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Zeaxanthin (dihydroxy-β-carotene) but not β-carotene rigidifies lipid membranes: a ^{1}H-NMR study of carotenoid-egg phosphatidylcholine liposomes

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Abstract

^{1}H-NMR technique was applied to study liposomes formed with egg-yolk phosphatidylcholine containing as an additional component two carotenoid pigments: β-carotene or zeaxanthin (dihydroxy-β-carotene). A strong rigidifying effect of zeaxanthin but not of β-carotene with respect to hydrophobic core of lipid bilayer was concluded from the carotenoid-dependent broadening of the NMR lines assigned to $-CH_2-$ groups and terminal $-CH_3$ groups of lipid alkyl chains. A similar effect of zeaxanthin with respect to polar headgroups was concluded on the basis of the effect of the pigment on the shape of NMR lines attributed to $-N^+(CH_3)_3$ groups. In contrast, β-carotene increases motional freedom of lipid polar headgroups. The inclusion of both carotenoids to liposomes resulted in the enhanced penetration of Pr^{3+} ions to the polar zone of the external layer of a membrane monitored by the splitting of the $-N^+(CH_3)_3$ signal, the effect of β-carotene being much more pronounced. Differences in the effect on membrane structure and molecular dynamics observed for β-carotene and its polar derivative are discussed in terms of organization of a carotenoid-containing lipid membrane.

Keywords: β-Carotene; Zeaxanthin; Carotenoid; Lipid membrane; NMR, ^{1}H-

1. Introduction

Carotenoids are widespread pigments of bacteria, algae, plants and animals [1]. Some carotenoid pigments like β-carotene, zeaxanthin and lutein are also present in human blood serum [2–4]. Physiological importance of the presence of carotenoids in living organisms is connected to their action as accessory pigments absorbing light and transferring it to chlorophyll in photosynthesis [5] and protection of neighbouring biomolecules against oxidative damage [6]. The latter action is realised via the quenching of triplet states of photosensitizers and deactivation of singlet oxygen and free radicals [6]. A different physiological function of carotenoids is directly related to their molecular structure and is usually referred to as modifying effect with respect to biomembranes. The effect of carotenoid pigments on struc-

Abbreviations: Car, β-carotene; Zea, zeaxanthin; EYPC, egg-yolk phosphatidylcholine.

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tural and dynamic properties of biological membranes and model lipid membranes has been extensively studied in recent years by means of several experimental techniques: electron paramagnetic resonance (EPR) [7–12], light scattering in liposome suspension [13,14], differential scanning calorimetry [7,15,16], X-ray diffractometry [17–19] and nuclear magnetic resonance applied to phosphorous nuclei (^{31}P-NMR) and carbon nuclei (^{13}C-NMR) of phospholipid molecules [20]. The effect of carotenoid pigments on structural and dynamic properties of lipid membranes observed in those biophysical studies may be summarised as follows:

(1) The chromophore of carotenoid pigment molecules is located in the hydrophobic core of a membrane. Xanthophyll pigments with their polar groups located at the opposite ends of a rigid, rod-like molecule are oriented to placing polar groups in two opposite polar zones of the bilayer.

(2) The effect of carotenoid pigments on molecular dynamics of a lipid membrane is based on a hydrophobic, van der Waals interaction between rigid pigment molecules and alkyl lipid chains undergoing continuous *gauche–trans* isomerization and hydrogen bonding formation between polar groups of xanthophylls and lipid heads. These interactions result in the fluidization of the well-ordered structure of a lipid membrane (usually observable at temperatures below the main phase transition) and the rigidifying effect to a membrane in its fluid state. The effect of the increase of motional freedom of lipid molecules in a membrane referred to as the fluidization is reported to be more distinctly pronounced in the case of β-carotene while the opposite – the rigidifying effect – is reported in the case of polar xanthophylls.

