α-Tocopherol Prooxidant Effect in Aqueous Media: Increased Autoxidation Rate of Linoleic Acid

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ABSTRACT

The prooxidant effect of α-tocopherol was investigated during linoleic acid autoxidation in an aqueous media, at pH 6.9. α-Tocopherol (1.25×10^{-4} M) was added to linoleic acid (2.5×10^{-3} M) and the linoleic acid autoxidation rate was evaluated using 2 methods: spectrophotometric measurement at 234 nm exhibited an important increase of conjugated dienes after α-tocopherol addition, especially during the first 4 days, and gas chromatographic measurement of unoxidized linoleic acid showed an important degradation of linoleic acid in the presence of α-tocopherol. During the prooxidant reaction, α-tocopherol was rapidly oxidized; it was detected as traces by thin layer chromatography after 4 days of experimentation. Two oxidation products of α-tocopherol have been identified: α-tocopherylquinone and a dimer of α-tocopherol.

INTRODUCTION

α-Tocopherol (AH) is commonly used as an antioxidant. It works as a radical scavenger and breaks down the propagation of lipids (RH) autoxidation according to the mechanism proposed by Mahoney (1),

$$R\text{-}R \xrightarrow{k1} 2\,R^{\bullet} \qquad [I]$$

$$R^{\bullet} + O_2 \xrightarrow{k2} ROO^{\bullet} \qquad [II]$$

$$ROO^{\bullet} + RH \xrightarrow{k3} ROOH + R^{\bullet} \qquad [III]$$

$$ROO^{\bullet} + AH \underset{k-4}{\overset{k4}{\rightleftharpoons}} ROOH + A^{\bullet} \qquad [IV,-IV]$$

$$A^{\bullet} + RH \xrightarrow{k5} R^{\bullet} + AH \qquad [V]$$

where R - R is the initiator and R• and ROO• represent the carbon radical and the chain carrying peroxyl radical, respectively. A• is the free radical derived from α-tocopherol.

Mahoney notes that reaction -IV and reaction V can take place with the nonhindered phenols as antioxidants and the phenoxy radical A• efficiently restarts the chains by reaction -IV and/or V.

The antioxidant activity of α-tocopherol can be reversed to a prooxidant activity especially when the concentration of the antioxidant increases (2,3).

This prooxidant effect of α-tocopherol has been described in oils such as grape seed oil (4), soybean oil (5) or cottonseed oil (6). We have shown a prooxidant effect of α-tocopherol in aqueous media during linoleic acid autoxidation (7-8). Available quantitative analysis of fatty acid autoxidation rates entailed difficulties because the autoxidation process was complex; primary oxidation lead to hydroperoxides which are unstable compounds and secondary oxidation rapidly took place involving the formation of aldehydes, alcohols and acids. Various procedures have been described to measure fatty acid autoxidation. We only reported the principal methods applied to emulsified oils: (a) the measurement of oxygen consumption, which was achieved by manometric methods (9-12); (b) the measurement of increasing conjugated dienes at 234 nm, which permitted the evaluation of fatty acid autoxidation in the early stages. This method was useful for emulsified oils (13-14).

The purpose of this work was to study a quantitative analysis of the prooxidant effect of α-tocopherol during linoleic acid autoxidation in an aqueous media.

Two methods were compared: spectrophotometric measurement of conjugated dienes at 234 nm and gas chromatographic measurement of unoxidized linoleic acid, according to the Rogstad et al. method (15) described for testing antioxidants during enzymatic oxidation of linoleic acid.

EXPERIMENTAL PROCEDURE

Materials

Linoleic acid (9,12-octadecadienoic acid) was purchased from Koch Light England (≥ 99% pure). This acid was dispersed with 0.5% Tween 20 (Merck) in 0.025 M phosphate buffered aqueous solution (pH = 9.0), under nitrogen atmosphere. Linoleic acid concentration was 10^{-2} M and this stock dispersion was stored at 4 C.

α-Tocopherol was synthesized and supplied by Hoffmann LaRoche-France. It was dispersed with Tween 20 in phosphate buffer (pH = 7.0) according to the same procedure described for linoleic acid. The final concentration of α-tocopherol was 5×10^{-4} M and the dispersion was stored at 4 C.

Procedure

Aliquots of each stock dispersion were adjusted to pH 6.9 and mixed at time zero (Table I). Samples (100 ml each) were placed in glass tubes and left in the dark under air at room temperature. Controls without linoleic acid were placed in similar conditions.

