Behavior of Alpha, Gamma, and Delta Tocopherols with Linoleic Acid in Aqueous Media.

J. CILLARD and P. CILLARD, Laboratoire de Botanique Pharmaceutique,
U.E.R. "Médicament," Avenue du Pr. Léon-Bernard, RENNES Cédex 35043 FRANCE

ABSTRACT

Tocopherols can exhibit opposite effects in aqueous media on linoleic acid autoxidation rate. The effect which was observed depended on tocopherol concentration and on the tocopherol itself. At 0.05 mole of tocopherol per mole of linoleic acid, α-tocopherol was prooxidant while in similar conditions, δ-tocopherol was antioxidant as well as γ-tocopherol. However, this latter one exhibited a slight antioxidant activity. When tocopherol concentration decreased (twice as weak), α-tocopherol still exhibited the same prooxidant activity, while the antioxidant effect of γ and δ tocopherols was increased. The study of tocopherol stability by HPLC has shown that tocopherol oxidation increased in order $\delta<\gamma<\alpha$. There was a relationship between the ability for a tocopherol to be easily oxidized by air and its prooxidant activity. Tocopherol oxidation would enhance the formation of a perhydroxyl radical ([•OOH] or one of this type [$O_2^{\doteq}$, •OH]) which was responsible for the prooxidant effect.

INTRODUCTION

Tocopherols, and especially α-tocopherol, were commonly used as antioxidants in oil as well as in aqueous media (1). Such compounds were easily oxidized by air in order α-T>γ-T>δ-T and consequently the antioxidant activity was in reverse order (2). We know that in particular conditions these tocopherols can promote a prooxidant effect in oil as well as in aqueous media.

Dubois (3) reported a prooxidant effect in grape seed oil enriched with tocopherols. According to him tocopherols increased hydroperoxide decomposition which involved free radical formation (RO•). This free radical would enhance fatty acid autoxidation.

Hydroperoxide decomposition: $ROOH \rightarrow RO\bullet + \bullet OH$

Prooxidant effect:

$$RO\bullet + RH \rightarrow ROH + R\bullet$$

$$R\bullet + O_2 \rightarrow ROO\bullet$$

$$ROO\bullet + RH \rightarrow ROOH + R\bullet$$

Loury et al. (4), after investigating about the conditions for using α-tocopherol as antioxidant in fats, reported that prooxidant effect occurred in soybean oil when α-tocopherol was present at a concentration superior to 0.1%. According to these workers, α-tocopherol gave rise to a free radical (α-T•) which arose in the propagation chain of fatty acid autoxidation.

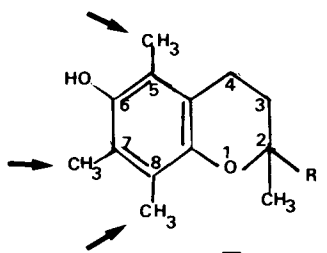
$$\alpha\text{-T}\bullet + \begin{matrix}RH\\ \text{or}\\ ROOH\end{matrix} \rightarrow \alpha\text{-TH} + \begin{matrix}R\bullet\\ \text{or}\\ ROO\bullet\end{matrix}$$

Similar observations have been reported by Khafisov et al. (5) about cottonseed oil. Tocopherols exhibited a maximum antioxidant effect at a concentration of 0.05 to 0.07%. γ-Tocopherol was a better antioxidant (0.025%) than α-Tocopherol (0.075%). At high concentration, prooxidant effect would take place according to the same mechanism (6) as the previous one described by Loury et al.

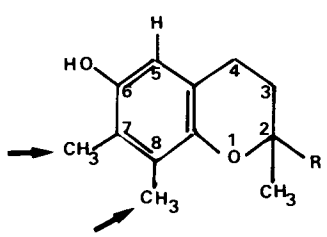
Prooxidant effect of tocopherol has also been observed in aqueous media by themselves (7-8). We noted that α-tocopherol could exhibit either an antioxidant effect when it was used at low concentration ($\leqslant 5 \times 10^{-3}$ mole α-T/mole of linoleic acid) or a prooxidant effect when it was used at high concentration ($> 5 \times 10^{-3}$ mole α-T/mole of linoleic acid). All the previous results concern prooxidant effect observed either with α-tocopherol or with the whole tocopherols without any distinction of the type of tocopherol.

The purpose of this paper is to determine whether other tocopherols such as γ and δ-tocopherols can promote the same prooxidant effect with linoleic acid as α-tocopherol does in aqueous media.