In the present study we present the results of ^{1}H-NMR measurements of liposomes formed with egg-yolk phosphatidylcholine, containing as an additional component either β-carotene or its 3,3′-dihydroxy derivative, zeaxanthin. This study was under-

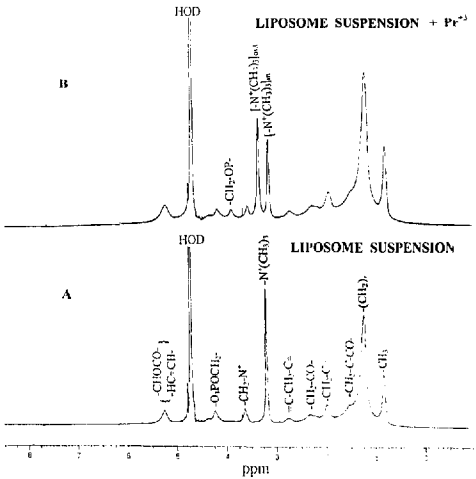

Fig. 1. Typical 300 MHz ^{1}H-NMR spectrum with assignments of the sample of EYPC liposome suspension in D_2O (A) and the same suspension after addition of $PrCl_3$ to the final concentration of 4.06 mM (B).

taken in order to assess the effect of polar groups of carotenoids in their modification of dynamic and structural properties of lipid membranes. In the present work membranes were formed with natural lipid and the effect of both carotenoids was studied by means of the same technique and under the same experimental conditions. In our opinion the differences in the effect of carotenes and xanthophylls on biomembranes may provide crucial arguments for the

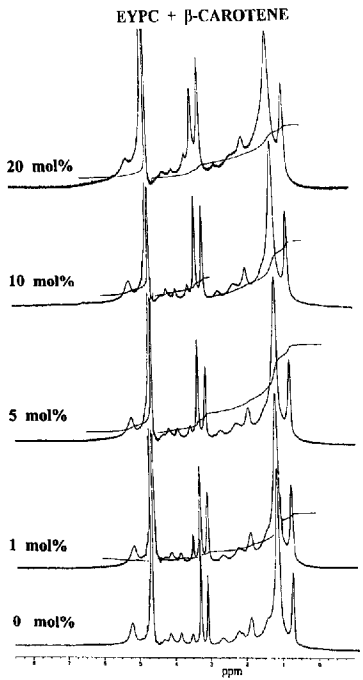

Fig. 2. Typical ^{1}H-NMR spectra of liposome suspension formed with EYPC and β-carotene in various molar percentages, as indicated. $PrCl_3$ was added to the samples before measurement.

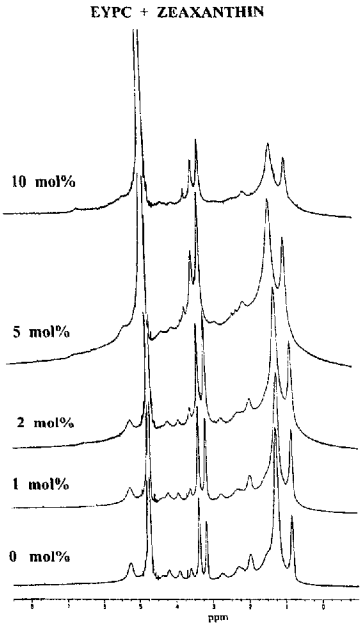

Fig. 3. Typical ^{1}H-NMR spectra of liposome suspension formed with EYPC and zeaxanthin in various molar percentages, as indicated. $PrCl_3$ was added to the samples before measurement.

dispute on a drug effect and biological action of carotenoids. According to some very recent reports, the presence of β-carotene in human organism is not a health-promoting factor but even increases the risk of cancer (see discussion in Ref. [21] and [22]).

2. Materials and methods

Egg-yolk phosphatidylcholine was prepared according to the method described by Singleton et al.

[23]. Crystalline carotenoid pigments: β-carotene (β,β-carotene) and zeaxanthin ((3R,3'R)-β,β-carotene-3,3'-diol) were a generous gift from Hoffmann–La Roche, Basel. Praseodymium chloride ($PrCl_3 \cdot 6H_2O$) was purchased from Sigma and heavy water D_2O was obtained from the Institute of Nuclear Research, Świerk (Poland). All chemicals were of the analytical grade.

The stock solution of lecithin in chloroform was stored at −20°C. The lecithin was mixed with a carotenoid, dried under nitrogen and dispersed in D_2O added. The final concentration of lecithin was 25 mg per ml. The concentrations of carotenoids were 0.5, 1.0, 5.0, and 10.0 mol% with respect to the lipid. The suspensions were then sonicated under nitrogen for 30 min with a 20 kHz sonicator with a titanium probe. During the sonication the samples were thermostated at 0–2°C. The sonication was followed by the centrifugation for 5 min at 690 × *g* in order to remove titanium particles. NMR data were collected for samples of 0.5 ml vesicle suspension with 4.06 mM $PrCl_3$ in 5-mm NMR tubes. The ratio of the $N^+(CH_3)_3$ downfield signal to the upfield signal after the addition of praseodymium was approx. 1.40.

