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RADICAL EXCHANGE REACTIONS BETWEEN VITAMIN E, VITAMIN C AND PHOSPHOLIPIDS IN AUTOXIDIZING POLYUNSATURATED LIPIDS

PIERRE LAMBELET*, FRANÇOISE SAUCY and JÜRG LÖLIGER

Nestec SA, Nestlé Research Center, Vers-chez-les-Blanc, 1000 Lausanne 26, Switzerland

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Antioxidant reactions of mixtures of vitamin E, vitamin C and phospholipids in autoxidizing lipids at 90 °C have been studied by ESR spectroscopy. When the phospholipid contained a tertiary amine (e.g. phosphatidylcholine), the vitamin C and the vitamin E radicals were successively observed as these two vitamins were sequentially oxidised during lipid oxidation. In the presence of the primary amine contained in phosphatidylserine, the vitamin E oxidation was delayed for a few hours. In this case neither the vitamin C, nor the vitamin E radicals but a nitroxide radical derived from the phospholipid was observed. Similar results to those obtained with PS were obtained in the presence of either phosphatidylethanolamine or soybean lecithin. The participation in the radical reactions of phospholipids possessing a primary amine can therefore explain the synergistic effect of these phospholipids in a mixture of vitamins E and C.

KEY WORDS: ESR, antioxidant, vitamin E, vitamin C, phospholipid.

INTRODUCTION

Phospholipids occur ubiquitously in living organisms. As constituents of cell walls and active participants in metabolic processes they appear to be essential to life. In food science, phospholipids are recognised as edible products which have nutritional value as well as specific functional properties.[1] Phospholipids are also used as a functional ingredient in many food systems. The major functional properties include: emulsifying, antispattening; instantizing, wetting, dispersing, releasing, parting agent and viscosity modifying.[2] Antioxidant properties have also been attributed to phospholipids.[3,4] These properties were first ascribed to their metal scavenging ability.[5] It has been shown, however, that phospholipids act principally in a synergistic way by enhancing the activity of primary antioxidants.[6-8] The phospholipids have different synergistic tendencies. While phosphatidylcholine (PC) has a poor synergistic activity, phosphatidylethanolamine (PE) appears to have a good synergistic activity with either natural[9] or synthetic antioxidants.[10]

The co-operative action of vitamins E and C in the protection against peroxidation of lipids in biological as well as in food systems is abundantly documented.[11-23] A regeneration scheme between vitamin E and vitamin C has been shown to take place. To begin with the vitamin E reacts with lipid peroxyl radicals to form tocopheroxyl radicals. These tocopheroxyl radicals are then scavenged by the vitamin C which in turn is transformed into the ascorbyl radical by a hydrogen

*To whom correspondence should be sent.

atom transfer reaction. Since vitamin C and vitamin E are hydro-and lipophilic respectively, the mechanism of reaction between these two vitamins was mainly studied with lipid water binary systems using phospholipids as emulsifiers. No participation of the phospholipid in the antioxidant activity has been reported in the literature. Very good oxidative protection of polyunsaturated lipids has been observed with a mixture containing vitamin E, vitamin C and lecithin.[24] The present study was therefore undertaken to improve the understanding of the role of phospholipids in such a mixture, i.e. whether they are only emulsifiers allowing the two vitamins to be in contact, or if they participate more actively in the antioxidant process. For this purpose, radical reactions occurring during oxidation of lipids protected by systems containing vitamins E, C and various phospholipids were investigated by ESR spectroscopy. The disappearance of the two vitamins during the oxidation was determined by polarography.

MATERIALS AND METHODS

Materials

Vitamin C, and methyl linolenate were purchased from Fluka (Buchs, Switzerland). They were of analytical grade purity. Vitamin E (d,l-α-tocopherol) was a gift from Hoffmann-La Roche (Basel, Switzerland). Soybean lecithin (Topcithin®) was obtained from Lucas Meyer (Hamburg, Germany). Tocopherol levels measured in the lecithi by HPLC were: α: 0.004%; β and γ: 0.074%; δ: 0.028%. 1,2-Dioleoyl-sn-glycero-3-phospholipids (phosphatidylcholine, PC; phospatidylethanolamine, PE; phosphatidylserine, PS; phosphatidylinositol, PI) were purchased from Avanti Polar Lipids (Pelman, Alabama, USA). Whereas PC, PE and PI were in the free form PS was obtained as its sodium salt. The salt was extracted with 0.01 N HCl from the phospholipid dissolved in $CHCl_3/CH_3OH$ (2:1).