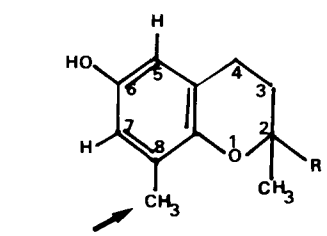
α, γ and δ-Tocopherol are homologous. They differ by the number of methyl substituents on the chromanoxy ring.



α_T



γ_T



δ_T

R = $C_{16}H_{32}$

EXPERIMENTAL PROCEDURE

Reagents

α-, γ- and δ-Tocopherols were synthesized and supplied to us by Hoffmann-LaRoche, France. Linoleic acid was purchased by Koch-Light, England (99/100 puriss); surfactant

TABLE I

Reaction Mixtures for Investigation of α, γ, δ Tocopherols Behavior in Aqueous Media

	So	Sα-T	Sγ-T	Sδ-T	Co	Cα-T	Cγ-T	Cδ-T
Linoleic acid (Mole)	2.5×10^{-3}	2.5×10^{-3}	2.5×10^{-3}	2.5×10^{-3}				
α-tocopherol (Mole)	—	1.25×10^{-4}	—	—	—	1.25×10^{-4}	—	—
γ-tocopherol (Mole)	—	—	1.25×10^{-4}	—	—	—	1.25×10^{-4}	
δ-tocopherol (Mole)	—	—	—	1.25×10^{-4}	—	—	—	1.25×10^{-4}
Concentration ratio[a]		5×10^{-2}	5×10^{-2}	5×10^{-2}				
Tween 20 (ml)	5	5	5	5	5	5	5	5
Solvent[b] up to	1000	1000	1000	1000	1000	1000	1000	1000

[a]Mole number of tocopherols (α or γ or δ) for one mole of linoleic acid.
[b]Phosphate-buffered aqueous solution, 0.025M, pH 6.9.

was tween 20, B.D.H.; the solvent was deionized water; the salt was disodium phosphate, Prolabo R.P.

APPARATUS

A Pye Unicam Model 104 was used for gas chromatography of linoleic acid. High performance liquid chromatograph L.D.C. was purchased from Sopares France and equipped with a constametric II G pump, a Valco 7 000 psi injector and a spectromonitor II UV as detector. This material was used to measure the variations of tocopherol concentration during the experiment.

METHODS

Linoleic acid was dispersed with 0.5% tween 20 (B.D.H.) in phosphate-buffered aqueous solution 0.025 M at pH 9, under nitrogen atmosphere. Linoleic acid concentraton was 10^{-2} M, and it was stored at + 4 C.

Each tocopherol (α,γ,δ) was dispersed with 0.5% tween 20 in phosphate-buffered aqueous solution 0.025 M, at pH 7 under nitrogen atmosphere. Final concentration of each tocopherol was 5×10^{-4}M; the solutions were stored at + 4 C.

Aliquots of each stock solution were adjusted to pH 6.9 and used just starting from zero. The reaction mixtures (Table I) were placed in glass tubes (100 ml) and left in darkness, exposed to air, at room temperature. Controls without linoleic acid were placed in similar conditions.

ANALYSIS

Linoleic acid autoxidation rate in absence and in presence of tocopherols has been followed by gas chromatography measurement of unoxidized linoleic acid. This latter one was extracted from the aqueous media by organic solvent (chloroform/methanol, 1:1, v/v). and after methylation it was measured by gas chromatography on DEGS 10% (8).

Tocopherol oxidation rate was evaluated by an indirect method based on the measure of tocopherol content in samples and controls by high performance liquid chromatography (HPLC). 20 μl of an aqueous solution of tocopherol were added to 20 μl of internal standard. This latter one was composed of a solution of γ-tocopherol at a concentration of 50 μg/ml in a mixture methanol/water, 92.5:7.5, v/v, for α and δ-tocopherols assays. For γ-tocopherol assays we chose a solution of α-tocopherol at 50 μg/ml in the same solvent as previously mentioned. The mixture tocopherol-internal standard was directly injected internally at the top of the column (200 mm x 6.35 mm) and chromatographed on an inverse phase of spherisorb O.D.S. C_{18} 5μ. Tocopherols (α, γ, δ) were eluted with a mixture of methanol/water, 92.5:7.5, v/v. The flow rate was 2 ml/mm. The detection of tocopherols was made at

