Phospholipids Increase Radical-Scavenging Activity of Vitamin E in a Bulk Oil Model System

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The effect of phospholipids on the radical-scavenging activity of vitamin E in a bulk oil model was studied to clarify the mechanism of their antioxidant synergism. Phospholipids retarded the consumption of vitamin E and elongated the induction period arising from vitamin E in the mixture of methyl linoleate and methyl laurate when exposed to a water-soluble radical initiator. However, they accelerated the consumption of vitamin E in methyl laurate alone. On the other hand, phospholipids little affected the consumption of vitamin E when lipid peroxidation was initiated by a lipid-soluble radical initiator. These results implied that phospholipids enhanced the accessibility of vitamin E toward the chain-initiating radicals in an aqueous microenvironment, resulting in the interruption of chain initiation. Phospholipids could act as neither the free radical scavengers nor the regenerators for vitamin E in this model system. Thus, it is concluded that the enhanced effect of phospholipids for vitamin E in bulk oil originated from the elevation of its radical-scavenging activity.

Keywords: *Vitamin E; phospholipid; antioxidant; synergistic effect*

INTRODUCTION

Considerable studies suggest that phospholipids exert a synergistic effect with vitamin E in inhibiting oxidation of edible oils (Privett and Quackenbush, 1954; Olcott and Veen, 1963; Linow and Mieth, 1976; Bhatia et al. 1978; Hildebrand et al., 1984; Ishikawa et al., 1984; Hudson and Ghavami, 1984; Dziedzic and Hudson, 1984; King et al., 1992; Oshima et al., 1993; Lambelet et al., 1994; Segawa et al., 1994). There are several ideas to explain this synergistic effect, for instance, enhancement of the antioxidant activity of vitamin E (Hildebrand et al., 1984; Ishikawa et al., 1984), regeneration of vitamin E (Lambelet et al., 1994; Segawa et al., 1994), and chelation of pro-oxidant metal ion (Hudson and Mahgoub, 1981). The mechanism of the synergistic effect between vitamin E and phospholipids is still a subject of argument, because oxidation conditions and phospholipid classes used are different in the individual studies.

We recently reported that a novel phosphatidyl derivative of vitamin E, in which the phytyl chain was replaced by a phosphatidyl moiety (Koga et al., 1994), acted as an antioxidant more effectively than vitamin E in lard and in its model oil (Koga and Terao, 1994). Its superiority seems to involve the enhanced effect of phospholipids for vitamin E activity. Furthermore, the antioxidant activity of this derivative against autoxidation of lard was more effective than that obtained by the addition of both vitamin E and phosphatidylcholine (PC) (Koga and Terao, 1994). These findings prompted us to investigate the mechanism of interaction of phospholipids and vitamin E. In the present study, we studied the effect of phospholipids on the radical-scavenging activity of vitamin E in a model system of bulk oil with a water-soluble or a lipid-soluble radical initiator. The results strongly suggest that phospholipids enhance the radical-scavenging activity of vitamin E by increasing the accessibility of vitamin E toward the chain-initiating radicals in an aqueous microenvironment where the initiation takes place.

MATERIALS AND METHODS

Chemicals. Dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), dicaprylphosphatidylcholine (DCPC), dibutyrylphosphatidylcholine (DBPC), dipalmitoylphosphatidylethanolamine (DPPE), and dimyristoylphosphatidylethanolamine (DMPE) were purchased from Sigma Chemical Co. (St. Louis, MO). Vitamin E (*dl*-α-tocopherol) was obtained from Eisai Co. (Tokyo, Japan). 2,2′-Azobis(2-amidinopropyl) dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) were the products of Wako Pure Chemical Co. (Osaka, Japan). Methyl linoleate (99%) and methyl laurate (99%) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Methyl linoleate was purified to remove contaminant hydroperoxide by column chromatography (Terao and Matsushita, 1986). Distilled water was further purified by the Milli-Q reagent system (Millipore Corp., Bedford, MA). The solvents for chromatography were of HPLC grade (Nacalai Tesque Inc., Kyoto, Japan). Other chemicals and solvents were of reagent grade.