Methods

The antioxidant mixtures in lipids were prepared by adding vitamin E and vitamin C in ethanol to methyl linolenate containing the phospholipid to obtain final concentrations of 500 μg/g and 1000 μg/g for vitamin E and vitamin C, respectively. The ethanol was eliminated by heating the mixture under a continuous flux of nitrogen. The lipid mixtures were autoxidised by heating in air at 90°C under stirring. Samples were removed during the reaction for vitamin E and vitamin C assays, and for ESR analysis. Hydrating the sample which can be useful for radical identification in some instances, was achieved by keeping the mixture in a water-saturated atmosphere for a couple of hours at room temperature. Subsequent drying was obtained by bubbling dry nitrogen through the sample.

Quantitative determinations of vitamins E and C were carried out by Differential Pulse Polarography (DPP) using a 3 electrode polarograph E 506 (Metrohm, Herisau, Switzerland). The fat samples were dissolved directly in the electrolyte solution (toluene/ethanol (2:1), 0.1 M H_2SO_4). The reference electrode was Ag/AgCl in ethanol saturated with LiCl. The auxiliary electrode was a platinum wire. ESR spectra were run on a Varian E109 Century series Mark III spectrometer (X band) using 100 MHz magnetic field modulation. The microwave frequency was measured using a HP 5342 frequency counter and the magnetic field with a Varian

E-500-2 NMR Gaussmeter calibrated with perylene radical cation.[25] The temperature of the samples was controlled by means of a Varian E-257 variable temperature unit.

RESULTS

According to the phospholipid used, various responses were recorded during the reaction of vitamin E (500 μg/g), vitamin C (1000 μg/g) and phospholipid (2500 μg/g) with methyl linolenate at 90°C.

Reactions occurring in the presence of vitamin E, vitamin C and either PC or PI

The contents of vitamin E and vitamin C (337 μg/g and 153 μg/g respectively) measured in the unheated vitamin E, vitamin C and PC mixture were lower than expected (500 μg/g and 1000 μg/g), the discrepancy being much greater for the vitamin C. Within the first two hours of the reaction the vitamin C was oxidised whereas the content of vitamin E remained almost constant (Figure 1A). During this reaction period no radical could be detected under the present conditions. Increasing the sample temperature to 100°C led, however, to the recording of an asymmetrical singlet (Figure 1B) at $g = 2.005$. It could be observed for a short period of time (~ 10 min) following aeration of the sample only, but could be recorded once again after a subsequent oxygenation of the sample.[26] This signal can be understood as an immobilised ascorbyl radical.[27] This radical is presumably also produced at 90°C, but cannot be detected because its steady state concentration at this temperature is too low. As soon as the vitamin C was exhausted, the level of vitamin E began to decrease and a seven line ESR spectrum (hyperfine coupling constant = 5.5 Gauss) was recorded (Figure 1B). This ESR spectrum can be ascribed to the tocopheroxyl radical.[28] Contrary to the ascorbyl radical, the tocopheroxyl radical observed under the present conditions is as mobile as it is in a pure lipid environment.

Except for the level of vitamin C observed by DPP which was very low from the very beginning of the reaction, similar results were obtained with the vitamin E, vitamin C and PI mixture.

Reactions occurring in the presence of vitamin E, vitamin C and PE

Minute quantities of vitamin C were detected at the beginning of the reaction (Figure 2A). This could be due to the formation of a complex between the vitamin C and the phospholipid.[a]) One hour after the start of the reaction, the vitamin C could no longer be detected. The level of vitamin E, on the contrary, remained almost

[a] In the presence of a large excess of methyl linolenate, the linewidth of the proton-decoupled 145.78 MHz ^{31}P-NMR signal of PE in acetone-d_6 solution was found to be ca. 20 Hz for PE alone and for PE + vitamin E, while a linewidth of 70–90 Hz was observed for PE + vitamin C and for PE + vitamin C + vitamin E. This broadening effect is probably caused by chemical interaction between PE and vitamin C.