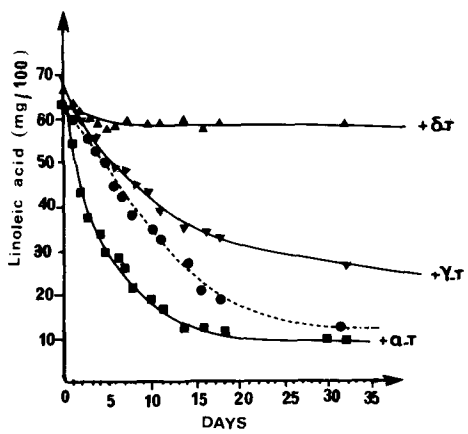


FIG. 1. Measurement of linoleic acid degradation by GLC during its autoxidation in aqueous solution. • = linoleic acid without any tocopherol; ■ = linoleic acid with α-tocopherol (5×10^{-2}M); ▼ = linoleic acid with γ-tocopherol (5×10^{-2}M); ▲ = linoleic acid with δ-tocopherol (5×10^{-2}M).

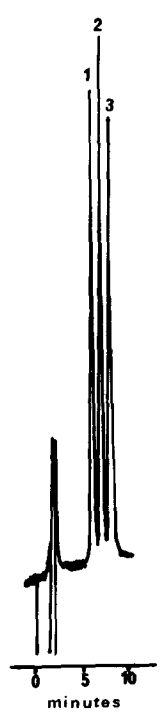


FIG. 2. High performance liquid chromatogram for α, γ and δ tocopherols assays. 1 : δ-tocopherol; 2 : γ-tocopherol; 3 : α-tocopherol.

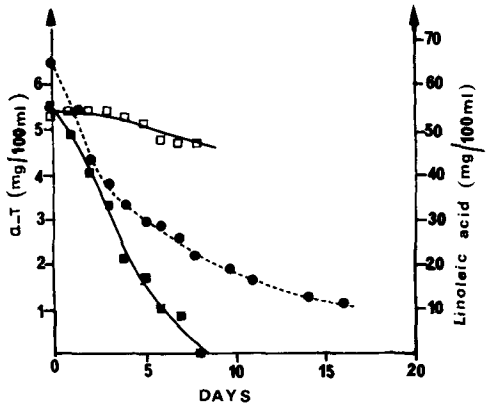


FIG. 3. Measurement of α-tocopherol degradation in aqueous solution by HPLC. ■ = α-tocopherol with linoleic acid (S α-T).□ = α-tocopherol without linoleic acid (Cα-T); ● = linoleic acid degradation in presence of α-tocopherol (S α-T).

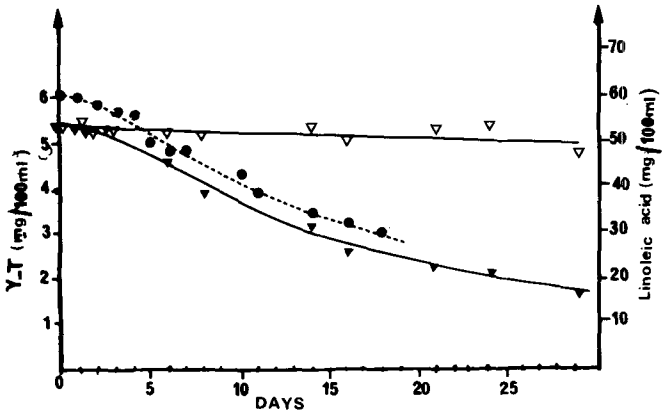


FIG. 4. Measurement of γ-tocopherol degradation in aqueous solution by HPLC. ▼ = γ-tocopherol with linoleic acid (S γ-T); ▽ = γ-tocopherol without linoleic acid (C γ-T); ● = linoleic acid degradation in presence of γ-tocopherol (S γ-T).

294 nm.

The oxidation products of the different tocopherols were detected by thin layer chromatography on silica gel GF_{254} (0.25 mm). Aliquots (5 ml) of aqueous solution of tocopherol were concentrated by evaporating the water under reduced pressure on a rotary evaporator. The dry residue obtained was dissolved with 0.5 ml of chloroform, and 20 µl of this solution was spotted on the plate and chromatographed with chloroform C.S. as solvent. The revelation was obtained by spraying sulfomolybdic reagent and after heating at 120 C for 5 min, the compounds revealed themselves as blue spots.

