been a matter of interest. Since α-tocopherol is oxidized to α-tocopherylquinone in autoxidizing fat, a study was undertaken of the action of various acids on solutions of α-tocopherylquinone under reflux. In the presence of phosphoric, citric or tartaric acid, a dismutation takes place: α-tocopherol is formed along with the red o-quinone and other oxidation products, separable by chromatographic methods. It may therefore be assumed that the regeneration of α-tocopherol from α-tocopherylquinone is involved in the synergistic action of these acids in prolonging the induction period of fats. Pure α-tocopherylquinone was conveniently prepared from α-tocopherol by oxidation with lead tetraacetate. This reaction can also be applied in the quantitative determination of α-tocopherol. A dimer was formed by oxidation of α-tocopherol with potassium permanganate in the cold.

Introduction

The antioxygenic action of o- and p-phenols and of α-tocopherol in fat substrates is prolonged by the addition of certain acids (phosphoric, sulfuric, citric, tartaric, etc.) which are not active alone.[3] Their role is not well understood. Apparently they have some action as metal scavengers,[4] precipitating traces of contaminants which would otherwise act as catalysts of fat peroxide formation. However, since purified esters of lard fatty acids are similarly stabilized,[3] these acids must have some other action also. Certain other acids, such as oxalic and gallic, are not only synergists but are also stabilizers by themselves—oxalic probably because of the nature of the bond between the two carbons which provides the equivalent of two OH groups in the ortho position[5] and gallic because of its phenolic nature.[6]

The synergistic action of ascorbic acid appears to be dependent on its oxidation in the presence of phenolic inhibitors, keeping them in the reduced state by providing hydrogen.[7,8]

With the exception of pyruvic acid, the organic acids which act as synergists are generally di- or tricarboxylic acids with a functional group (keto, hydroxy, unsaturation, etc.) adjacent to the carboxyl. It has been suggested[9] that the synergism of these acids (and of phosphoric and sulfuric acids) is owing to the presence of two or more hydroxyl groups, through which complexes can be formed with quinone or quinol and an activated fat acid, thereby deactivating the latter.

Since acidity favors the reaction quinol⇌quinone toward the quinol, this might explain the synergism of acids with quinones and hydroquinones. However, in the case of tocopherylquinone, the reaction is more complicated, because the corresponding hydroquinone, tocopherylhydroquinone, has no antioxygenic action *in vitro* and no biological activity in rats. However, a tocopherol-like substance, responding to the Emmerie and Engel test and possessing biological activity, was formed from tocopherylquinone in the presence of H₃PO₄

in purified esters of lard fatty acids.[10] Thus, reduction as well as cyclization must have taken place in order to regenerate tocopherol.

In the experiments here described, pure α-tocopherylquinone dissolved in pure methanol and refluxed overnight in the presence of H_3PO_4 (0.2 mole/liter of methanol) changed from a yellow to a dark red oil which was active as an antioxidant and had 15–20% of the biological activity of dl-α-tocopherol, thus confirming the former observations.[10] The isolated oil contained no phosphorus; when H_3PO_4 containing P^{32} was used, the oil had no appreciable radioactivity. Such treatment produced no change in α-tocopherol, even after 24 hours of refluxing. In the absence of H_3PO_4 tocopherylquinone remained unchanged and in a solution containing equimolar quantities of tocopherol and the quinone, refluxed without acid for 24 hours, the characteristic absorption maxima of both substances were still present and the reducing properties were unchanged.

The dark red oil was fractionated by chromatographic methods. The proportions of the various fractions varied with the time of refluxing. Thus, after five hours, the isolated oil gave an Emmerie and Engel[11] (E. and E.) value of 20% of that of α-tocopherol. Most of the fractions of the chromatogram showed maxima around 260 mμ corresponding to quinones. The lowest fraction had an E (1%, 1 cm.) of 80 at 298 mμ and an E. and E. value of 100%, both data indicating α-tocopherol.