Generation of Free Radicals in Bulk Oils. Vitamin E and phospholipids were dissolved in a solution of chloroform and methanol (2:1 v/v) and placed in a test tube. The solvent was removed with a stream of nitrogen and then *in vacuo*. The residue was dissolved in a model oil followed by mixing with a vortex mixer. In the experiments with a water-soluble radical initiator, oxidation was started by the addition of 10 μL of 400 mM AAPH dissolved in water to 1 mL of the mixture and dispersed by a vortex mixer for 30 s followed by ultrasonic irradiation in a Heat System sonifier (Model W-380, Farmingdale, NY) for 1 min. In the experiments with a lipid-soluble radical initiator, AMVN was directly mixed with the solution of vitamin E and phospholipids before removal of the solvent. The mixture was then incubated in the dark at 50 °C with continuous shaking at 120 rpm. At appropriate intervals, aliquots of the reaction mixture were withdrawn at regular

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0021-8561/95/1443-1450$09.00/0

intervals to measure the formation of methyl linoleate hydroperoxides (MeL-OOH) and the residual amounts of vitamin E.

Generation of Free Radicals in the Organic Solvent of Phospholipids with Vitamin E. Free radicals were generated from lipid-soluble azo compounds in the organic solvent of phospholipids with vitamin E to measure the consumption of vitamin E and phospholipids by free radicals. The mixture of chloroform and methanol (1:1 v/v) containing DMPC and DMPE (500 μL) was added to 450 μL of acetonitrile/ethanol (3:2 v/v) containing vitamin E. The oxidation was initiated by adding 50 μL of AMVN in acetonitrile/ethanol (3:2 v/v). Finally, the reaction system consisted of vitamin E (1 mM), AMVN (4 mM), and phospholipids (5 mM) in the mixture of chloroform/methanol/acetonitrile/ethanol (5:5:6:4 v/v/v/v). This mixture was incubated at 50 °C with continuous shaking at 120 rpm in the dark and aliquots were withdrawn at regular intervals to measure the contents of vitamin E and phospholipids.

Measurement of MeL-OOH. The formation of MeL-OOH was measured by HPLC with UV detection at 235 nm, as described previously (Terao and Matsushita, 1986). Samples were injected into a silica column (Inertsil SIL, 6 × 150 mm, 5 μm; GL Sciences, Tokyo, Japan) and eluted with hexane/2-propanol (99:1 v/v) at a flow rate of 2.0 mL/min. The retention time of MeL-OOH was 2.2–2.5 min.

Determination of Vitamin E. Vitamin E was measured by HPLC on an octadecane-bonded silica column (YMC-pack ODS, 6 × 150 mm, 5 μm; Yamamura Chemical Laboratories, Kyoto, Japan) with the eluting solvent of acetonitrile/ethanol (3:2 v/v) at the flow rate of 2.5 mL/min. The eluent was monitored fluorometrically at an excitation wavelength of 298 nm and an emission wavelength of 325 nm using a JASCO 821-FP spectrofluorometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan). Vitamin E was eluted at 4.5 min.

Determination of Phospholipids. DMPC and DMPE were measured by HPLC using a silica column (TSK gel silica 60, 4.6 × 250 mm, 5 μm; TOSOH, Japan) with acetonitorile/methanol/85% H_3PO_4 (900:95:5 v/v/v) as eluent at the flow rate of 1.2 mL/min. The eluent was monitored at 210 nm. DMPC and DMPE were eluted at 13.8 and 8.1 min, respectively.

RESULTS

Effect of Phospholipids on Antioxidant Activity of Vitamin E in the Bulk Oil Models. The mixture consisting of methyl linoleate and methyl laurate (15:85 by mol) was used as a model for edible oil in bulk phase. The concentration of methyl linoleate was adjusted to the linoleic acid content in lard (Koga and Terao, 1994). This model oil was oxidized by a water-soluble azo compound (AAPH) dissolved in a trace amount of water and dispersed in bulk phase. The water content in this model oil is 1% by volume. All phospholipids used here consisted of saturated fatty acids since phospholipids composed of unsaturated fatty acids may exert a pro-oxidant effect (Husain et al., 1986; Oshima et al., 1993; Koga and Terao, 1994). Figure 1 shows the profile of MeL-OOH accumulation and the loss of vitamin E during the AAPH-initiated oxidation of the model oil. In the absence of vitamin E, the oxidation proceeded without any induction period and MeL-OOH accumulated linearly. Vitamin E retarded the accumulation of MeL-OOH with a clear induction period, and MeL-OOH accumulated after vitamin E was mostly consumed. The rate of oxidation without an antioxidant ($R_p = 2.45 \times 10^{-7}$ M/s) was obtained from the slope of the curve for MeL-OOH formation. The rate of chain initiation (R_i) was determined to be 1.63×10^{-9} M/s according to the conventional inhibitor method using vitamin E, which was assumed to trap two radicals (Burton and Ingold, 1981). The kinetic chain length, that is the number of chain propagations per chain-initiating radical, is given by the ratio R_p/R_i and was 1.50×10^2 ($2.45 \times 10^{-7}/1.63 \times 10^{-9}$). This large value indicated that most of the MeL-OOH was formed by the chain propagation reaction in which lipid-peroxyl radicals mediate the chain reaction by attacking another unsaturated lipids. DPPC and DPPE showed little effect on the accumulation of MeL-OOH. However, the combination of vitamin E and DPPC or DPPE apparently retarded the decrease of vitamin E and elongated the induction period by vitamin E as compared with the case in vitamin E alone. DPPC was more effective than DPPE in the retardation of vitamin E consumption and the elongation of its induction period.