RESULTS

Linoleic Acid Autoxidation Rate

The addition of a same amount (5×10^{-2}M) of α or γ or δ-tocopherol to one mole of linoleic acid has not produced the same effect on the linoleic acid autoxidation rate. In presence of α-tocopherol, linoleic acid concentration largely decreased especially during the first 4 days because it was rapidly oxidized (Fig. 1). This prooxidant effect has been previously described (7,8). In presence of γ-tocopherol, in similar conditions, linoleic acid concentration decreased in minor ratio than in the sample α-tocopherol and even than in the sample without any tocopherol (Fig. 1). When γ-tocopherol was used at a concentration of 5×10^{-2}M per one mole of linoleic acid, it exhibited a slight antioxidant effect.

The addition of δ-tocopherol in identical manner has shown that linoleic acid concentration remained almost constant during all the experimentation; we have only noted a loss of linoleic acid ca. 10% during the first 2 days (Fig. 1). Linoleic acid autoxidation was greatly inhibited in presence of δ-tocopherol. This latter one exhibited a strong antioxidant activity compared to the other tocopherols. When α, γ and δ-tocopherols are used at a concentration twice as weak (2.5×10^{-2} mole/mole of linoleic acid), we noted that α-tocopherol always exhibited a prooxidant effect; its intensity was identical to the previous one obtained with a concentration twice as strong. On the other hand, the antioxidant effect observed with γ-tocopherol was increased as well as the one observed with δ-tocopherol.

Tocopherols Oxidation Rate

α, γ and δ-Tocopherols were eluted in order: δ (5 min), γ (6 min) and α (7 min) (Fig. 2). These tocopherols exhibited different oxidation rate in presence of linoleic acid as well as in absence of linoleic acid.

α-Tocopherol was the most oxidizable. Its concentration decreased rapidly in presence of linoleic acid. After 3 days of experimentation, 40% of α-tocopherol disappeared and after 9 days, it was completely oxidized (Fig. 3).

α-Tocopherol in aqueous solution without linoleic acid was more stable; however, after 9 days of experimentation nearly 10% of α-tocopherol was oxidized (Fig. 3). In Figure 3 we also reported the curve of linoleic acid autoxidation in presence of α-tocopherol. This latter one revealed an identical profile obtained in the curve of α-tocopherol oxidation in the same sample.

With regards to γ-tocopherol, its oxidation rate in presence of linoleic acid was less important than the previous one observed with α-tocopherol. After 9 days, only 30% of γ-tocopherol was oxidized (Fig. 4).

γ-Tocopherol in aqueous solution without linoleic acid exhibited a good stability since less than 5% of γ-tocopherol oxidized after 29 days of experimentation (Fig. 4). Considering linoleic acid autoxidation rate in presence of γ-tocopherol, the curve showed a similar profile to the γ-tocopherol oxidation curve in the same sample.

On the other hand, δ-tocopherol exhibited a great stability in presence of linoleic acid as well as in its absence (Fig. 5). In these two cases the δ-tocopherol concentration remained constant during the entire lapse of the experimen-

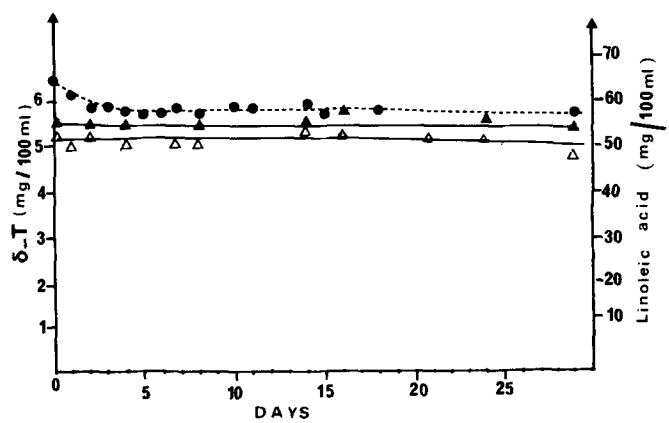


FIG. 5. Measurement of δ-tocopherol degradation in aqueous solution by HPLC. ▲ = δ-tocopherol with linoleic acid (S δ-T); △ = δ-tocopherol without linoleic acid (C δ-T; ● = linoleic acid degradation in presence of δ-tocopherol (S δ-T).

tation. In parallel, the linoleic acid concentration remained unchanged after the first 2 days of experimentation.