After 14 hours of refluxing the E. and E. value increased to 57%. The visible spectrum showed the presence of red o-quinone and chromatographic separation yielded quinones and about 50% α-tocopherol. After 24 hours of refluxing the E. and E. value was 55–70%, and one of the chromatographic fractions was again α-tocopherol.

The use of CCl₄ as a solvent gave no red o-quinone, in accordance with the findings of Smith, et al.[12] After 24 hours, however, the E. and E. value was 34%. The same figure was obtained after refluxing for 20 hours in glacial acetic acid (20 moles/liter). Chromatographic separation gave various zones which could not be identified.

The action of HCl (0.4 mole/liter) in a methanol solution of α-tocopherylquinone seemed to be similar to that of phosphoric; tocopherol and some red o-quinone were formed. Citric and tartaric

(1) Supported in part by a grant from Swift and Company, Chicago, Illinois.

(2) Institute for Enzyme Research, University of Wisconsin, Madison.

(3) H. S. Olcott and H. A. Mattill, THIS JOURNAL, **58**, 2204 (1936).

(4) H. J. Dutton, A. W. Schwab, H. A. Moser and J. C. Cowan, *J. Am. Oil Chem. Soc.*, **15**, 385 (1948).

(5) H. A. Mattill, *Oil & Soap*, **22**, 1 (1945).

(6) C. Golumbic and H. A. Mattill, *ibid.*, **19**, 144 (1942).

(7) C. Golumbic and H. A. Mattill, THIS JOURNAL, **63**, 1279 (1941).

(8) V. P. Calkins and H. A. Mattill, *ibid.*, **66**, 239 (1944).

(9) V. P. Calkins, *ibid.*, **69**, 384 (1947).

(10) C. Golumbic, *Oil & Soap*, **19**, 181 (1942).

(11) L. Emmerie and C. Engel, *Rec. trav. chim.*, **57**, 1351 (1938).

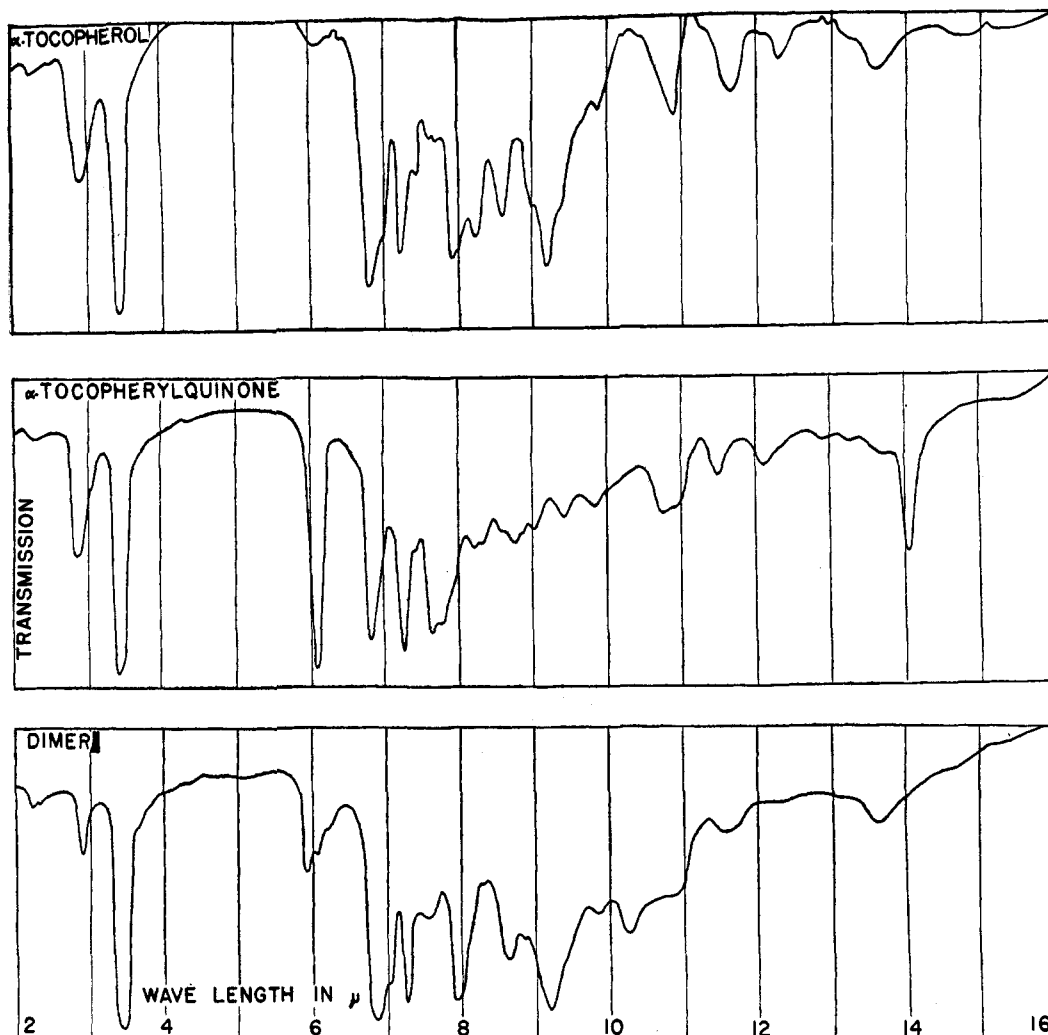(12) L. I. Smith, W. B. Irwin and H. E. Ungnade, THIS JOURNAL, **61**, 2424 (1939).

