Oxidation of Lipids. VIII. Synergistic Inhibition of Oxidation of Phosphatidylcholine Liposome in Aqueous Dispersion by Vitamin E and Vitamin C

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Vitamin E acted as an efficient antioxidant in the oxidation of soybean phosphatidylcholine liposome in an aqueous dispersion initiated by free radicals generated initially either in the aqueous phase or in the lipid phase. Vitamin E decayed linearly with time, and when it was exhausted, the oxidation proceeded rapidly at a similar rate to that in the absence of vitamin E. Vitamin C was also effective by itself in scavenging radicals in an aqueous phase, but it could not scavenge the radicals efficiently in a lipid bilayer. However, when vitamin E was located in phosphatidylcholine bilayer, the addition of vitamin C into an aqueous phase prolonged the period of suppression of oxidation and vitamin C reduced the rate of decay of vitamin E markedly even when the radicals were generated initially in the lipid region. Vitamin C was predominantly consumed linearly at first and vitamin E began to decay after vitamin C was exhausted. It was suggested that vitamin C could regenerate vitamin E by reacting with vitamin E radical during the oxidation of phosphatidylcholine liposome in an aqueous dispersion as observed in a homogeneous solution.

Vitamin E is now accepted to function as a chain-breaking antioxidant during the non-enzymatic oxidation of lipids *in vivo*.[1-5] It scavenges the chain-carrying peroxyl radicals very quickly[6-12] and a small concentration of vitamin E protects the biomembranes from peroxidation efficiently. Vitamin E is also popular as a natural and safe antioxidant for foods. A synergistic inhibition of oxidation by the combination of vitamin E and vitamin C has long been suggested[10,13-22] and it has been found experimentally that vitamin E radical reacts with vitamin C,[21,22] glutathione,[22,23] and cysteine.[23] We have recently found[10] that, when both vitamin E and vitamin C are present, the oxidation of methyl linoleate solution is suppressed quite efficiently and that vitamin E remains almost unchanged while only vitamin C is consumed at the initial stage and vitamin E is consumed after vitamin C is exhausted. A question still remains whether vitamin C which resides largely in an aqueous phase can interact with vitamin E radical that must be located in a lipid phase in heterogeneous liposome system or *in vivo*. We wish to report here the evidence that shows the synergistic inhibition of oxidation by vitamin E and vitamin C in the oxidation of soybean phosphatidylcholine (PC) as a bilayer liposome in an aqueous dispersion.

Experimental

Commercial soybean PC purchased from Daigo Chemicals Co. (Osaka) was purified with alumina and silica-gel columns. The purified PC gave only one spot on thin-layer chromatography and no conjugated diene was observed before oxidation. The composition of fatty acids in PC measured by gas-liquid chromatography after hydrolysis and esterification with HCl-methanol was as follows: palmitic acid 12.1, stearic acid 1.6, oleic acid 9.6, linoleic acid 69.6, and linolenic acid 7.1 mol%. Dimyristoyl PC was purchased from Sigma Chemical Co. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) were used as a water-soluble and oil-soluble radical initiator respectively. Both AAPH and AMVN were provided by Wako Chemicals (Tokyo) and used as received. These water-soluble and lipid-soluble initiators enable us to generate radicals initially outside or within the bilayer as requested and initiate free radical chain oxidation at a constant rate, which is essential for the fundamental and quantitative study.

Vitamin E (*d*-α-tocopherol) was kindly supplied from Eisai Co. (Tokyo) and commercial vitamin C (L-ascorbic acid) was used as received.

The liposome was prepared as follows as reported previously.[11] PC and oil-soluble additives such as AMVN and vitamin E were dissolved in chloroform and the solution was taken into a small flask. Chloroform was removed by evacuation on a water aspirator using a rotary vacuum evaporator to obtain a thin film on a flask wall. Appropriate amount of 0.1 M (M=mol dm^{-3}) NaCl aqueous solution was added and the film was slowly peeled off by shaking to obtain a white and milky liposome solution. It must be noted that the liposome we have prepared in this study is a multilamellar vesicles.

