response of the *In(3R)AFA,e* stock to quinine salt; *In(3R)AFA,e* males are attracted to quinine sulfate in contrast to *Canton-S* males, which avoid quinine salt (see the third line of the part a of table 1). Siddiqi and Rodrigues[22] have analyzed the electrophysiological responses of taste sensillae to quinine sulfate in *Drosophila melanogaster;* unlike salt and sugar, quinine does not appear to excite individual neurons; 'the only effect quinine seems to have is to inhibit the firing of the other chemosensory neurons. Since the S cell is inhibited more strongly than the two L cells, the presence of quinine is likely to favor rejection by changing the ratio of S spikes to L spikes'[22]. *gust-M_1* responds mistactically to NaCl and quinine sulfate in contrast to *wild-type* males that avoid both compounds; this phenomenon can be accounted for assuming that *gust-M_1* could be a mutation perturbing functions in the central nervous system affecting the responses to both compounds. Alternatively, *gust-M_1* could be defective in a common part of the peripheral gustatory machinery that might bind both salt and quinine sulfate. The existence of *gus* mutations which alter the proboscis extension response is of practical significance, because mutations that are detectable in individual flies can be analyzed with genetic mosaics to locate anatomical foci of mutant behavior[9]. These mutants are useful because they provide us with a way of studying the organization of the chemoreceptor system which in *Drosophila* is sufficiently simple, for a correlated study of electrophysiological and behavioral responses of mutants to be likely to throw interesting light on the sensory code, whose exact nature so far remains unknown[7,18].

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Chemical evidence for interactions between vitamins E and C

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Summary. Experimental proof is provided for interactions between radicals of vitamin E/vitamin C as generated by air-oxidized lipids (liquid fraction of subcutaneous chicken fat). Using ESR spectroscopy, hydrogen atom exchange is shown to take place between vitamin C and the radical of vitamin E. Sequential consumption of these two vitamins in oxidized lipid, first vitamin C then vitamin E, is demonstrated by means of differential pulse polarography. These results elucidate the in vitro radical scavenging functions attributed to vitamin E and vitamin C as well as their synergism in lipid antioxidation.
Key words. ESR; vitamin E; vitamin C; antioxidation; radical; exchange; mechanism.

Introduction

More and more evidence accumulates suggesting the overwhelming importance of radical species in many vital biological processes in the living cell. Cancer tissue formation and many age-related processes have been associated with reactions involving radicals[3, 10, 24, 29, 30, 32]. Understanding mechanisms of formation of radicals and the body defence systems could be an important contribution to cancer and age-related diseases therapy as radicals can interfere with metabolic pathways. Radicals are formed as a result of irradiation or form a number of exogenous precursors stemming from the environment[2, 12]. But one of the most important sources of radical intermediates are reactions associated with the generation of usable energy from dioxygen reduction[11, 15]. Only a minor 'slipping' in one of the numerous defence reactions (involving superoxide dismutase[13, 14], glutathione peroxidase[28], catalases[32], tocopherols[26], Vitamin C[9], uric acid[3, 27], β-carotene[8, 24], etc.) against the toxicity of dioxygen reduction products can have disastrous effects[15]. Antioxidants play their role of this defence in deactivating hydroxyl radicals formed as the last step in the reduction chain of dioxygen to water.

A number of authors have discussed mechanisms of vitamin E and vitamin C interactions and their importance as chain breaking antioxidants in the in vivo autoxidation of polyunsaturated lipids of cellular membranes. Speculations and experiments have been published to show the importance of vitamin C and vitamin E interactions during lipid autoxidations. Tappel[28] proposed a regeneration scheme whereby the primary radical formed from vitamin E can be intercepted by vitamin C, which itself becomes oxidized by a hydrogen atom transfer reaction. The radical of vitamin C (ascorbyl radical) then further undergoes oxidation to form dehydroascorbic acid. By generating the one electron oxidizing reagent trichloromethylperoxy radical by pulse radiolysis in a water/isopropanol/acetone solution containing carbon tetrachloride, Packer[23] showed that a hydrogen atom transfer reaction takes place between ascorbic acid and α-tocopheroxyl radicals. Bascetta[6] showed the quenching by vitamin C of α-tocopheroxyl radicals generated by oxidizing lipids on silica gel in an electron spin resonance (ESR) experiment. Niki[20, 21] generated α-tocopheroxyl radicals in solution using 2,2-diphenyl-1-picrylhydrazyl as a radical chain initiator and showed that upon exposure of these radicals to glutathione or vitamin C, the ESR signals of the α-tocopheroxyl radical disappeared. Niki[20, 22] and Barclay[4, 5] showed that there was a synergism between α-tocopherol and ascorbic acid using their respective chemically induced radical generating systems in an oil in water emulsion.