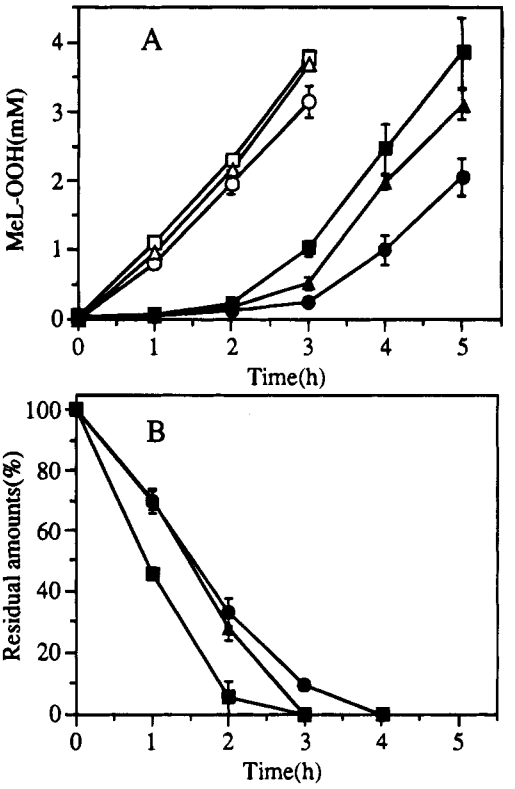

Figure 1. Accumulation of MeL-OOH (A) and loss of vitamin E (B) in the mixture of methyl linoleate and methyl laurate with phospholipids exposed to AAPH. The reaction system consisted of vitamin E (10 nmol) and phospholipids (100 nmol) in a mixture of methyl linoleate and methyl laurate (1 mL, 15:85 by mol) and a trace amount of water (10 μL) with AAPH (4 μmol). The mixture was incubated with continuous shaking at 50 °C in the dark. (□) No addition; (○) DPPC; (△) DPPE; (■) vitamin E; (●) vitamin E in the presence of DPPC; (▲) vitamin E in the presence of DPPE.

Effect of Phospholipids on the Consumption of Vitamin E by Peroxyl Radicals Generated from Azo Compound in the Bulk Phase of Methyl Laurate Alone. The model oil composed of methyl laurate alone was exposed to AAPH or AMVN to exclude the participation of the chain propagation reaction and the chain-propagating lipid-peroxyl radicals in the consumption of vitamin E. In this experiment, vitamin E was decreased by scavenging the radicals generated from AAPH or AMVN directly. When the free radicals were generated by the addition of AAPH with a trace amount of water, vitamin E was consumed more rapidly in the presence of each phospholipid than in the presence of vitamin E alone (Figure 2). On the other hand, little difference was observed in the consumption rate of vitamin E with and without phospholipids in this bulk phase exposed to lipid-soluble radical initiator, AMVN (Figure 3). Thus, phospholipids accelerated the reactiv-