The oxidation was carried out at either 37 or 50 °C under air in a Pyrex glass ampoule. The reaction conditions such as temperature and concentrations of substrate, initiator, and additives were determined so as to obtain appropriate rates of oxidation and disappearance of reactants. The reaction mixture was agitated with a magnetic stirrer. The rate of oxygen uptake was measured continuously in an automatic recording gas absorption apparatus with a pressure transducer.[10] In some oxidations, the rate of build-up of conjugated diene was followed spectrometrically at 233 nm. Since linoleic acid is the major fatty acid in soybean PC and its oxidation gives conjugated diene hydroperoxides quantitatively,[24-29]

this spectrometric analysis should give a good measure of the rate of oxidation. Since the extinction coefficient of conjugated diene is large, ε=28000 $M^{-1}cm^{-1}$, the rate of oxidation can be followed at very early stages.

The rates of consumption of vitamin E and vitamin C were followed by high performance liquid chromatography (HPLC).[10] A Finepak SIL C18 column was used and methanol was delivered as an eluent at 1 cm^3 min^{-1}. Vitamin C (eluted at 2.7 min) was measured at 275 nm and vitamin E (eluted at 7.7 min) was measured at 290 nm.

Results

When water soluble AAPH is used as a radical initiator, the radicals are formed initially in an aqueous phase, whereas water-insoluble and oil-soluble AMVN incorporated into PC bilayers generates free radicals within the lipid region. In both cases, the soybean PC liposome was oxidized smoothly at a constant rate without any noticeable induction period with long kinetic chain length, suggesting that the free radicals initially formed either in aqueous phase or in lipid phase induce the autoxidation of liposome similarly as observed previously.[11] In the absence of radical initiator, the rates of oxygen uptake and conjugated diene formation and that of vitamin E disappearance were negligibly small. Figure 1 illustrates the profile of oxygen uptake during the oxidations of soybean PC

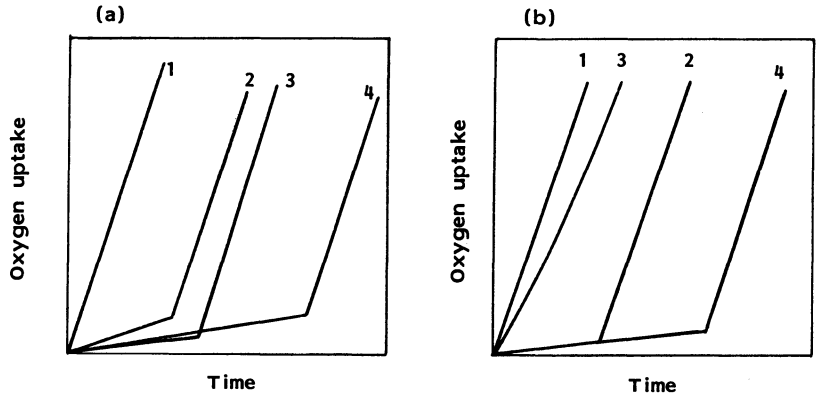


Fig. 1. Profile of the oxygen uptake during the oxidations of soybean PC liposome in aqueous dispersions initiated with (a) water soluble AAPH and (b) lipid soluble AMVN, respectively.
1: without antioxidant; 2: with vitamin E; 3: with vitamin C; 4: with both vitamin E and vitamin C

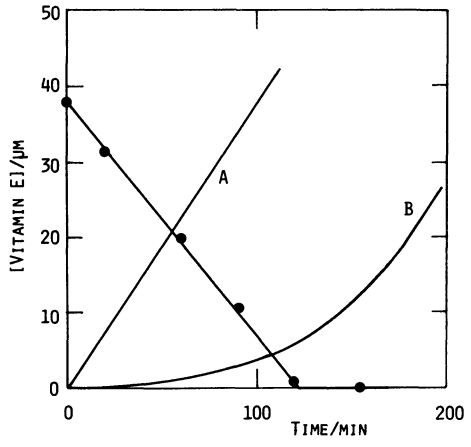


Fig. 2. Consumption of vitamin E (●) during the oxidation of 20 mM soybean PC liposome in 0.1 M NaCl aqueous dispersion at 37 °C under air, [AAPH]=50 mM, [vitamin E]=36.8 μM. Lines A and B show oxygen uptake without and with vitamin E, respectively.

