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Rate of Free-Radical Oxidation of C_{18} Diene and Triene Fatty Acids in Aqueous Micellar Solutions and Effectiveness of β-Carotene as an Inhibitor of Their Oxidation

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Abstract—The rate of accumulation of conjugated dienes of polyunsaturated fatty acids was measured during free-radical oxidation of linoleic acid (18:2n-6, LA), α-linolenic acid (18:3n-3, α-LNA), and γ-linolenic acid (18:3n-6, γ-LNA) initiated by 2,2′-azo-bis-(2-amidinopropane) hydrochloride in aqueous micellar solutions of sodium dodecyl sulfate and sodium cholate. It was shown that, unlike homogeneous solutions, the oxidative stability of PUFAs in aqueous dispersions increased with an increase in the extent of unsaturation. The rate of LA oxidation was more than tenfold greater than that of α- and γ-LNA. The antioxidant activity of β-carotene, in contrast to homogeneous solutions, in both micellar systems studied depended on the degree of PUFA unsaturation. We found that 5 μM β-carotene effectively inhibited the LA oxidation (almost by 90%), whereas the oxidation of α-LNA and γ-LNA was not inhibited by β-carotene even at much greater concentration (30 μM). The paradoxical discrepancy between the extent of unsaturation and the PUFA oxidation rate, as well as a decrease in the efficiency of β-carotene-dependent inhibition of oxidation of more polyunsaturated fatty acids in reactions conducted in aqueous dispersions is consistent with the model according to which the peroxyl radicals of LA and fatty acids with the double-bond number greater than two exhibit different polarity.

Key words: linoleic acid, linolenic acid, β-carotene, conjugated dienes, lipoperoxides, antioxidants, free-radical oxidation, micelle

Currently, studies of the role of carotenoids in preventive maintenance and treatment of some pathologies (including cardiovascular and oncological diseases) attract considerable attention [1, 2]. However, the results of clinical use of carotenoids are fairly controversial [1, 2], which may be due to the concentration-dependent effect of β-carotene-containing drugs. Indeed, earlier we found that, depending on the dose administered, β-carotene in mammalian tissues *in vivo* may have both antioxidant and prooxidant effect [3], with its positive therapeutic effect being exerted only at a concentration that causes inhibition of free-radical oxidation [3, 4]. In model systems, β-carotene effectively suppresses free-radical oxidation of polyunsaturated fatty acids (PUFAs), presumably forming adducts with the lipid peroxyl radicals (low activity carbon-centered radicals) [5].

These adducts may undergo degradation to form lipid alcoxyl radicals, which allows β-carotene to act as a prooxidant, inducing co-oxidation of other unsaturated substrates, such as PUFAs [5, 6].

The majority of works on the antioxidant effect of β-carotene was performed using free-radical oxidation modeling in homogeneous systems. However, it is obvious that lipid oxidation should be studied in aqueous dispersions to understand the oxidative processes occurring in biomembranes and other cell structures. In view of this, the purpose of this work was to study the effect of β-carotene on induced free-radical oxidation of micro-heterogeneous aqueous dispersions formed by fatty acids with identical length of the carbon chain but different extent of unsaturation. The oxidation rates of diene and triene C_{18} fatty acids in micellar solutions of sodium cholate and sodium dodecyl sulfate, as well as the effi-

Abbreviations: PUFA) polyunsaturated fatty acids; LA) linoleic acid; LNA) linolenic acid; ABAP) 2,2′-azo-bis-(2-amidinopropane) hydrochloride.

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ciency of inhibition of their oxidation by β-carotene, were studied. It is shown that the rate of free-radical oxidation of PUFAs and the extent of its inhibition by β-carotene reciprocally depend on the extent of unsaturation of the substrate oxidized.