We have been developing an experimental system for studying antioxidant radical reactions in a physiological lipid type environment[16]. Our results with vitamin E and vitamin C give additional support to the hypothesis of Tappel[28] already mentioned.

Materials and methods

Chicken fat liquid fraction (CFLF) was prepared by a dry fractionation procedure. Deodorized chicken fat (prepared by passing nitrogen through the molten chicken fat at 140 °C for 3 h, conditioned in nitrogen gazed tins and stored at −20 °C) was molten at 40 °C for 24 h. The cristallization of the solid fraction was initiated at 15 °C during 3 days and then continued for 3 more days at 0 °C. The separation of the liquid fraction was achieved by rapidly pressing the microcristalline mass at room temperature using pre-cooled equipment. The composition with respect to fatty acids of CFLF is reported in the table. The content of tocopherols (α, β, γ and δ) was below the detection limit e.g. smaller than 0.5 ppm.

2.5 g CFLF was oxidized in an open 20 ml beaker in an air circulation oven at 150 °C for 3 h (peroxide values reached about 130 meq. O_2/kg of fat). The cooled oil was then treated with the indicated quantities of antioxidants dissolved in a minimum amount (approximately 20%) of a 9/1 n-butanol/methanol mixture.

Ascorbyl palmitate (AP) was obtained from Hoffmann-La Roche, Basel, and used without further purification. dl-α-Tocopherol (analytical grade) was purchased from E. Merck, Darmstadt.

The samples of CFLF containing the antioxidants were stored in the dark at room temperature in stoppered 20 ml Pyrex bottles, from which they were taken for periodical analysis. These manipulations assured abundant oxygen supply.

First derivative ESR spectra were recorded at ambient temperature on a Varian E-109 Century series Mark III spectrometer (X band) with 100 kHz magnetic field modulation. The relative radical concentration was determined by adding the peak-to-peak amplitudes for all the lines of the first derivative ESR spectra recorded at high magnetic field modulation[16].

Quantitative determinations of dl-α-tocopherol and AP were carried out using Differential Pulse Polarography[18] (DPP) by dissolving the fat samples directly in the electrolyte solution (toluene: ethanol 2:1, 0.1 M H_2SO_4). The reference electrode used for these determinations and for determining the oxidation potentials was Ag/AgCl in ethanol saturated with LiCl. The auxiliary electrode utilized with the 3 electrode Polarograph E 506 (Metrohm, CH-9100 Herisau) was a platinum wire.

Results

Adding 2.3 mM AP to oxidized CFLF led to the observation of the two lines ESR spectrum reported in figure 1. The ESR parameters obtained from this spectrum (hy-

Fatty acid composition of CFLF

Fatty acid	%
C14:0	0.75
C14:1	0.2
C15:0	0.15
C16:0	14.4
C17:0	4.6
C17:1 + C18:branched	0.4
C18:0	4.0
C18:1n-9	51.7
C18:2n-6	21.8
C18:3n-3	1.6
C22:0	< 0.1

perfine coupling constant and g-factor equalled to 1.8 Gauss and 2.0053, respectively) are closely related to those of the radical of ascorbic acid[17]. Furthermore, the molecular fragments over which the unpaired electron is delocalized are identical in both radicals derived from ascorbic acid and AP. The ESR spectrum reproduced in figure 1 can therefore be attributed to the radical of AP. In the same way, adding 2.4 mM dl-α-tocopherol to oxidized CFLF resulted in the observation, directly after mixing, of the ESR spectrum reported in figure 2. This ESR spectrum can be unambiguously attributed to the dl-α-tocopheroxyl radical[16]. The oxidized CFLF did not show any ESR signal under the conditions used for observing the radicals of AP and of dl-α-tocopherol.

On the other hand, about equimolar mixtures (2.4 mM dl-α-tocopherol and 2.3 mM AP), in oxidized CFLF gave three different types of ESR spectra, according to the time the reaction was allowed to proceed. At the onset, shortly after mixing (about 15 min), the ESR spectrum corresponding to the radical of AP (fig. 1) was only observed. After this solution was left at room temperature for about 10 days, the ESR signals completely changed and the ESR spectrum corresponding to the tocopheroxyl radical (fig. 2) was observed. In between these two extremes, about 6 days after the start of the experiment, the badly resolved ESR spectrum represented in figure 3 was obtained. It could only be speculated that it represented a mixture of the two spectra shown in figures 1 and 2. At different molar concentrations, e.g. 5 mM each, the sequence of the radicals formed was identical, but the time required for the radical of AP to be replaced by the dl-α-tocopheroxyl radical was longer (14 days). Much lower concentrations than approximately 2.5 mM were not very practical for ESR spectroscopy.