Conjugated Dienes Measurement at 234 nm

Diene measurement was performed using a Pye Unicam SP 800 spectrophotometer. Readings were done at 2 h intervals during the first 10 hr and subsequently every 24 h.

Linoleic Acid Gas Chromatography

Gas chromatography was carried out at the same time as the spectrophotometric readings.

Extraction from aqueous media. A 10-ml sample was mixed with 6 ml of a chloroform-methanol mixture (1:1, v/v) and then stirred for 3 min with a Vortex Vibrator. After centrifugation, the lower phase was recovered and the upper phase was reextracted twice with 3 ml chloroform. The organic fractions were combined and evaporated to dryness under reduced pressure at low temperature on a rotary evaporator.

TABLE I

Experimental Conditions for Investigation of α-Tocopherol Prooxidant Effect in Aqueous Media

Samples	L.A.[a] (mol)	α-T[b] (Mol)	Tween 20 (ml)	Solvent[c] up to	Mol α-T/ mol of linoleic acid	pH
S	2.5×10^{-3}	0	5	1,000	0	6.9
$S_{\alpha-T}$	2.5×10^{-3}	1.25×10^{-4}	5	1,000	5×10^{-2}	6.9

[a]L.A. = linoleic acid.

[b]α-T = α-tocopherol.

[c]Solvent = phosphate buffered aqueous solution, 0.025 M, pH 6.9.

TABLE II

Standard Mixtures for Linoleic Acid Gas Chromatography

	Standards					
	1	2	3	4	5	6
Methyl arachidate[a] (ml)	1	1	1	1	1	1
Methyl linoleate[b] (ml)	0.25	0.50	0.75	1	1.25	1.50
Chloroform up to (ml)	50	50	50	50	50	50
Methyl arachidate concentration (mg/100 ml)	46	46	46	46	46	46
Methyl linoleate concentration (mg/100 ml)	11.5	23	34.5	46	57.5	69
$\frac{hL^c}{hA^d}$	0.30	0.60	0.90	1.20	1.47	1.68

[a]Stock solution of methyl arachidate dissolved in chloroform (23 mg/ml).

[b]Stock solution of methyl linoleate dissolved in chloroform (23 mg/ml).

[c]Peak height of methyl linoleate on the gas chromatogram.

[d]Peak height of methyl arachidate on the gas chromatogram.

Preparation of the methyl ester. The dry residue was dissolved in 2 ml boron trifluoride methanol (14% BF_3 in methanol, Merck) and refluxed for 3 min. One ml distilled water was put through the condenser to stop the reaction.

At this stage, 200 μl internal standard solution was added to the previous mixture. The standard solution was composed of methyl arachidate (methyl eicosanoate, Merck) dissolved in methanol at a concentration of 23 mg/ml. Methyl esters of fatty acids were extracted with 3 x 2 ml chloroform. The chloroform extracts were evaporated to dryness and the residue was dissolved with 10 ml chloroform. This solution (0.5:1 μl) was directly injected into the gas chromatograph.

Gas chromatography. GC was achieved using a Pye Unicam 104 Model fitted with a flame ionization detector and a 5 ft x 1/8 in. glass column packed with 10% DEGS on 80/100 mesh Chromosorb W HP. Operating conditions were: column temperature, 180 C; injector-detector temperature, 240 C; carrier gas, nitrogen "U"; flow rate, 60 ml/min.

Standard curve: Standards of varying concentrations of methyl linoleate and fixed concentrations of methyl arachidate were prepared by volumetric dilution of the methyl ester stock solutions with chloroform (Table II).

The ratio of the peak height of methyl linoleate and methyl arachidate was calculated and plotted against the concentration of linoleic acid. A least squares linear regression was performed; the correlation coefficient obtained was 0.998.