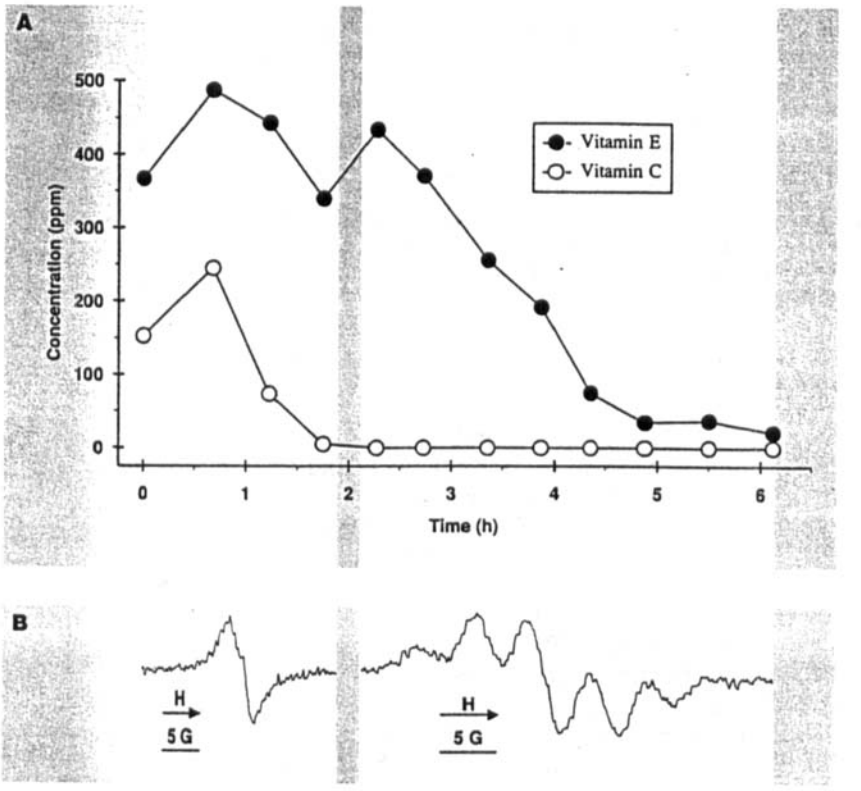


FIGURE 1 Reaction of vitamin E, vitamin C and PC with methyl linolenate at 90°C. A. Concentration time-course of vitamin E and vitamin C. B. ESR spectra observed. During the reaction the spectrometer settings were: microwave power, 50 mW; modulation amplitude, 1.25 G; receiver gain, 4×10^4 for the left-hand spectrum and microwave power, 100 mW; modulation amplitude, 2.5 G; receiver gain, 4×10^4 for the right-hand spectrum. The left-hand spectrum was recorded at T = 100°C.

constant ($\sim$ 500 μg/g) for the first 2 hours of the reaction. During this reaction stage, no radicals could be observed under experimental conditions. However, increasing the sample temperature to 110°C allowed us to record a weak quartet ESR spectrum (hyperfine splitting = 7.9 Gauss; g-factor = 2.0036) (Figure 2B). These signals, which could only be detected immediately after the aeration of the sample, are tentatively assigned to a nitroxide radical bearing a hydrogen atom on the nitrogen (see forthcoming discussion). 2.5 hours after the start of the reaction, the level of vitamin E started to decrease and the tocopheroxyl radical could be observed.

Reactions occurring in the presence of vitamin E, vitamin C and PS

As with PE, and presumably for the same reasons, only traces of vitamin C were detected in the presence of PS. After an initial increase over the first half an hour, the level of vitamin E remained almost stable during the first three hours of the reaction and then steadily decreased (Figure 3A). A strong ESR spectrum composed of three broad unsymmetrical lines (Figure 3B) was recorded during the entire reaction. The same ESR spectrum could be observed for temperatures ranging from -20°C to