In addition to this general behavior of the oxidized CFLF/dl-α-tocopherol/AP system, a number of similar experiments revealed interesting results. The addition of AP to oxidized CFLF containing dl-α-tocopherol and showing a neat tocopheroxyl radical ESR spectrum led to the observation of ESR signals which could be attributed to the ascorbyl radical. On the other hand, running the oxidation with a much higher ratio of dl-α-tocopherol compared to AP (e.g. 100:1) resulted, after about 15 min, in an ESR spectrum suggesting a mix of radicals from these two antioxidants.

In parallel with this qualitative investigation of radicals formation a quantitative study was conducted by ESR spectroscopy and DPP to determine the relative radical concentrations and absolute antioxidants concentrations during their reaction with oxidized CFLF. Figure 4 shows the relationship of the AP and the dl-α-tocopheroxyl radicals as a function of time for about equimolar concentrations of AP and dl-α-tocopherol in oxidized CFLF. As was said before, the radical of dl-α-tocopherol was not detectable at the beginning of the reaction, only the radical of AP could be observed during the first 6

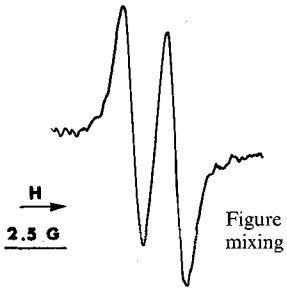

Figure 1. ESR spectrum recorded shortly after mixing 2.3 mM AP with oxidized CFLF.

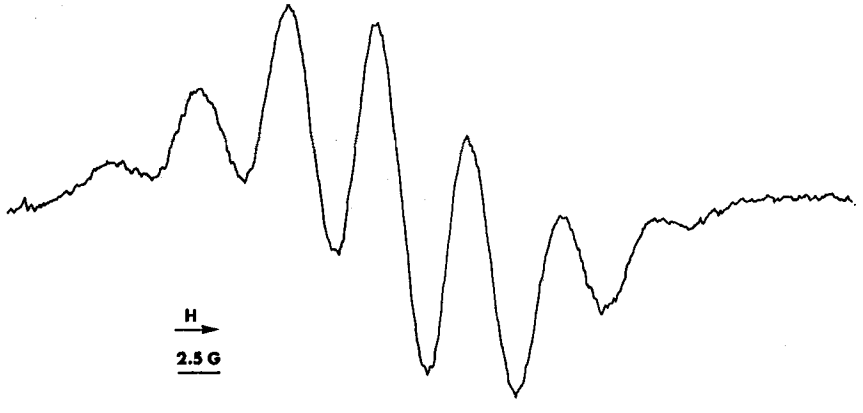

Figure 2. ESR spectrum recorded shortly after mixing 2.4 mM dl-α-tocopherol with oxidized CFLF.

Figure 3. ESR spectrum of a mixture of 2.3 mM AP and 2.4 mM dl-α-tocopherol in reaction with oxidized CFLF.

days. Within this period of time the relative concentration of the radicals of AP continuously decreased right from the beginning of the reaction. After about 6 days the radical of AP was replaced by the dl-α-tocopheroxyl radical which stayed unchanged in concentration over the time scale presented. It was only after approximately 150 days that the dl-α-tocopheroxyl radical concentration had started to diminish.

On the other hand, the quantities of AP and dl-α-tocopherol determined by DPP are shown in figure 5. Throughout the 10 days of the experiment represented, no appreciable change in dl-α-tocopherol concentration could be seen. However, a substantial decrease in AP concentration occurred during the first 2 days of the experiment, and after 6 to 7 days no AP could be detected any longer. This time corresponds roughly to the time required for the radical of AP to disappear and for the dl-α-tocopheroxyl radical to be formed. DPP showed that the concentration of dl-α-tocopherol began diminishing only after more than 100 days of oxidation.

Discussion

Many authors[4-6, 20-23] have described experiments for elucidating interactions between vitamin C and vitamin E. Packer[23] first demonstrated under short time conditions in a rather dynamic model the interactions of dl-α-tocopheroxyl radicals with vitamin C. The approach described here has the advantage of employing a system (CFLF) which more closely simulates the lipid type structures encountered in living organisms. On the other hand, it has the disadvantage of being less well defined. CFLF was chosen as lipid mainly for two considerations: it contains highly unsaturated lipid (> 75%) and almost no antioxidants. AP was chosen instead of ascorbic acid for solubility reasons. It was shown by Pongracz[25] and Cort[9] to have chemical properties very similar to those of ascorbic acid with respect to its radical scavenging reactivities.