MATERIALS AND METHODS

In the study we used linoleic (18:2n-6, LA), α-linolenic (18:3n-3, α-LNA), and γ-linolenic (18:3n-6, γ-LNA) acids. The micelles were prepared by injecting the PUFA alcoholic solutions (the final concentration of alcohol did not exceed 2%) with constant stirring on a Vortex mixer into medium containing 50 mM K,Na-phosphate buffer (pH 7.4) and either 5 mM sodium cholate or 40 mM (or 100 mM) SDS as micelle-forming detergents. Oxidation was performed under aerobic conditions at 37°C in 50 mM phosphate buffer (pH 7.4) supplemented with 10 μM diethylenetriaminepentaacetic acid (a transition-metal chelating agent). Oxidation was induced by addition of 2,2′-azo-bis-(2-amidinopropane) hydrochloride (ABAP), a water-soluble azoinitiator. The PUFA oxidation rates were monitored by the increase in the optical density of conjugated dienes at 233 nm on a Hitachi-557 spectrophotometer. The concentration of conjugated dienes was calculated using the molar extinction coefficient $\varepsilon = 2.7 \cdot 10^4$ liter·mol^{-1}·cm^{-1} [7]. β-Carotene was added to the oxidation medium in the form of a hydrosol (BASF). All PUFAs used in the study were obtained from Sigma (USA), and ABAP was from Polysciences Inc (USA).

RESULTS

ABAP-induced oxidation of linoleic acid in SDS-containing micelles in the absence and presence of β-carotene. It is known that the critical concentration of micelle formation by SDS is 7-8 mM [8]. Therefore, the major portion of SDS (used at concentrations 40 and 100 mM) was apparently incorporated in micelles. Because one SDS micelle contains approximately 60 SDS molecules [9], the concentrations of micelles in our experiments (at the specified SDS concentrations) were approximately 0.5 and 1.5 mM, respectively. The LA concentration in the mixed micelles varied from 0.4 to 5 mM. The kinetic curves of conjugated diene accumulation during the ABAP-induced LA oxidation in all experiments were linear for at least the first 40 min (Fig. 1, curve *1*). The dependence of the rate of the conjugated diene accumulation on the LA concentration in micelles is shown in Fig. 2 (curves *1* and *2*). As is seen from this figure, when the number of LA molecules per micelle was less than two, the rate of the conjugated diene accumulation increased proportionally to the PUFA concentration. At

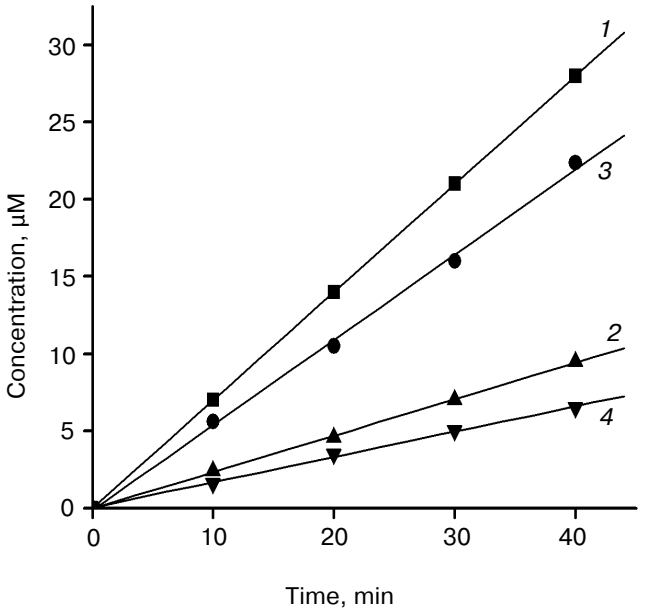


Fig. 1. Kinetics of ABAP-induced oxidation of micelles formed by 1 mM linoleic acid in the presence of 40 mM SDS (*1*, *2*) and 5 mM sodium cholate (*3*, *4*) in the absence of β-carotene (*1*, *3*) and in the presence of 2.5 μM β-carotene (*2*, *4*) (2.4 mM ABAP).