^{1}H-NMR spectra were recorded on Bruker Avance DRX 300 spectrometer, 300 MHz ^{1}H-NMR parameters were as follows spectral window 6173 Hz; digital resolution 0.188 Hz; pulse width 4.5 μs (30° flip angle); acquisition and delay times were 2.65 s and of 1 s, respectively; acquisition temperature 300 K.

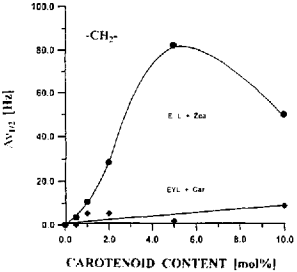

Fig. 5. Carotenoid related increase in a half-width ($\Delta\nu_{1/2}$) of the ^{1}H-NMR maximum corresponding to a CH_2 group of alkyl chains of EYPC in liposomes as a function of the molecular percentage of β-carotene or zeaxanthin, as indicated.

3. Results

Fig. 1A shows the complete 300 MHz ^{1}H-NMR spectrum with assignments for EYPC vesicles in

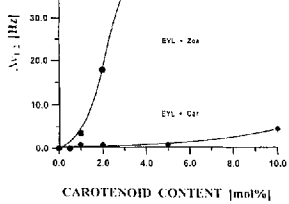

Fig. 4. Carotenoid related increase in a half-width ($\Delta\nu_{1/2}$) of the ^{1}H-NMR maximum corresponding to a CH_3 group of alkyl chains of EYPC in liposomes as a function of the molecular percentage of β-carotene or zeaxanthin, as indicated.

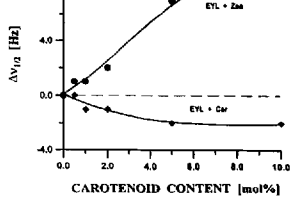

Fig. 6. Carotenoid related increase in a half-width ($\Delta\nu_{1/2}$) of the ^{1}H-NMR maximum corresponding to a $-N^+(CH_3)_3$ choline polar headgroup of EYPC in liposomes as a function of the molecular percentage of β-carotene or zeaxanthin, as indicated.

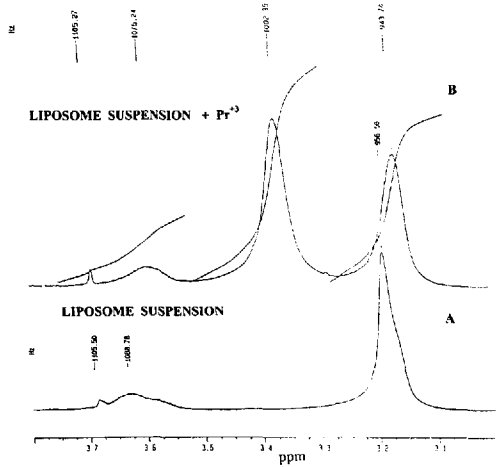

Fig. 7. Typical 300 MHz ^{1}H-NMR spectrum in the region of the resonance of proton in a $-N^+(CH_3)_3$ choline group of the sample of EYPC liposome suspension in D_2O (A) and the same suspension after addition of $PrCl_3$ to a final concentration of 4.06 mM (B).

D_2O. The major $(CH_2)_n$, CH_3, and choline $N^+(CH_3)_3$ resonance of phosphatidylcholines are well recognised, [24–27]. Fig. 1B shows the spectrum of EYPC vesicles in D_2O after the addition of Pr^{3+} with an extravesicular concentration of 4.06 mM. The initial single peak of $N^+(CH_3)_3$ resonance is split by the addition of 'shifts reagents' into the resolved downfield and upfield components. The downfield signal comes from the extravesicular (out) and the upfield resonance from intravesicular (in) choline headgroups [27–29]. Figs. 2 and 3 present typical ^{1}H-NMR spectra of liposome suspension formed with EYPC and containing as an additional component β-carotene and zeaxanthin, respectively, in different molecular fractions, as indicated. As it may be seen at first glance, carotenoid addition affects the ^{1}H-NMR spectrum of the lipid. The effect of carotenoids may be analysed precisely by comparison of a half-width of a certain NMR maximum in spectra recorded from pigmented liposomes and from the control [20]. Such