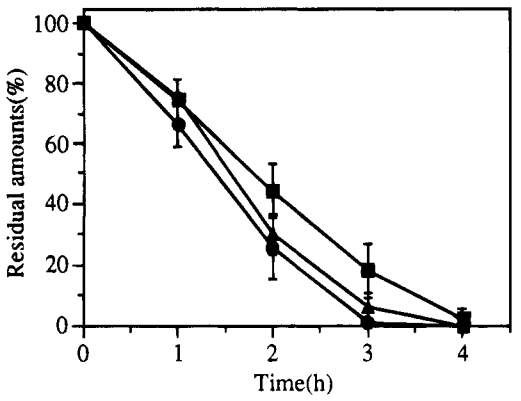

Figure 2. Loss of vitamin E in methyl laurate with phospholipids exposed to AAPH. The reaction system consisted of vitamin E (10 nmol), phospholipids (100 nmol), and AAPH (4 μmol) in methyl laurate (1 mL) and a trace amount of water (10 μL) with AAPH (4 μmol). The mixture was incubated with continuous shaking at 50 °C in the dark. (■) vitamin E; (●) vitamin E and DPPC; (▲) vitamin E and DPPE.

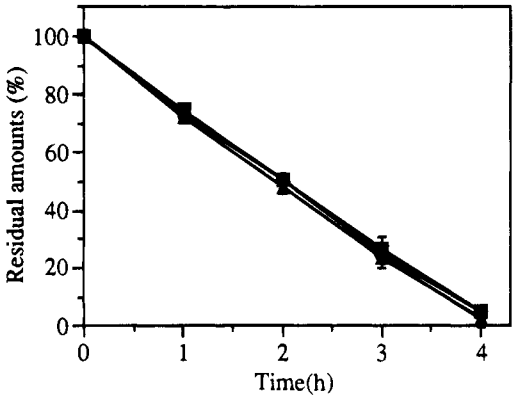

Figure 3. Loss of vitamin E in methyl laurate with phospholipids exposed to AMVN. The reaction system consisted of vitamin E (10 nmol), phospholipids (100 nmol), and AMVN (250 nmol) in a mixture of methyl laurate (1 mL). The mixture was incubated with continuous shaking at 50 °C in the dark. (■) Vitamin E; (●) vitamin E in the presence of DPPC; (▲) vitamin E in the presence of DPPE.

ity of vitamin E with the radical generated from azo compounds only when water-soluble radical generators were added with a trace of water.

Effect of Phospholipids on the Consumption of Vitamin E by Free Radicals Generated from Azo Compound in the Organic Solvent. Figure 4 shows the effect of phospholipids on the consumption of vitamin E by scavenging the free radicals generated from AMVN in a homogeneous system. DMPC and DMPE were used because of their solubility in the organic solvent. Although vitamin E was consumed linearly, no change was observed for DMPC and DMPE. The rate of disappearance of vitamin E was not affected by the presence of phospholipids in this reaction system.

Effect of Hydrocarbon Chain Length in Phospholipids on Antioxidant Activity of Vitamin E in the Bulk Oil Model. To investigate the effect of acyl moieties in phospholipids on the antioxidant activity of vitamin E in the bulk oil model with the water-soluble radical initiator, we tested phospholipids containing various lengths of hydrocarbon chains. As shown in Table 1, DBPC, which contains a short hydrocarbon chain, slightly elongated the induction period of vitamin E. The induction period apparently increased with increasing hydrocarbon chain length of acyl moieties in phospholipids.

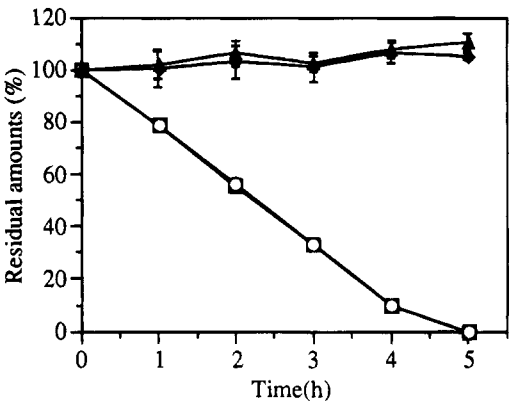

Figure 4. Loss of vitamin E and phospholipids in a homogeneous system exposed to AMVN. The reaction system consisted of AMVN (4 μmol) and vitamin E (1 μmol) and/or phospholipids (5 μmol) in chloroform/methanol/acetonitrile (1 mL, 2:2:1 by vol). The mixture was incubated with continuous shaking at 50 °C in the dark. (□) loss of vitamin E; (○) loss of vitamin E in the presence of DMPC and DMPE; (●) loss of DMPC; (▲) loss of DMPE.