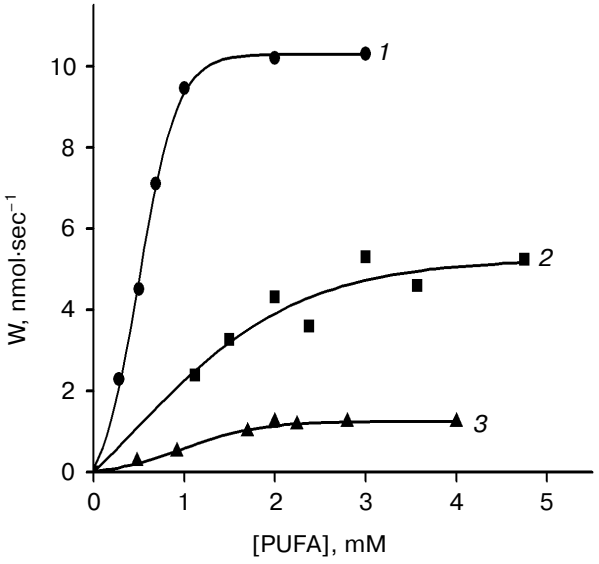


Fig. 2. Dependence of the rate of accumulation of conjugated dienes on the PUFA concentration during ABAP-induced oxidation of micelles formed by (*1*) linoleic acid in the presence of 40 mM SDS (2.4 mM ABAP), (*2*) linoleic acid in the presence of 100 mM SDS (2.4 mM ABAP), and (*3*) γ-linolenic acid in the presence of 40 mM SDS (3.6 mM ABAP).

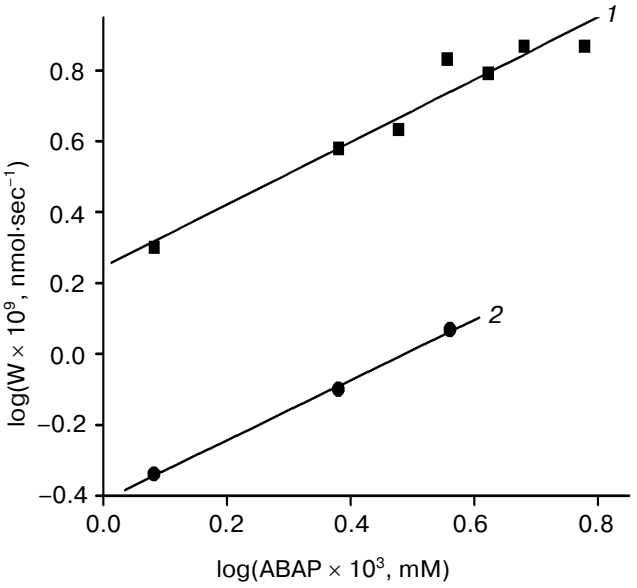


Fig. 3. Dependence of the rate of the conjugated diene accumulation on the ABAP concentration during oxidation of micelles formed in the presence of 40 mM SDS by (*1*) 2.38 mM linoleic acid and (*2*) 2.24 mM γ-linolenic acid. The data are plotted in double logarithmic coordinates.

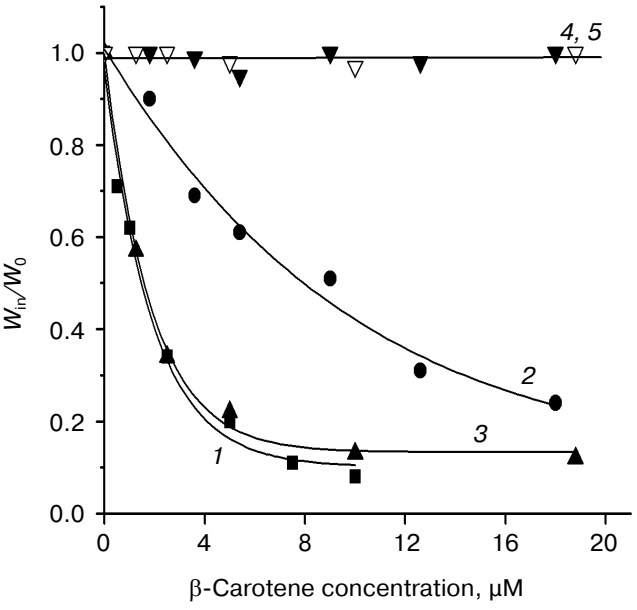


Fig. 4. Inhibition by β-carotene of ABAP-induced oxidation of micelles formed by (*1*) 1.0 mM linoleic acid in the presence of 40 mM SDS, (*2*) 2.36 mM linoleic acid in the presence of 100 mM SDS, (*3*) 1.0 mM linoleic acid in the presence of 5 mM sodium cholate, (*4*) 1.0 mM α-linolenic acid in the presence of 40 mM SDS, and (*5*) 1.0 mM α-linolenic acid in the presence of 5 mM sodium cholate. The ordinate shows the ratio between the oxidation rates in the presence (W_{in}) and absence (W_0) of β-carotene (2.4 mM ABAP).