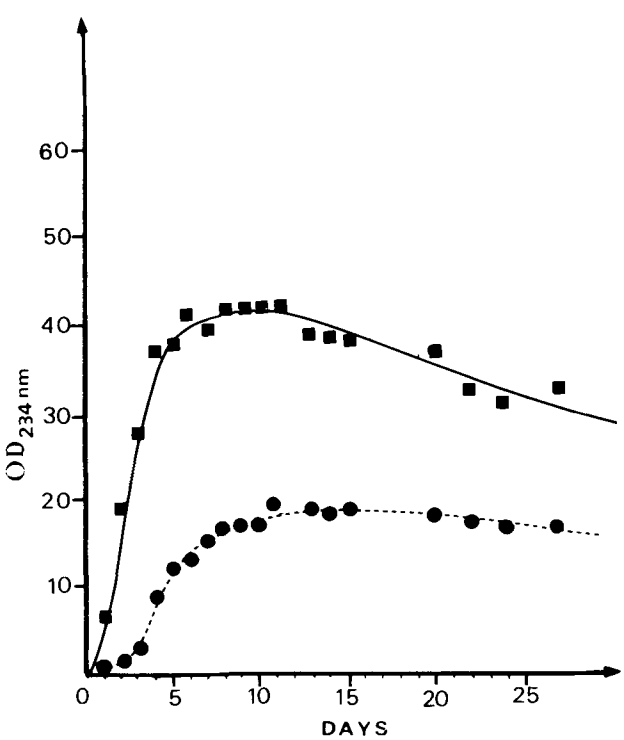

FIG. 1. Conjugated diene measurement during linoleic acid autoxidation in aqueous media. •: linoleic acid without α-T; ▪: linoleic acid with α-T (5×10^{-2} M).

Thin Layer Chromatography of α-Tocopherol

Thin layer chromatography (TLC) was carried out on Silica-Gel F_{254}, 0.25 mm thick. The solvent was chloroform in a saturated chamber. Aliquots of the samples were evaporated to dryness under reduced pressure on a rotary evaporator. The dry residue was dissolved with 0.5 ml chloroform and 20 µl of this solution was chromatographed.

α-Tocopherol and its oxidation products were visualized by spraying with a sulfomolybdic reagent and after heating at 120 C for 5 min, the compounds appeared as blue spots.

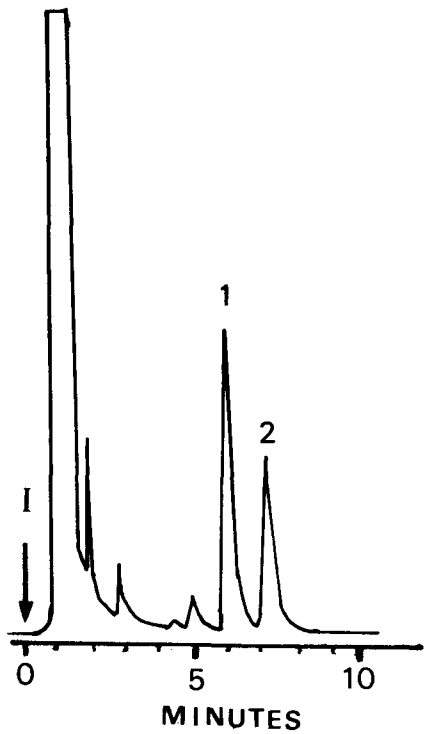

FIG. 2. Gas liquid chromatogram of methyl linoleate. 1: Methyl linoleate; 2: methyl arachidate (reference).

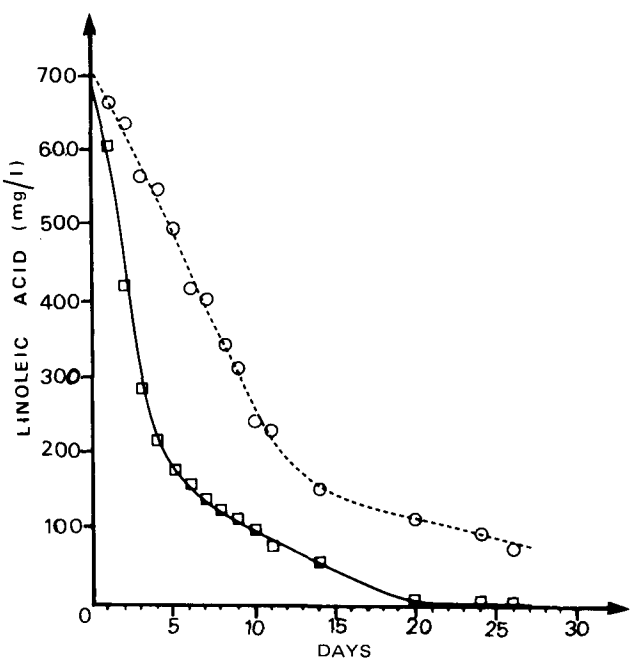

FIG. 3. Linoleic acid measurement by GLC after extraction from aqueous media and methylation. ○: linoleic acid without α-T; □: linoleic acid with α-T (5×10^{-2} M).