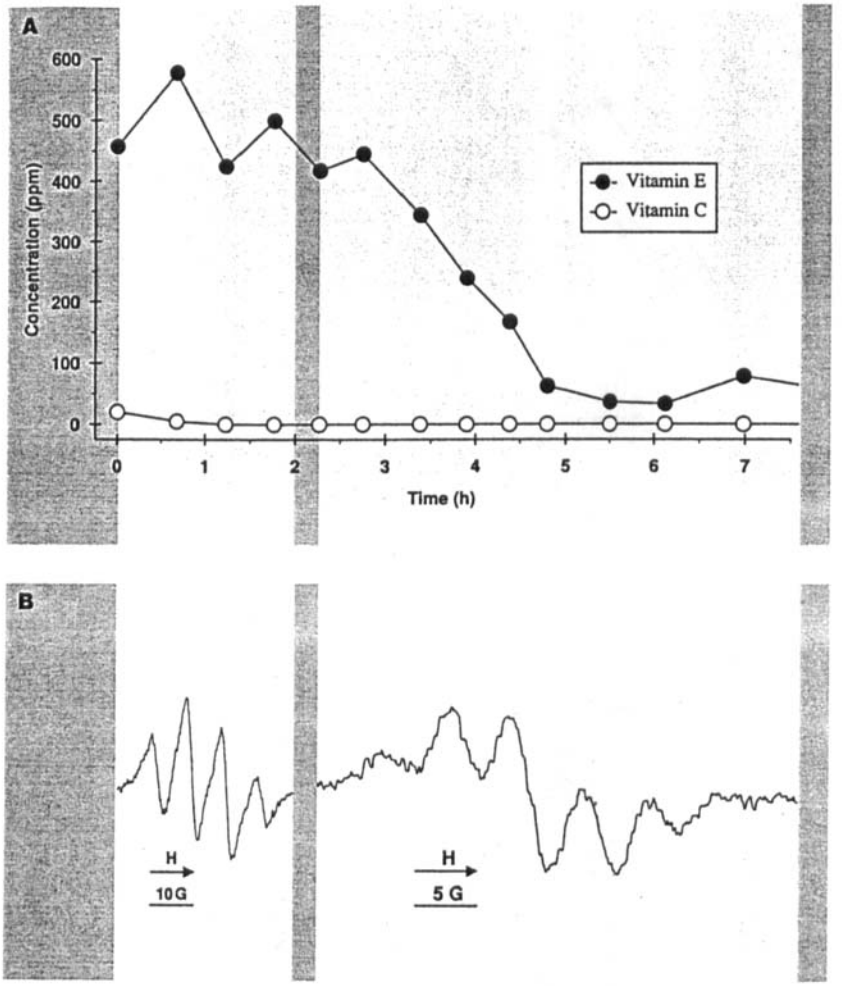


FIGURE 2 Reaction of vitamin E, vitamin C and PE with methyl linolenate at 90°C. A. Concentration time-course of vitamin E and vitamin C. B. ESR spectra observed. During the spectrometer settings were: microwave power, 100 mW; modulation amplitude, 2.5 G; receiver gain, 3.2×10^4 for the left-hand spectrum and microwave power, 100 mW, modulation amplitude, 2.5 G; receiver gain, 4×10^4 for the right-hand spectrum. The left-hand spectrum was recorded at T = 110°C.

120°C, and without aeration of the sample. It corresponds to a strongly immobilised radical. Hydrating the sample gave a more mobile radical (Figure 4). This process was reversible: drying the sample led to the re-observation of the ESR spectrum depicted in the Figure 3B. The ESR spectrum reported in Figure 4 is characterised by a coupling to a single nitrogen (a_N = 8.2 (Gauss) without a_H splitting and by a g-factor = 2.0037. Most likely, these ESR features are associated with a nitroxide radical.[29] The ESR spectra shown in Figures 3B and 4 are tentatively ascribed to the persistent iminoxyl radical *[I]* derived from the phospholipid. The small coupling constant associated with the nitrogen atom can be explained by delocalisation of the spin over the carboxylic group.