Our work under almost steady-state conditions for the formation/destruction of intermediates of radical nature supplies further evidence for the interactions between dl-α-tocopheroxyl radicals and vitamin C. It was rather surprising, however, to find that in oxidized CFLF short lived radicals could build up in the presence of antioxidants to steady state radical concentrations which were detectable by ESR spectroscopy. We did not measure the half-life of these radicals, but we have shown that these interact at steady state concentrations. It was clearly shown that dl-α-tocopheroxyl radicals were quenched by AP and that the radicals of AP were subsequently formed. In addition to these ESR results, polarographic half-wave oxidation potentials of AP and dl-α-tocopherol (962mV and 768 mV vs. sat. LiCl in ethanol/toluence) reflects the difference in energy required for the one electron oxidation (formation of radicals) of the two antioxidants[7]. This difference of 194 mV showed the greater ease with which dl-α-tocopherol was oxidized. However, the determination of the concentrations of AP and dl-α-tocopherol during oxidation experiments with CFLF showed that the first species consumed was AP. This in turn implied that dl-α-tocopherol which was the antioxidant most readily oxidized, was regenerated by AP.

Radicals generated by reactions initiating autoxidation within structural cell membrane lipids are very easily intercepted by the tocopherols present there. To be able to regenerate the tocopherols, ascorbic acid needs to approach the cell walls as it is a hydrosoluble component of the cell fluids. Exchange reactions would have to take place at the interface between hydrophilic and hydrophobic sites. With this regeneration scheme it can easily be understood why in man no marked characteristic pathologic picture of a deficiency in vitamin E is known[32]. Vitamin E, even in small concentrations, undergoes a one electron oxidation and can then be regenerated by vitamin C or other reducing agents (glutathione, NADH, etc.).

Further investigations with liposomes and other non homogeneous lipid[19] containing systems make it possible to elaborate a model which more closely mimics the radical type reactions within a living cell membrane. These systems should give answers to questions about inter-

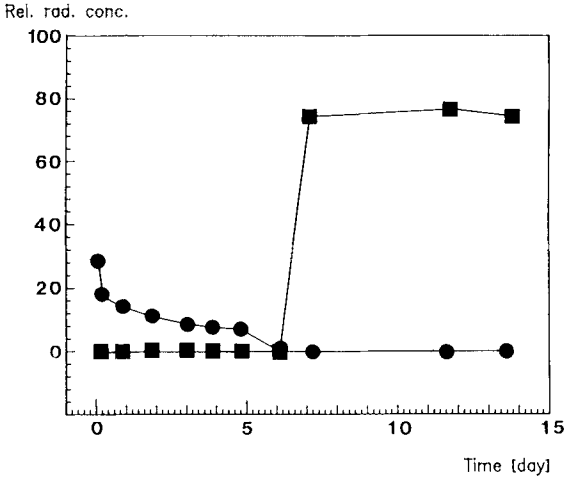

Figure 4. Variation of relative concentrations of the dl-α-tocopheroxyl radical (■) and the radicals from AP (●) during 14 days after mixing dl-α-tocopherol (2.4 mM) and AP (2.3 mM) with oxidized CFLF.

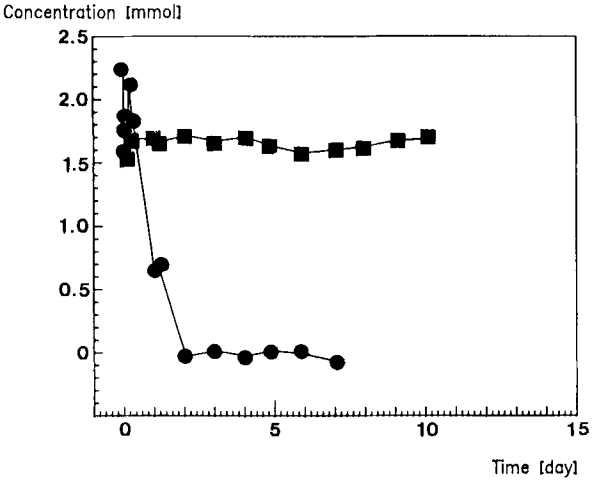

Figure 5. Variation of concentrations of dl-α-tocopherol (■) and AP (●) during 10 days after mixing dl-α-tocopherol (2.4 mM) and AP (2.3 mM) with oxidized CFLF.

actions between radicals and other constituents of the living cell, in particular uric acid[2, 27], salicic acid[31], glutathione[32], carotenoids[8, 24].

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