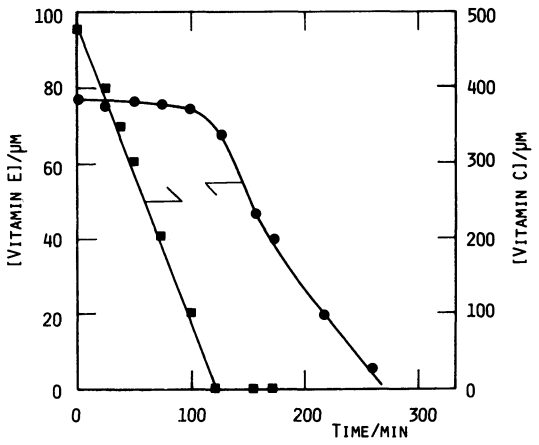


Fig. 3. Consumption of vitamin E (●) and vitamin C (■) during the oxidation of 22 mM soybean PC liposome in 0.1 M NaCl aqueous dispersion initiated with 50 mM AAPH at 37 °C under air, [vitamin E]=77 μM, [vitamin C]=480 μM.

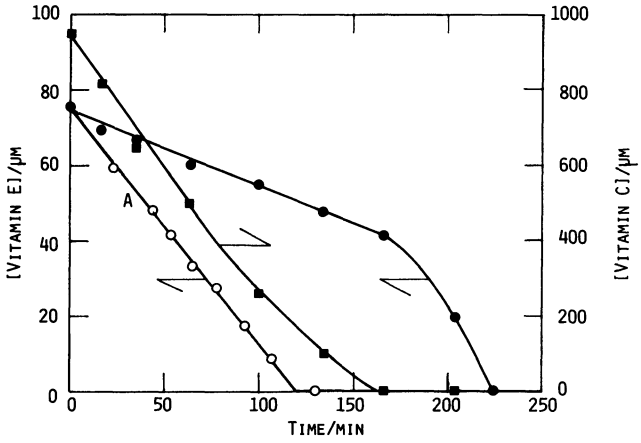


Fig. 4. Consumption of vitamin E (●) and vitamin C (■) during the oxidation of 20 mM soybean PC liposome in 0.1 M NaCl aqueous dispersion initiated with 4.0 mM AMVN at 50 °C under air, [vitamin E] = 77 μM, [vitamin C] = 950 μM. Line A (○) shows the rate of decay of vitamin E in the absence of vitamin C.

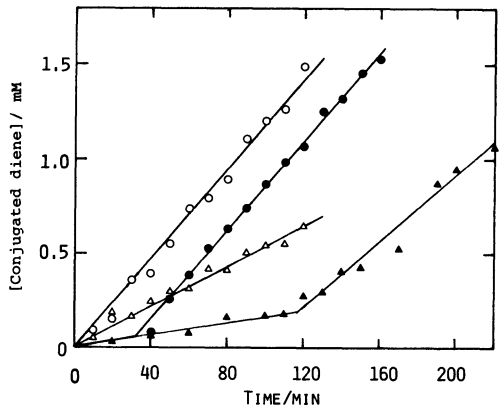


Fig. 5. Formation of conjugated diene in the oxidation of 10.7 mM soybean PC liposome dispersed in 10 cm³ of 0.1 M aqueous NaCl at 50 °C under air initiated with 2.53 mM AMVN.
○, without antioxidant; ●, with 14.5 μM vitamin E; △, with 929 μM vitamin C; ▲, with 14.5 μM vitamin E and 929 μM vitamin C.

liposome initiated with AAPH and AMVN.