a greater LA content in micelles, the rate of conjugated diene accumulation did not depend on the PUFA concentration. At a constant LA concentration, the rate of oxidation decreased as the SDS concentration increased (Fig. 2, curves *1* and *2*). A nonlinear dependence of the rate of the conjugated diene accumulation on the PUFA concentration observed in our experiments (Fig. 2) indicates that the kinetics of the ABAP-induced LA oxidation in micelles is inconsistent with the classical kinetic scheme of free-radical oxidation of hydrocarbons in homogeneous solution, according to which the rate of chain oxidation (W) of unsaturated substrates is determined by the equation $W = k_2/2k_3^{0.5}[C]\ W_i^{0.5}$, where k_2 and k_3 are the rate constants of propagation and termination of the oxidation chain, respectively, [C] is the substrate concentration, and W_i is the initiation rate [10]. When studying the dependence of the rate of LA oxidation on the ABAP concentration (Fig. 3, curve *1*), we found that W is proportional to the azoinitiator concentration (the slope is 0.88 ± 0.1 in the double-logarithmic coordinate system). This is also inconsistent with the classical kinetic scheme, according to which the slope should be equal to 0.5. Hence, in our experiments we recorded predominantly linear termination of chain oxidation. Similar kinetic tendencies were observed in [11] describing methyllinoleate oxidation in SDS micelles in the presence of 2,2′-azo-bis(4-carboxyisovaleronitrile), another water-soluble inducer.

Kinetics of LA oxidation in the presence of 2.5 μM β-carotene is shown in Fig. 1 (curve *2*). As is seen from this figure, β-carotene efficiently inhibits LA oxidation, which proceeds at constant rate without an induction period. The relative values of the rate of β-carotene-inhibited LA oxidation depended both on the β-carotene and SDS concentrations (Fig. 4, curves *1* and *2*). Note that the concentration of micelles and the inhibitory concentration of β-carotene in our experiments were approximately 1 mM and 5 μM, respectively (i.e., one molecule of β-carotene per approximately 200 micelles). This is indicative of a rapid exchange of β-carotene molecules between the micelles and suggests that the time of the β-carotene molecule retention in a micelle is much shorter than the lifetime of the LA peroxyl radical.

ABAP-induced oxidation of α- and γ-linolenic acid in SDS-containing micelles in the absence and presence of β-carotene. Figure 2 (curve *3*) shows the dependence of the rate of conjugated diene accumulation on the concentration of γ-linolenic acid. The dependence of the rate on the azoinitiator concentration plotted in double logarithmic coordinates is shown in Fig. 3 (curve *2*). The results confirm that the tendencies in ABAP-induced oxidation of γ-linolenic acid in the presence of 40 mM SDS are similar to those observed for LA (Figs. 2 and 3), i.e., the oxidation of γ-linolenic acid in SDS-containing micelles is also characterized by a competition between the linear and the quadratic chain termination (the slope is 0.84 ± 0.4).

The results show that, at equal PUFA concentrations, the absolute values of the rates of accumulation of conjugated dienes during γ-LNA oxidation were almost an order of magnitude lower than those recorded during the LA oxidation. It should be noted that the azoinitiator concentration in the experiments with γ-LNA was even somewhat higher (3.6 mM) than in the corresponding experiments with LA (2.4 mM)—i.e., at equal ABAP concentrations the differences between the absolute values of the rates of oxidation of γ-LNA and LA should be even greater. Because the rate of α-LNA oxidation was twofold lower than the rate of γ-LNA oxidation (data not shown), the differences between the rates of α-LNA and LA oxidation were even more significant.

Unlike the inhibited LA oxidation, β-carotene at a concentration range from 1 to 30 μM did not suppress the accumulation of conjugated dienes during α-LNA and γ-LNA oxidation (Fig. 4, curve *4*).