Fig. 1.

acids produced the same results when they were used in higher concentrations (0.4 and 0.3 mole/liter, respectively).

Oxygen is not concerned in this reaction, because the red o-quinone was formed by refluxing in the presence of acids even in a nitrogen atmosphere; when methanol freshly distilled over Mg turnings was used, the formation of formaldehyde was demonstrated, an indication that the solvent might have taken part in the reaction.

An explanation for the formation of both α-tocopherol and the red o-quinone from tocopherylquinone is that the latter undergoes a dismutation. The solvent seems to play a role somewhat as in the formation of the red o-quinone by oxidation of tocopherol with HNO_3. A portion of tocopherylquinone is reduced to tocopherylhydroquinone which in the presence of acids cyclizes to form α-tocopherol, whereas another portion is oxidized to the red o-quinone and other oxidation products.

Since hydrogen has been found in the gaseous products of rancidifying fats,[13] the reduction of α-tocopherylquinone to the hydroquinone and the

cyclization to tocopherol in the presence of acids have a likely explanation.

However, in the present experiments there was no fat. In observations on fat substrates,[14] the red o-quinone was not produced when α-tocopherol, added as a stabilizer, was oxidized during the autoxidation of the fat. On the other hand, during the autoxidation of vegetable fats containing mixed tocopherols, the red o-quinone appeared in variable amounts and was considered to have its origin in γ-tocopherol.[15] This compound lacks a methyl group on carbon 5. The ease of formation of the red o-quinones by oxidation of any of the tocopherols with silver nitrate depends on the substituent on carbon 5 and was found to decrease in the order γ, β, α.[16] It may therefore be concluded that the formation of the red o-quinone from α-tocopherylquinone is due to the more ready displacement of the methyl group on carbon 5 in the presence of certain acids and possibly also to the availability of traces of hydrogen from the solvent. Whether these factors are concerned with a possible regeneration of α-tocopherol and with the syner-

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(14) C. Golumbic, *Oil & Soap*, **20**, 105 (1943).

(15) C. E. Swift, G. E. Mann and G. S. Fisher, *ibid.*, **21**, 317 (1944).