RESULTS AND DISCUSSION

Conjugated Diene Measurement (od 234 nm)

The addition of α-tocopherol to linoleic acid involved an important increase of conjugated dienes, especially during the first 4 days of experimentation (Fig. 1). The conjugated diene level reached a maximum as early as the 6th day in the sample with added α-tocopherol and only after 11 days in the sample without α-tocopherol. In the sample without α-tocopherol, the maximal conjugated diene concentration was twice as weak as the one observed after α-tocopherol addition.

Unoxidized Linoleic Acid Measurement

Linoleic acid extraction and methylation yielded 100% recovery of linoleic acid. Methyl linoleate was eluted after 6.5 min (Fig. 2).

The linoleic acid concentration decreased rapidly in the presence of α-tocopherol, especially during the first 4 days (Fig. 3). On the 4th day of experimentation, 70% of the linoleic acid was oxidized in the presence of α-tocopherol whereas in the absence of α-tocopherol, only 25% of the linoleic acid was oxidized. After the 4th day, we noted a decreased linoleic acid autoxidation rate in the sample with α-tocopherol since 30% of linoleic acid still present was completely oxidized only after 13 days of experimentation.

Thin Layer Chromatography of α-Tocopherol

α-Tocopherol was rapidly oxidized during the prooxidant reaction (Fig. 4). On the 4th day of experimentation only traces of α-tocopherol could be detected on the chromatogram. Two oxidation products have been identified: α-tocopherylquinone, which was the major oxidation product, and a dimer of α-tocopherol, detected at the

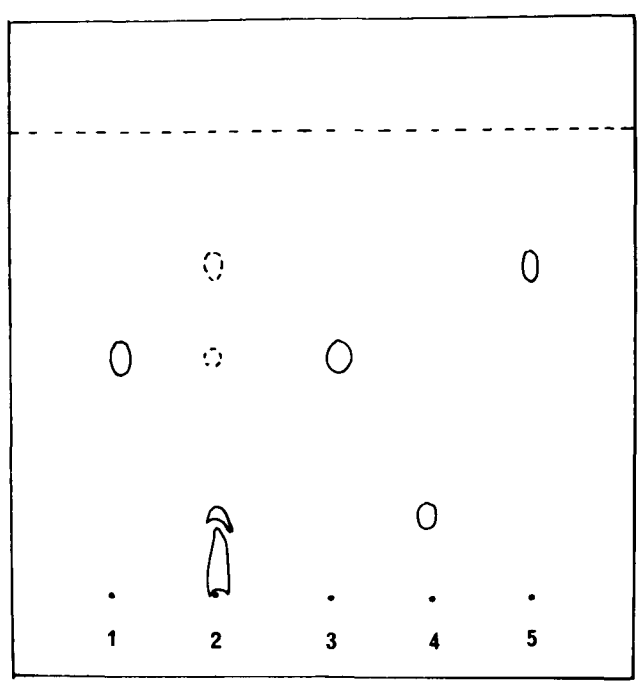

FIG. 4. Thin layer chromatogram of α-tocopherol and its oxidation products after 4 days of experimentation. 1: control α-tocopherol in aqueous solution; 2: sample α-tocopherol with linoleic acid in aqueous solution; 3: reference α-tocopherol; 4: reference α-tocopherylquinone; 5: reference dimer of α-tocopherol.

beginning of experimentation.

Both methods used for quantitative analysis of linoleic acid autoxidation rate exhibited a prooxidant effect of α-tocopherol that took place during the first 4 days of experimentation. At the end of this period, α-tocopherol was oxidized for the most part and we noted a decreased autoxidation rate of linoleic acid.

GC measurement of unoxidized linoleic acid was the best method for quantitative analysis of fatty acid autoxidation rate in the presence and in the absence of α-tocopherol. In addition, during the prooxidant effect of α-tocopherol (first 4 days), conjugated diene measurement permitted an available evaluation of fatty acid autoxidation rate because the rate of conjugated dienes degradation was negligible compared to the rate of conjugated dienes formation. This method has the advantage of being easy and rapid. After the first 4 days, the conjugated diene levels rapidly reached a maximum while gas chromatographic measurement of linoleic acid indicated a further oxidation of the fatty acid.