ABAP-induced oxidation of linoleic and α-linolenic acids in solutions containing 5 mM sodium cholate in the absence and presence of β-carotene. It is known that sodium cholate in aqueous solutions forms aggregates (atypical micelles) with a low number of associated molecules ($n = 2$, 4, and more, depending on the detergent concentration) [12]. According to the data obtained by different researchers, the critical concentration of micelle formation for sodium cholate varies within the range of 3-16 mM, depending on the method of measurement [12]. Thus, in our experiments, the concentration of micelles formed in the presence of 5 mM sodium cholate was approximately 1 mM, which is comparable with the PUFA concentration used (0.6-3 mM LA and 1-2.5 mM α-LNA). It can be assumed that, under these conditions, we observed the formation of mixed micelle-like aggregates of PUFAs and sodium cholate.

The results of experiments on the oxidation of LA and α-LNA in micelles formed in the presence of 5 mM sodium cholate are shown in Fig. 5. One can see that, under the conditions used, the rate of the conjugated diene accumulation linearly depends on the PUFA concentration. Figure 6 shows the dependences of the rate on the initiator concentration plotted in double logarithmic coordinates. We found that, in the sodium cholate-containing micelles, the rate of the conjugated diene accumulation during the oxidation of LA and α-LNA was proportional to the azoinitiator concentration at a power of 0.5. These results led us to conclude that the kinetics of the PUFA oxidation in the presence of 5 mM sodium cholate obeys to the classical kinetic scheme of free-radical chain oxidation in homogeneous medium. Similarly to the SDS-containing micelles, the rate of α-LNA oxidation was by an order of magnitude lower than the rate of LA oxidation.

The conjugated diene accumulation during LA oxidation in the sodium cholate-containing micelles, as well as in the SDS-containing micelles, was effectively inhibited by β-carotene, with the oxidation proceeding at constant rate without an induction period (Fig. 1, curves *3* and *4*). The relative rate of β-carotene-inhibit-

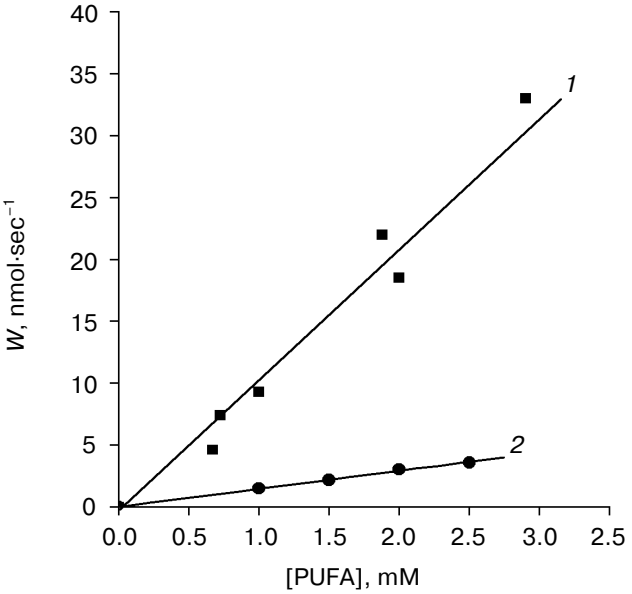


Fig. 5. Dependence of the rate of the conjugated diene accumulation on the PUFA concentration during ABAP-induced oxidation of micelles formed in the presence of 5 mM sodium cholate by (*1*) linoleic acid and (*2*) α-linolenic acid (2.4 mM ABAP).