Oxidation Products of Tocopherols

They have been identified by thin layer chromatography. As early as the first day of experimentation, oxidation products of α-tocopherol were revealed in the presence of linoleic acid. They were identified as α-tocopherylquinone essentially, and traces of a dimer of α-tocopherol (Fig. 6). α-Tocopherylquinone was also detected in the control Cα-t without linoleic acid but only after the 5th day of experimentation. Thin layer chromatography (TLC) was a less sensitive method than HPLC, since after 5 days only traces of α-tocopherol could be detected by TLC while HPLC revealed that 20% of α-tocopherol was still present.

The first oxidation product of γ-tocopherol was detected in the sample with linoleic acid, after only 4 days of experimentation. It was identified as probably a γ-tocopherylquinone. This latter one was observed in the control without linoleic acid after 8 days. No oxidation products of δ-tocopherol were detected in our experiment.

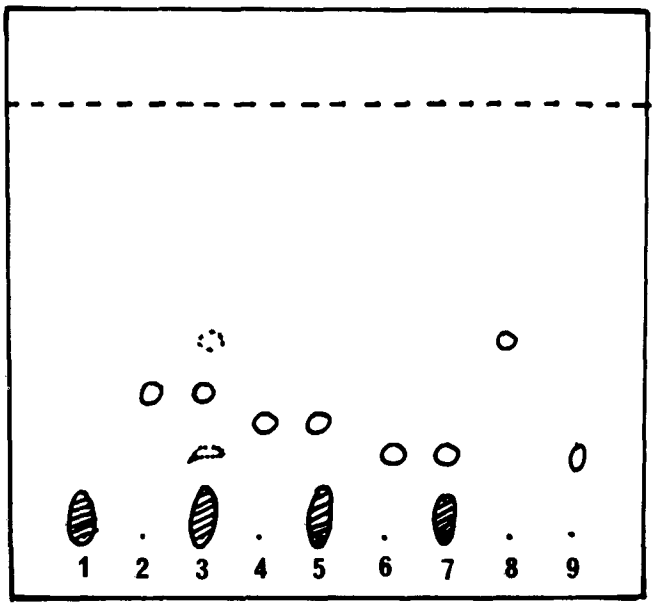


FIG. 6. Thin layer chromatogram of tocopherols and their oxidation products after 1 day of experimentation. 1 : linoleic acid; 2 : control α-tocopherol in aqueous solution; 3 : sample α-tocopherol with linoleic acid in aqueous solution; 4 : control γ-tocopherol; 5 : sample γ-tocopherol with linoleic acid; 6 : control δ-tocopherol; 7 : sample δ-tocopherol with linoleic acid; 8 : reference dimer of α-tocopherol; 9 : reference α-tocopherylquinone.

DISCUSSION

The addition of the same amount (5 x 10^{-2}M) of α or γ or δ-tocopherol to one mole of linoleic acid has exhibited opposite effects on linoleic acid autoxidation rate in aqueous media. α-Tocopherol was prooxidant, while in similar conditions γ-tocopherol has arised as a slightly antioxidant as well as δ-tocopherol, which has exhibited the greatest antioxidant activity.

Considering the oxidation rate of these three tocopherols in aqueous solution, we have noted that the oxidation rate increased in order $\delta < \gamma < \alpha$. Ikeda and Fukuzumi (2) have mentioned the same phenomenon during air oxidation of α, γ and δ-tocopherols. The different oxidation rate of these three tocopherols was related to the number of methyl substituents on the chromanoxy ring which would influence hydrogen mobility of hydroxyl function on C_6. The main oxidation product of tocopherols, which has been identified, was a tocopherylquinone.

The relationship between tocopherol oxidation and prooxidant activity has been previously studied in our laboratory (8). Tocopherol oxidation enhanced the formation of a perhydroxyl radical (•OOH) or one of the same type ($O_2 \doteq$, •OH). Such a radical would be responsible for the prooxidant effect. At a concentration of 5 x 10^{-2}M, only α-tocopherol was able to produce sufficient amount of perhydroxyl radicals to get the prooxidant reaction because it was the most easily oxidizable. One can think that γ and δ-tocopherols at concentrations higher than the previous one would be able to give enough perhydroxyl radicals to enhance a prooxidant reaction. The decrease of γ-T and δ-T concentration has exhibited an increase of antioxidant activity while α-tocopherol was still prooxidant.

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