Table 1. Effect of Hydrocarbon Chain Length in the Phospholipid on Antioxidant Activity of Vitamin E

	induction period (min)	rel antioxidant effect[a]
control[b]	97.1	1.00
DBPC (C4:0)	99.7	1.03
DCPC (C10:0)	122.7	1.26
DMPC (C14:0)	179.9	1.85
DPPC (C16:0)	204.6	2.11

[a] Values were calculated on the basis of the induction period treated *vs* control. [b] Without phospholipid.

DISCUSSION

We have studied the effect of phospholipids for the antioxidant activity of vitamin E in a simple model system in which the composition of oil and the initiation mechanism of lipid peroxidation are clearly understood. Since we used relatively high concentrations of azo compounds as a radical initiators, the oxidation was proceeded immediately by a free radical chain mechanism (Niki, 1990) and it excludes the other initiation factors such as metal ions. In addition, the incubation at relatively low temperature (50 °C) avoids the formation of antioxidative browning products from phospholipids (Husain et al., 1986; King et al., 1992). Hence, the model system used here is suitable for evaluating the effectiveness of phospholipids on the radical-scavenging activity of vitamin E in edible oils under storage conditions.

Our preceding work demonstrated that egg yolk PC showed neither inhibitory effect in its own right nor synergistic effect with vitamin E when the same model oils were exposed to lipid-soluble radical initiator, AMVN (Koga and Terao, 1994). The results of this work also showed that DPPC and DPPE had little effect on the consumption of vitamin E in the bulk phase of saturated fatty esters and in organic solvent exposed to AMVN (Figures 3 and 4). This means that no effects of phospholipids are exerted on the action of vitamin E when radical chain reaction is initiated in the lipid phase of bulk oil and in organic solvent. However, the result that the combination of vitamin E and phospholipids was superior as an antioxidant to vitamin E alone on the AAPH-induced oxidation clearly indicates that phospholipids can exert "*the enhanced action*" for vitamin E when the oxidation is initiated in an aqueous

phase. We prefer *the enhanced action* to *the synergistic action*, because the actions of phospholipids presented are different from the real synergistic action (Ingold et al., 1961; Mahoney, 1969) such as the interaction between vitamins E and C (Doba et al., 1985; Niki et al., 1985). In this case, a trace amount of water is dispersed in bulk oil, and AAPH generates the chain-initiating free radicals in this aqueous microenvironment because of its water solubility. Figure 1 also shows that DPPC or DPPE retarded the consumption of vitamin E in bulk oil comprising methyl linoleate and methyl laurate exposed to AAPH. The opposite trend was found in the bulk oil comprising methyl laurate alone, where DPPC and DPPE accelerated the consumption of vitamin E (Figure 2). Since methyl laurate is a saturated fatty acid, most of the free radicals generated from AAPH react with vitamin E directly, resulting in the consumption of vitamin E. The results in the bulk oil comprising methyl laurate alone imply that phospholipids enhance the accessibility of vitamin E toward the free radicals generated in an aqueous microenvironment. In the mixture of methyl linoleate and methyl laurate, the main target for the scavenging activity of vitamin E is the chain-propagating peroxyl radicals generated in the lipid phase because vitamin E is a lipid-soluble antioxidant and the chain propagation reaction seems to proceed rapidly in the lipid phase as indicated by the kinetic chain length (1.50×10^2). Under this condition, one chain-initiating radical produces an enormous number of hydroperoxides by free radical chain mechanism. Therefore, the interruption of the chain propagation by scavenging of the chain-initiating radical is likely to be of great advantage in the suppression of the accumulation of hydroperoxides in bulk oils. In the presence of phospholipids vitamin E may scavenge not only the chain-propagating peroxyl radicals in the lipid phase but also the chain-initiating free radicals generated in an aqueous microenvironment. According to this action, phospholipids enhance the inhibitory effect of vitamin E against the chain initiation and the following accumulation of MeL-OOH by chain propagation in bulk oil comprising methyl linoleate and methyl laurate.