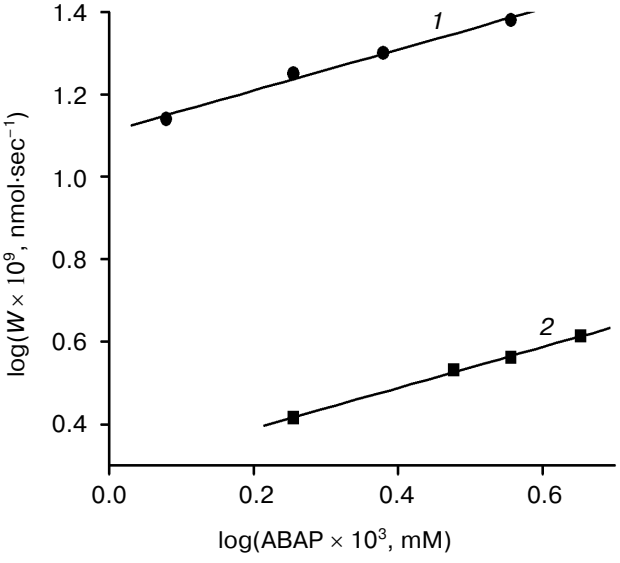
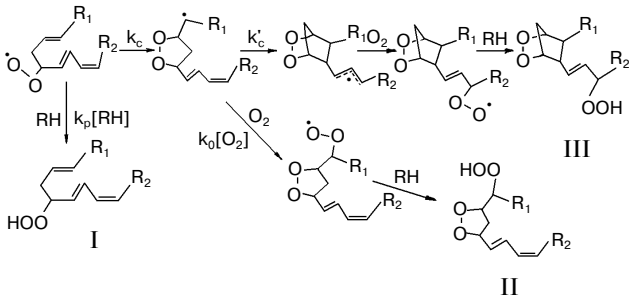


Fig. 6. Dependence of rate of conjugated diene accumulation on ABAP concentration during oxidation of micelles formed in the presence of 5 mM sodium cholate by (*1*) 2.38 mM linoleic acid and (*2*) 2.24 mM α-linolenic acid (2.4 mM ABAP). The data are plotted in double logarithmic coordinates.

ed oxidation of LA in the sodium cholate-containing micelles, similar to the SDS-containing micelles, markedly depended on the concentration of β-carotene (Fig. 4, curve *3*). However, β-carotene had no effect on the α-LNA oxidation in both types of micelles within the concentration range of 1-20 μM (Fig. 4, curves *4* and *5*).

DISCUSSION

Currently, the mechanism of oxidation of PUFAs containing more than two double bonds in homogeneous systems can be illustrated by the following scheme [13]:



One can see that the oxidation of such PUFAs yields not only aliphatic hydroperoxides (the only primary product of LA oxidation, product I), but also cyclic peroxides containing a conjugated double bond (product II) and bicyclic endoperoxides without a conjugated double bond (product III). Thus, taking into account the characteristic features of oxidation of trienes and more unsaturated fatty acids, it can be assumed that the rate of conjugated diene accumulation (the method of oxidation monitoring used in this work) does not completely reflect the rate of the PUFA oxidation measured by a decrease in the content of the unoxidized substrate [14]. Nevertheless, as was shown in [15], the oxidation of γ-LNA-containing micelles did not lead to considerable production of endoperoxides without a conjugated double bond (product III on the scheme), which is characteristic of oxidation in homogeneous systems. This finding assumes that, owing to the high microviscosity of micellar media [16], a repeated cyclization of the PUFA peroxyl radical in micelles, which is accompanied by the product III formation, is hampered. Thus, it can be concluded that the process of PUFA oxidation in aqueous systems can be satisfactorily characterized by the rate of the conjugated diene accumulation. This conclusion is also confirmed by the data published in [14]. The authors showed that, in the course of oxidation of ethyl linoleate and ethyl docosahexaenoate in Tween-20-containing micelles, utilization of both substrates correlated with accumulation of the conjugated dienes. It is well known that the oxidizability of PUFA in homogeneous systems increases with an increase in the number of double bonds in the fatty-acid molecule [17]. However, our results show that the rate of accumulation of conjugated dienes in micelles during induced oxidation of diene LA is more than tenfold greater than that recorded during oxidation of triene LNA (Figs. 2 and 5). These results are consistent with the data obtained in [18], where it was shown that the rate of utilization of α-LNA and γ-LNA during induced oxidation in Tween-20-containing micelles was approximately tenfold and fivefold lower, respectively, than the rate of the LA utilization. In addition, it was found that the PUFA resistance to induced oxidation in the micelles containing Tween-20 [18] and Triton X-100 [19] increases in the series $C_{18:2} < C_{18:3} < C_{20:4} < C_{20:5} < C_{22:6}$.