(16) J. G. Baxter, C. D. Robeson, J. D. Taylor and R. W. Lehman, THIS JOURNAL, **65**, 918 (1943).

gistic action of polyhydroxy acids in fat substrates remains to be further explored.

Occasionally, following the action of phosphoric acid on tocopherylquinone, a compound appeared in the chromatographic column having an absorption spectrum resembling that of α-tocopherol (max. 290–292 mμ) but with little or no reducing properties. The amounts formed were variable and rather small.

Information was at hand[17] that two compounds, having similar spectra—max. at ca. 290 mμ— and both of them dimers, could be prepared, one of them by heating α-tocopheroxide,[18] which was non-reducing, the other by oxidizing α-tocopherol in alkaline solution; the latter had 50% of the reducing properties of α-tocopherol.

Molecular weight determination on this present compound showed that it too was a dimer, and oxidation of an acetone solution of α-tocopherol with potassium permanganate in the cold gave the compound in 50% yield along with some epoxy compound, red o-quinone and α-tocopherylquinone. Shortly after its preparation, the E (1%, 1 cm.) max. at 290 mμ was 106, and the E. and E. value was 70% of that of α-tocopherol. The latter value increased during the determination and reached 90% after 5–10 min. Several days after the preparation, both the E (1%, 1 cm.) max. and the E. and E. value decreased without any direct correlation. Analysis of the compound gave the empirical formula $C_{13}H_{21}O$, or a multiple, while the molecular weight indicated that it was probably four times that. The compound differed in the ultraviolet and infrared spectra[19] from that of the lactone, $C_{21}H_{40}O_2$,[20] obtained by refluxing an acetone solution of α-tocopherol with potassium permanganate.

Experimental

Preparation of Pure α-Tocopherylquinone.—Of the various methods used for the oxidation of α-tocopherol to α-tocopherylquinone (FeCl$_3$, AuCl$_3$, Ce(SO$_4$)$_2$), a procedure employing lead tetraacetate in acetic acid was found to be most convenient.

3.4 grams of dl-α-tocopherol[21] was dissolved in 70 ml. of glacial acetic acid and Pb(OCOCH$_3$)$_4$ was added until the diphenylamine indicator turned blue (3.5 g.). The solution was immediately diluted with 100 ml. of water and extracted with petroleum ether. The petroleum ether layer was washed several times with water, 5% NaHCO$_3$ solution and water and dried with Na$_2$SO$_4$. The petroleum ether was evaporated and the yellow oil usually required no further purification. It was dissolved in Skellysolve B (SSB) and chromatographed through a column of silicic acid and celite (1:1).

Zone I, E (1%, 1 cm.) at 268 mμ, 422; E. and E. 1%; yield 3.15 g., 90%.

Zone II, E (1%, 1 cm.) at 268 mμ, 390; E. and E. 1%; yield 0.20 g.

For quantitative determination of tocopherol a method similar to the one described[22,23] for the determination of

glycols was used. The iodine liberated from potassium iodide by the excess lead tetraacetate was titrated with thiosulfate. The end-point is sharper than in other titrimetric methods.

Fifty to one hundred mg. of dl-α-tocopherol was dissolved in 10 ml. of purified glacial acetic acid. Ten ml. of a 0.1 N of Pb(OCOCH$_3$)$_4$ solution in acetic acid was added and immediately thereafter 10 ml. of a KI solution containing 20 g. of KI and 500 g. of sodium acetate in 1000 ml. of water. For the titration 0.02 N Na$_2$S$_2$O$_3$ was used. The blank consisted of 10 ml. of 0.1 N Pb(OCOCH$_3$)$_4$ diluted with 10 ml. of acetic acid. Determination: amount, 97.5 mg., found, 95.5 mg. Amount, 60 mg., found, 58.5 mg. Accuracy, about 2%.