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REFERENCES

1. Mahoney, L.R., Angew. Chem. Int. Ed. Eng. 8:547 (1969).
2. Labuza, T.P., CRC Crit. Rev. Food Technol. 2:355 (1971).
3. Naudet, M., Labo-Pharma. Prob. Tech. 215:61 Nov. (1972).
4. Dubois, P., Ann. Technol. Agric. 13:97 (1964).
5. Loury, M., C. Bloch and R. Francois, 13:747 (1966).
6. Khafisov, R., N.I. Dzhura and N.K. Nadirov, Izv. Vyssh. Uchebn. Zaved Pishch. Tekhnol. 4:37 (1975).
7. Cillard, J., M. Cormier and L. Girre, C.R. Acad. Sc. Paris, t. 281, 4, 11, 18 (1975).
8. Cillard, J., M. Cormier, P. Cillard and L. Girre, Ann. Nutr. Alim. 31:27 (1977).
9. Morita, M., M. Tanaka, Y. Takayama and Y. Yamamoto, JAOCS 53:489 (1976).
10. Labuza, T.P., H. Tsuyuki and M. Karel, Ibid. 46:409 (1969).
11. Marcuse, R. and P.O. Fredriksson, Ibid. 46:262 (1969).
12. Chan, H.W.S., Ibid. 54:100 (1977).
13. Sengupta, A. and S.P. Mehta, Indian J. Technol. 3:254 (1965).
14. Rhodes, C.T., Can. J. Pharm. Sci. 2:16 (1967).
15. Rogstad, A. and R. Reinton, JAOCS 54:282 (1977).

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Effect of Experimental Factors on the Prooxidant Behavior of α-Tocopherol

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ABSTRACT

We have investigated the effect of experimental factors on the prooxidant effect of α-tocopherol during the autoxidation of linoleic acid. The prooxidant effect depended on two factors: the concentration of α-tocopherol ($\geq 5 \times 10^{-3}$ mol α-tocopherol/1 mol linoleic acid) and the solvent, an aqueous system in which the prooxidant effect occurred more easily. On the other hand, the prooxidant behavior of α-tocopherol was unaffected by the type of surfactant used in water as well as by the presence of different salts. The initial content of hydroperoxides affected the intensity of the prooxidant effect which varied in an inverse ratio to the initial hydroperoxide level.

INTRODUCTION

Most of the observations on the prooxidant effect of α-tocopherol have been established in oils (1-3). It is a general fact that some phenols used as antioxidants can promote a prooxidant reaction, especially beyond a certain limit of concentration (1-4). Loury et al. (2) reported a limit of 0.1% α-tocopherol in soybean oil. Witting (5) showed that during the autoxidation of methyl or ethyl linoleate, the addition of α-tocopherol as antioxidant was ineffective when the ratio linoleate: α-tocopherol was inferior to 10^3. He founded that, in this case, the concentration of hydroperoxides increased. At high concentrations, these phenols did not act as free radical scavengers but as free radical chain breakers (4):

Antioxidant $\quad ROO^{\bullet} + AOH \rightarrow ROOH + AO^{\bullet} \quad [I]$

Prooxidant $\quad ROOH + A^{\bullet} \rightarrow ROO^{\bullet} + AH \quad [II]$

or

$\quad RH + A^{\bullet} \rightarrow R^{\bullet} + AH \quad [III]$

Thus, the solvent system has significant effects on lipid autoxidation rate; it affected the metal catalysis in lipid autoxidation. The solvent could inhibit metal catalysis either by formation of a solvent-metal complex (6) or by formation of hydrogen bond between solvent and hydroperoxide preventing hydroperoxide decomposition (4,7). This inhibition was observed with polar solvents such as ethanol (7,8), ethyl acetate (8), acetic acid (7) and water at low moisture content (4). On the other hand, a solvent such as water at high moisture content (4) could enhance the metal catalysis by increasing the mobility of the metal catalyst. Moreover, some solvents such as ethanol, formate and benzoate can act as hydroxyl scavengers (9). During the autoxidation of fatty acids, hydroxyl radicals were formed by the decomposition of hydroperoxides. This work studied the influence of the solvent and of the concentration of α-tocopherol on the advent of the prooxidant effect of α-tocopherol. The effect of water was largely investigated because of its important role in food oxidation. Inherent factors in the use of water were thereby studied; with emphasis on the effect of the surfactant which is required to disperse linoleic acid and α-tocopherol, as well as the effect of the presence of different salts.

EXPERIMENTAL

Material

Solvents and surfactants. Linoleic acid autoxidation with and without α-tocopherol has been carried out in different solvents such as polar protic solvents (deionized water or ethanol: Normapur, Prolabo-France), polar aprotic solvents