The question arises as to how phospholipids enhance the scavenging activity of vitamin E toward the free radicals. We now postulate that vitamin E is positioned in the aggregation form of phospholipids with the active center of antioxidant action, the phenolic head group, near the polar region where aqueous peroxyl radicals are generated and trapped. This hypothesis is based on previous observations of the antioxidant activity of vitamin E in organic solvent, in emulsion, and in phospholipid bilayers. Amphiphilic molecules such as phospholipids, when dissolved in organic solvent, aggregate to form reverse micelles (Barclay et al., 1984; Luisi et al., 1988), a so-called microemulsion (Han et al., 1991). In a microemulsion the polar head groups of phospholipids are directed toward the interior and a polar core can solubilize water where aqueous peroxyl radicals are generated. On the other hand, the lipophilic acyl chains are exposed to the apolar media. Table 1 shows that the effect of PC to enhance the action of vitamin E is elevated with increasing length of its hydrocarbon chain. The tendency to form aggregates appears to be affected by the hydrocarbon chain length and the hydrophobic to hydrophilic balance within molecules (Tardieu and Luzzati, 1973). In addition, the enhanced effects of phospholipids increased with increasing ratio of phospholipids to vitamin E (data not shown). Thus, the aggregation of phospholipids to form a microemulsion would be involved in their effects to enhance the action of vitamin E. According to the paper by Frankel (1994), lipophilic antioxidants such as vitamin E are sufficiently surface active to be oriented in the oil–water interface to better protect oil against oxidation in the emulsion system. Therefore, it is proper that vitamin E scavenges aqueous peroxyl radicals in a microemulsion more effectively when phospholipids are added together. In addition, the mechanism proposed here is not contrary to our previous observation that a novel phospholipid having the chromanol moiety of vitamin E at its polar head group located in the interior of the aggregate showed strong antioxidant activity in bulk phase. The experimental observations in phospholipid bilayers may also support our hypothesis that vitamin E is positioned near the polar region and can scavenge the aqueous peroxyl radicals in the aggregation of phospholipids. For instance, the polar OH group in vitamin E is suggested to be not located deeply in the hydrophobic region of membranes but near the membrane surface (Takahashi et al., 1989; Fukuzawa et al., 1992). A specific physicochemical interaction of vitamin E and phospholipids has also been proposed in biomembranes from ^1H, ^{13}C, and ^{31}P NMR analyses (Fragata and Bellemare, 1980; Srivastava et al., 1983; Perly et al., 1985; Kagan and Quinn, 1988). In addition, polar peroxyl radicals diffuse toward the polar aqueous region of PC membranes, where they are trapped at or near the aqueous region (Barclay and Vinqvist, 1994).

Some previous studies indicate that the effect of PE on the enhancement of vitamin E activity was stronger than that of PC (Hildebrand et al., 1984; Hudson and Ghavami, 1984; Segawa et al., 1994). However, this study shows that the effect of DPPE for vitamin E was inferior to that of DPPC. Lambelete et al. (1994) suggested that vitamin E is spared by PE when nitroxide radicals are generated from primary amines of PE in the mixture of vitamin E, vitamin C, and PE. However, the phenomenon that vitamin E decreased linearly without loss of DPPC and DPPE by the exposure to AMVN in organic solvent (Figure 4) suggests that phospholipids act as neither the radical scavengers nor the regenerators for vitamin E in homogeneous systems when the chain propagation reaction proceeds. The possibility of the phospholipids acting as regenerators for vitamin E in a heterogeneous system remained. Further investigations will be needed for characterization of the action of each phospholipid in a heterogeneous system.

In conclusion, the effect of phospholipids to enhance the antioxidant action of vitamin E in bulk oils can be explained, at least partly, by the idea that phospholipids enhance the accessibility of vitamin E into the aqueous microenvironment where the initiation reaction takes place, because edible oil usually contains a trace amount of water. Recently, Frankel et al. (1994) reported that the efficiency of antioxidants depends on their affinities toward the air–oil interfaces in bulk oil and the oil–water interfaces in emulsion where the initiation happens exclusively. We concluded that the antioxidant potency of vitamin E in bulk oil is strongly related to its effective concentration at the site where the oxidation is initiated.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropyl) dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); HPLC,

high-performance liquid chromatography; DBPC, dibutyrylphosphatidylcholine; DCPC, dicaprylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; MeL-OOH, methyl linoleate hydroperoxides.

ACKNOWLEDGMENT

We thank Ms. K. Moro for technical assistance.

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Received for review October 27, 1994. Revised manuscript received March 16, 1995. Accepted March 27, 1995.[⊗]

JF940608P

[⊗] Abstract published in *Advance ACS Abstracts,* May 15, 1995.