Oxidations Initiated with AAPH. When vitamin E or vitamin C was added in the oxidation of PC liposome initiated with AAPH, the rate of oxygen uptake was suppressed markedly as observed in the oxidation of methyl linoleate in homogeneous solution[10] or in water dispersion.[9,11] As shown in Fig. 2, vitamin E was consumed linearly with time and after all of vitamin E was consumed the oxidation proceeded rapidly at a similar rate to that in the absence of vitamin E. Figure 3 shows that, when both vitamin E and vitamin C were present, vitamin C was consumed first linearly with time but vitamin E remained almost constant at the initial stage and then it was consumed after vitamin C was exhausted.

Oxidations Initiated with AMVN. When oil-soluble AMVN was incorporated into liposome and the initiating radicals were generated within the liposomal lipid region, the oxidation of PC liposome proceeded smoothly at a constant rate and oxygen uptake was suppressed efficiently by a small amount of vitamin E which was also incorporated into lipid bilayer (Fig. 1b). Vitamin E decayed linearly with time as shown in Fig. 4 (Line A). However, although vitamin C alone added to an aqueous phase retarded the oxidation, it did not produce a clear induction period (Fig. 1b) which was observed when the oxidation was initiated with water-soluble AAPH (Fig. 1a). As shown in Fig. 5, similar results were observed when the rate of oxidation was followed by the formation of conjugated diene. These results show that, as observed previously in the oxidation of methyl linoleate micelle in aqueous dispersions,[9] vitamin C can not scavenge the radicals in the bilayer as efficiently as it scavenges radicals in aqueous phase.

When vitamin E and vitamin C were located in bilayer and in aqueous phase, respectively, the induction period was lengthened markedly as shown in Figs. 1b and 5. Furthermore, it must be pointed out that the rate of oxidation during the induction period was much smaller than that in the presence of vitamin C alone. Figure 4 shows how vitamin E and vitamin C decayed with time when both vitamins were present in the oxidation of PC liposome initiated with AMVN. Interestingly, the rate of consumption of vitamin E in liposome was reduced markedly by vitamin C in an aqueous phase even though the radicals were generated initially in the lipid region. When all of vitamin C was consumed, vitamin E began to decrease at a similar rate to that in the absence of vitamin C.

Discussion

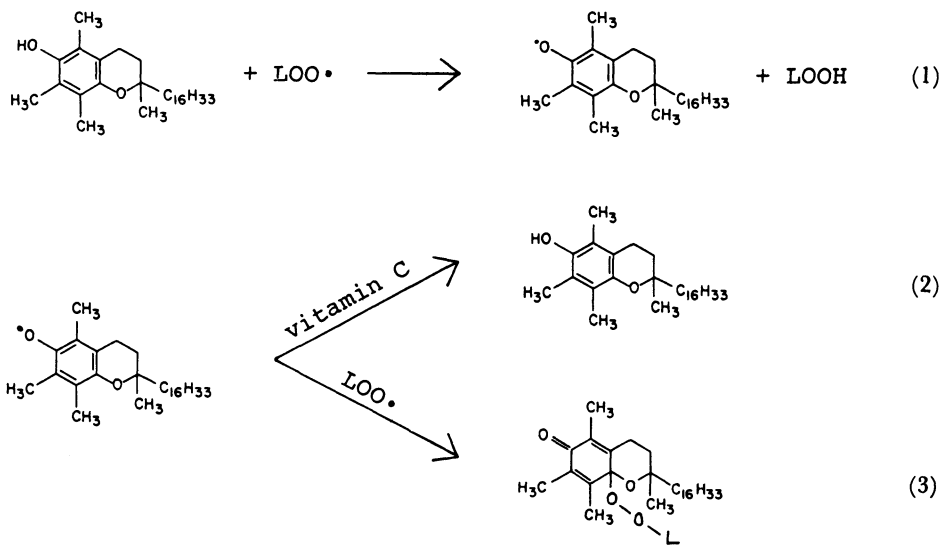
Both vitamin E and vitamin C can scavenge peroxyl radicals when they are accessible and interact.[10] However, Fig. 1b indicates that vitamin C can not efficiently scavenge peroxyl radicals formed from AMVN and PC within liposomal lipid region, probably because AMVN is buried in the bilayer and the peroxyl radicals from both AMVN and PC are not accessible to vitamin C.