Apparently, a marked decrease in the PUFA oxidizability upon an increase in the number of double bonds in the molecule is not related to the characteristic features of the micelle formation in the presence of different types of detergents, because it is observed in micelles formed using both ionic (Figs. 2 and 5) and nonionic [14, 18-20] detergents. Our results are consistent with the model suggested by a group of Japanese researchers [19], according to which the LNA peroxyl radicals after cyclization and attachment of two oxygen molecules (product II, see the scheme) become more polar than the LA peroxyl radicals after attachment of one oxygen molecule. Hence, the more polar LNA radicals should be displaced to the micelle periphery near the phase interface, whereas the less polar LA radicals should be concentrated predominantly inside the micelles. As a result, during the LNA oxidation the peroxyl radicals are located on the micelle surface, becoming spatially separated from the *bis*-allyl methylene groups of unoxidized LNA molecules (from which they should accept a hydrogen ion in the propagation reaction). This process should be accompanied by a decrease in the rate of chain propagation and an increase in the rate of chain termination as well, which is consistent with our data (Figs. 2 and 5). The effects observed may be also explained by other hypotheses. In particular, it was assumed that the oxidizability of PUFAs with different extent of unsaturation is determined by the differences in their conformation in the micelle [14, 18].

We found that the antioxidative activity of β-carotene in both micellar systems studied depends on the extent of saturation of the PUFAs oxidized (Fig. 4), which agrees with the differences in the sensitivity of diene and triene PUFAs to oxidation in micelles (Figs. 2 and 5). Indeed, β-carotene effectively inhibited oxidation of the LA micelles and, even at high concentrations, had no effect on oxidation of the α-LNA micelles (Fig. 4). An opposite effect was observed in a homogeneous system: the inhibitory activity of β-carotene increased with an increase in the extent of unsaturation of the substrate oxidized [21]. The mode of inhibition by β-carotene of free-radical oxidation of PUFAs with different extent of

unsaturation in micellar systems can be explained based on the notion of the effect of the peroxyl radical polarity on the PUFA oxidative stability in micelles [19]. It is known that the mechanism of inhibition of the PUFA oxidation by β-carotene is based on attachment of the peroxyl radicals involved in the oxidation chain reaction to the isoprenoid system of the conjugated double bonds in the β-carotene molecule. β-Carotene, as a hydrophobic molecule, is located in the hydrophobic nucleus of the micelle and should easily trap the less polar LA peroxyl radicals but not the LNA radicals located in more hydrophilic region of micelles. This hypothesis is corroborated by the experimental data on the efficiency of inhibition of the PUFA oxidation in micelles by antioxidants differing in lipophilicity and, hence, located in the micelle areas with different hydrophobicity [22]. It can be concluded that the efficiency of an antioxidant should be estimated with regard for the relative position of the antioxidant molecules and the fatty-acid peroxyl radicals in the structures studied. For example, β-carotene may inhibit free-radical reactions in the liposomal membrane even more effectively than α-tocopherol [23].

Thus, the results of this work and the data of other authors [14, 18-20] are indicative of a paradoxical discrepancy between the PUFA oxidation rate and the efficiency of inhibition by β-carotene of oxidation of PUFAs with different extent of unsaturation. The precise mechanism of this phenomenon remains obscure. Based on the notion of different polarity of the peroxyl radicals, it can be assumed that, in the course of oxidation of a PUFA mixture, PUFAs with greater extent of unsaturation will play a stabilizing role both in natural lipid–protein complexes and in model systems. This assumption is confirmed by the results on the oxidation of micellar mixtures comprised of LA and eicosapentaenoic acid [19] or LA and docosahexaenoic acid [18]. On induced *in vitro* oxidation of low-density lipoproteins containing various LA and polyunsaturated fatty acids at different ratios, lower oxidation rates were characteristic of those lipoproteins in which the relative content of triene and more unsaturated PUFAs was higher [24]. These results confirm that, in natural lipid–protein supramolecular complexes, oxidation of PUFAs with different extent of unsaturation may have a more complex, less predictable nature than in homogeneous systems. It cannot be ruled out that inconsistency between the oxidation rate and the extent of lipid unsaturation may be expressed in an intact organism, which was indirectly confirmed by our earlier data [25] on predominant detection of the cholesterol linoleate hydroxyl derivatives in the zones of atherosclerotic lesion of human aorta, despite the simultaneous presence in them of considerable amount of more unsaturated cholesterol esters.

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