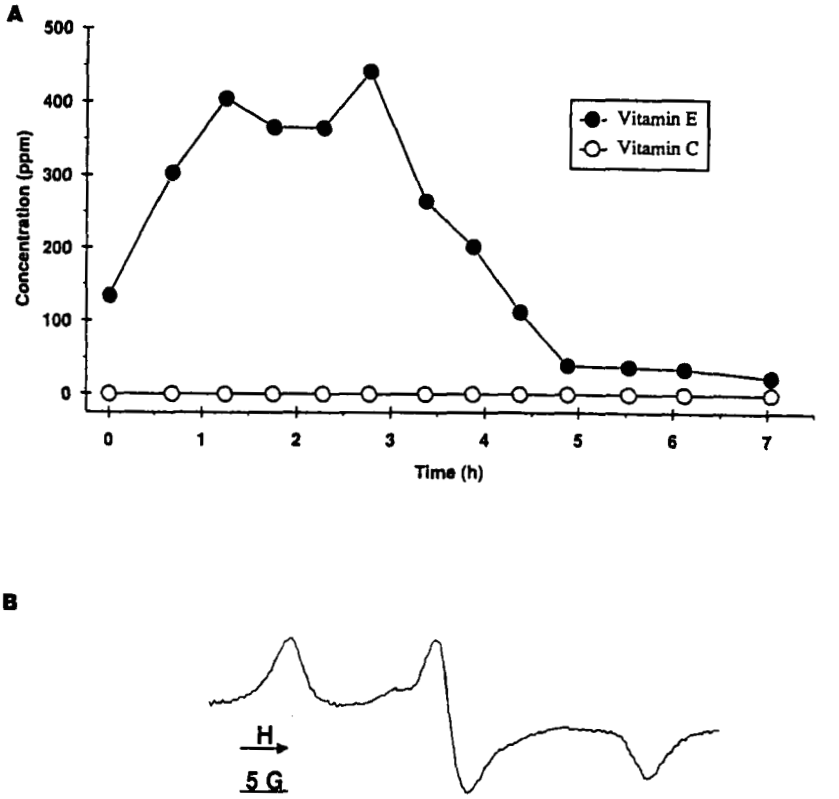


FIGURE 3 Reaction of vitamin E, vitamin C and PS with methyl linolenate at 90°C. A. Concentration time-course of vitamin E and vitamin C. B. ESR spectrum observed. During the reaction the spectrometer settings were: microwave power, 100 mW; modulation amplitude, 2.5 G; receiver gain, 4×10^4.

FIGURE 4 ESR spectrum recorded during the reaction of vitamin E, vitamin C and PS with methyl linolenate at 90°C, after hydration of the sample. The spectrometer settings were: microwave power, 20 mW; modulation amplitude, 2 G; receiver gain, 8×10^4.

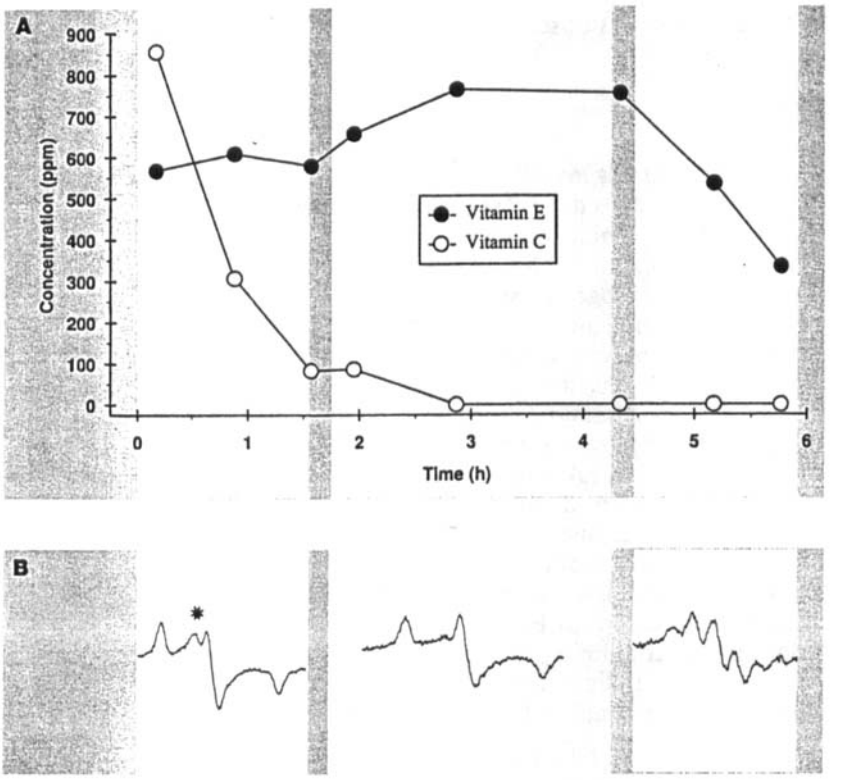
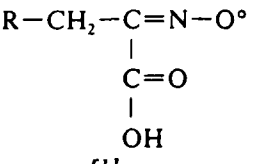