Refluxing of Tocopherylquinone with H$_3$PO$_4$ in Methanol. —One hundred mg. of α-tocopherylquinone was dissolved in 20 ml. of methanol and 0.2 ml. of 85% H$_3$PO$_4$ was added. There was no change in the spectrum when this solution stood overnight. After refluxing for 5 hours, the spectrum in the visible showed the presence of some red o-quinone. The methanol was evaporated under nitrogen, and the water layer was extracted with petroleum ether. The petroleum ether was washed with water, dried with Na$_2$SO$_4$ and evaporated under nitrogen. It was dissolved in SSB and chromatographed through a column of silicic acid and celite (1:1). Fractions I, II and III had maxima around 260 mμ and E and E values of about 10%. For fraction IV, E (1%, 1 cm.) was 80 at 298 mμ and E and E 100%. Its infrared spectrum was the same as that of α-tocopherol. Oxidation of this fraction with Pb(OCOCH$_3$)$_4$ gave almost pure tocopherylquinone. The procedures for the several 14- and 24-hour runs were essentially the same.

Biological Activity.—The biological activity of the product of the action of phosphoric acid on α-tocopherylquinone was determined on vitamin E-deficient rats according to the method described by Mason and Harris,[24] and two individual products were tested, each of them on about twenty animals, at three or four levels.

Oxidation of Tocopherol with KMnO$_4$.—Two grams of α-tocopherol was dissolved in 50 ml. of pure acetone. Two per cent. aqueous KMnO$_4$ was added until it did not decolorize immediately. After dilution with water and extraction with ether, the ether layer was washed several times with water, dried with Na$_2$SO$_4$, and the ether evaporated under nitrogen. The oil was dissolved in SSB and chromatographed through a column of silicic acid and celite (1:1).

Zone I, Yellow, 98 mg., E. and E. 1%, max. at 250 mμ. The compound liberated iodine from sodium iodide in acetic anhydride (epoxy compound).

Zone II, Red, 221 mg., E. and E. 1%, max. at 260–270 mμ. With ethylaniline a red color (tocopherylquinone).

Zone III, Red, 114 mg., E. and E. 1%, max. 260–270 mμ.

Zone IV, Red, 99 mg., E. and E., 5%. Small max. at 260 and 290 mμ.

Zone V, Red, 497 mg., E. and E., 9%, max. at 296 mμ.

Zone VI, Yellow, 900 mg., E. and E., 70%, increased to 90% during the determination. E (1%, 1 cm.) was 106 at 290 mμ (dimer). After standing for 6 days, E (1%, 1 cm.) was 70.5 and E. and E., 3%.

Molecular Weight of Dimer.—The Menzies–Wright apparatus as modified by Hanson was used with purified acetone as solvent and a cup of platinum foil to introduce the viscous liquid. The figures obtained were 879 and 887.6; average, 883.

Anal.[25]: Found: C, 80.17, 80.32; H, 11.34, 11.14.

Heating with 1% NaOH for five minutes did not change the spectrum; this remained unchanged after oxidation with Pb(OCOCH$_3$)$_4$ or hydrogenation with Pd on CaCO$_3$ for one-half hour under 20 pounds pressure. The compound gave no derivative with 2,4-dinitrophenylhydrazine and did not form hydroxamic acid on treatment with hydroxylamine.

(17) Personal communication from Dr. Paul D. Boyer.

(18) P. D. Boyer, M. D. Rabinovitz and E. Liebe, Ann. N. Y. Acad. Sci., 52, 188 (1949); P. D. Boyer, THIS JOURNAL, 73, 733 (1951).

(19) The infrared spectra of α-tocopherol, the quinone and the dimer were determined by S. P. Sadtler, Philadelphia, and we are indebted to Universal Oil Products for spectra of the dimer and the lactone.

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(21) Kindly supplied by Merck and Company, Inc., Rahway, New Jersey.

(22) R. Criegee, Ber., 64B, 260 (1931).

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(24) K. E. Mason and P. L. Harris, Biol. Symposia, 12, 459 (1947).

(25) Kindly made by Mr. C. E. Steinle.