The results shown in Fig. 3 that vitamin C retards the rate of consumption of vitamin E during the

oxidation of PC liposome initiated with AAPH suggest that vitamin C scavenges the initiating radicals from AAPH in an aqueous phase directly before they attack liposome or that vitamin C regenerates vitamin E by reacting with vitamin E radical as observed in homogeneous solution.[10] Probably some combination of these reactions may be involved. The use of water soluble initiator AAPH does not permit us to differentiate which reaction is more important.

On the other hand, the results of oxidation initiated with lipid soluble AMVN give us more information concerning the cooperative and synergistic inhibition of oxidation by vitamins E and C. The results in Figs. 1 and 5 show that, although vitamin C can not scavenge chain-carrying peroxyl radicals within liposomal lipid region efficiently, vitamin C can prolong the induction period in the presence of vitamin E. This strongly supports the interaction of vitamin C in an aqueous phase and vitamin E radical in the lipid region. Apparently, vitamin C is more accessible to vitamin E radical than to peroxyl radicals in the lipid region, since the phytyl side chain of vitamin E must be buried in the lipid region and polar chromanyloxyl head group must be located near the membrane surface.

In contrast to the oxidation initiated with AAPH (Fig. 3), vitamin E did not remain constant but it was consumed gradually (Fig. 4) even though some vitamin C was present in an aqueous phase. This may imply that vitamin C in an aqueous region can not react with vitamin E radical in the lipid layer as efficiently as in the homogeneous system and/or that vitamin E radical reacts with another peroxyl radical (Reaction 3)[30] faster than it reacts with vitamin C (Reaction 2).



Furthermore, the synergistic inhibition of oxidation of methyl linoleate micelle by the combination of vitamin C and 2,2,5,7,8-pentamethyl-6-chromanol (PMC, a vitamin E model) observed previously[32] supports the interaction of vitamin E radical with vitamin C. Vitamin C acts as an efficient antioxidant for the oxidation of methyl linoleate micelle in 0.01 M Triton X-100 aqueous dispersion initiated with AAPH. On the other hand, when AMVN was used as an initiator and the radicals were generated initially in the oil region, vitamin C could not act as an antioxidant. However, even in this case, vitamin C was found to lengthen the suppression period when PMC was located in the methyl linoleate micelle.

It must be also noteworthy that ESR spectrum of vitamin E radical disappeared quite rapidly when it reacted with vitamin C.[22] It was also found by ESR study and absorption spectroscopy that galvinoxyl, a stable phenoxyl radical, which was incorporated into dimyristoyl PC liposome could react with vitamin C in an aqueous phase.[23]

The results and discussion given above strongly suggest that vitamin C can contribute to the synergistic inhibition of oxidation and to the regeneration of vitamin E by reacting with vitamin E radical during the oxidation of PC liposome in an aqueous dispersion as well as in the homogeneous solution.

As shown in Figs. 1b and 5, vitamin C could retard, although slightly, the oxidation of soybean

PC liposome initiated with AMVN. This probably indicates that vitamin C could interact with peroxyl radicals at the membrane surface and/or that vitamin C could penetrate into the membrane slowly. It must be noteworthy that the liposome prepared in this study by soybean PC alone without choresterol may be more leaky than real biomembranes. In biological systems, the oxygen radicals generated in a lipid region must be scavenged by vitamin E while those present in an aqueous region must be scavenged by water soluble antioxidants such as vitamin C, uric acid, and cysteine, and the synergistic inhibition by vitamin E and vitamin C must contribute to maintaining the vitamin E level and also its antioxidant activity in biological membranes.

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