FIGURE 5 Reaction of vitamin E, vitamin C and lecithin with methyl linolenate at 90°C. A. Concentration time-course of vitamin E and vitamin C. B. ESR spectra observed during the reaction. For the three spectra the spectrometer settings were: microwave power, 150 mW; modulation amplitude, 2.5 G; receiver gain, 8×10^4.



[I]

Reactions occurring in the presence of vitamin E, vitamin C and soybean lecithin

During the first stage of the reaction the level of vitamin C rapidly decreased whereas that of the vitamin E increased slightly (most probably due to an analytical artefact) as a function of time (Figure 5A). Only traces of vitamin C could thus be detected during the two hours following the start of the reaction. During this reaction period the spectrum depicted on the left-hand part of Figure 5B was recorded. The main features of this spectrum correspond to the immobilised nitroxide radical whereas the supplementary absorption line (marked with an *) can be associated with the vitamin C radical. As the reaction progressed further the vitamin E concentration continued to increase and only the nitroxide radical could be observed (Figure 5B center). In a later stage of the reaction, the amount of vitamin E decreased rapidly and

the vitamin E radical was observed (Figure 5B right-hand spectrum).

DISCUSSION

Among the phospholipids investigated in the present study, one (PC) contains a tertiary amine, one (PI) does not possess any amine group at all, whereas two (PE and PS) have a primary amine group. The radical reactions observed during lipid protection by mixtures containing vitamin E, vitamin C and PC are similar to those already reported for analogous systems: the vitamin C radical (although less stable and less mobile than in homogeneous lipids) and then the vitamin E radical are sequentially observed while a successive disappearance of these two vitamins during the inhibition procedure is recorded. This indicates that in the presence of PC the vitamin E radical is regenerated by the vitamin C through a hydrogen atom exchange reaction.[11-23] Nitroxide radicals are never detected in systems containing PC.

A different mechanism takes place in the presence of either PE or PS, that is when primary amines are involved. In this case only minute quantities of vitamin C are measured in the mixture. Instead of the vitamin C radical (which is never detected) a nitroxide radical derived from the phospholipid is recorded at the beginning of the reaction. Although a transient species in the presence of PE, this nitroxide is a persistent radical in the presence of PS. Only the nitroxide radical can thus be detected in the reaction mixture containing PS. Secondary amines are known to produce nitroxide radicals by reaction with lipid hydroperoxides.[30-32] The first step in this reaction is the formation of an aminyl radical by reaction of the amine with a lipid peroxy radical.

$$R_2NH + R'OO^{\bullet} \rightarrow R_2N^{\bullet} + R'OOH$$

The aminyl radical reacts rapidly with oxygen to form, via an N-peroxy radical intermediate, the corresponding secondary nitroxide.[33-34]

$$R_2N^{\bullet} + O_2 \rightarrow R_2NOO^{\bullet}$$

$$2\ R_2NOO^{\bullet} \rightarrow 2\ R_2NO^{\bullet} + O_2$$

A similar mechanism can be hypothesised here. Reaction of primary amines, however, leads to the formation of primary nitroxide radicals which are transient species under the present conditions. Nevertheless, in the case of PS, the primary nitroxide radicals are transformed into long-lived iminoxyl radicals which are stabilised by conjugation between the iminoxyl and the carboxylic groups.

The present results clearly show that the vitamin E is spared when the nitroxide radical is formed. This indicates that the protective effect of the mixture, which ends when the vitamins are completely consumed, is prolonged. The formation of nitroxide radicals is therefore related to antioxidant activity. The participation of PE or PS in the radical reactions may account for the better synergistic effect of PE as compared to that of PC[8], and/or explain the good antioxidant activity of the vitamin E, vitamin C, lecithin mixture.[24]

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