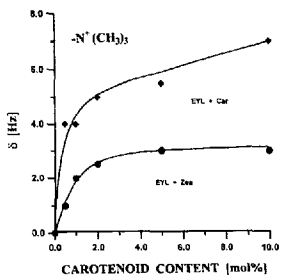

Fig. 8. Increase of splitting of the maxima (δ) related to the proton resonance in a $-N^+(CH_3)_3$ group in EYPC liposome suspension after addition of $PrCl_3$ as a function of the molar percentage of β-carotene or zeaxanthin present in the lipid phase (see Fig. 7).

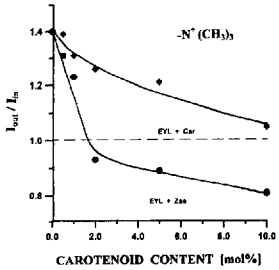

Fig. 9. Intensity ratio (I_{up}/I_{in}) of the upfield and downfield H^1-NMR maxima related to $-N^+(CH_3)_3$ group split after addition of $PrCl_3$ as a function of the molar percentage of β-carotene or zeaxanthin present in EYPC liposomes (see Fig. 7).

an analysis is presented in Fig. 4 for the proton resonance in a $-CH_3$ group, in Fig. 5 in $(-CH_2-)_n$ groups and in Fig. 6 for $-N^+(CH_3)_3$ choline headgroup. A different kind of information on a structural effect of carotenoids on EYPC liposome suspension supplemented with Pr^{3+} may be obtained from the analysis of the split of the $-N^+(CH_3)_3$ resonance (Figs. 7 and 8) and an intensity ratio of the downfield and upfield maxima (Fig. 9).

4. Discussion

The analysis of the position of absorption maxima of zeaxanthin and β-carotene in EYPC liposomes indicates that the chromophores of both pigments are localised within the hydrophobic core of a lipid bilayer [30]. However, there are significant differences in orientation of β-carotene and zeaxanthin with respect to the membrane: the long axis of Zea forming the narrow angle of 44 ± 3° with the axis normal to the plane of the membrane while Car being distributed homogeneously within the membrane without any preferred well defined orientation [30]. The comparison of the thickness of the hydrophobic core of EYPC membrane calculated on the basis of the diffractometric measurements like in Ref. [17] (22.6 Å, [30]) and the distance of the opposite hydroxyl groups of Zea (30.2 Å, [13]) indicates that, indeed, the orientation of 42° is required to fulfil the conditions, so that the hydrophobic portion of the pigment molecule is placed in the hydrophobic compartment of the membrane and at the same time both polar ends of Zea are in contact with the opposite polar zones of the bilayer. Such an organization of EYPC-Zea membrane in which pigment molecules are anchored in both polar sides of the bilayer provides exceptional conditions for a rigidifying action of the carotenoid with respect to lipid acyl chains realised via the van der Waals interactions. There are not similar possibilities in the case of β-carotene lacking polar groups responsible for the orientation of xanthophyll pigments in lipid bilayers [17,18] and the organization of lipid-carotenoid membranes (see Fig. 10 for an illustration). Differences between Car and Zea in their effect on EYPC membranes are very distinctly expressed by the differences in all parameters applied to the analysis of ^{1}H-NMR spectra (see Figs. 1–9). The effect of the broadening of spectral peaks which is directly related to limitations of the segmental movement of lipid molecules [20] is very strong in the case of CH_2 groups (Fig. 4) and terminal CH_3 groups (Fig. 5) of lipid alkyl chains in the zeaxanthin-containing samples but almost negligible in the samples containing β-carotene. This is an indication of the rigidifying effect of Zea but not Car to the hydrophobic part of the membrane. At 10 mol% Zea the effect observed is not as strong as that one observed at 5 mol%. This is most probably related to the process of the xanthophyll aggregation in the membrane which initiates at about 10 mol% carotenoid with respect to lipid [31]. The effect of

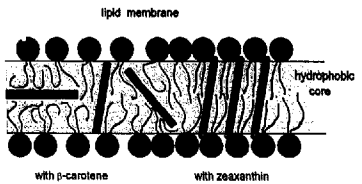

Fig. 10. Schematic drawing of the EYPC membrane containing β-carotene and zeaxanthin, indicated. Please note the differences in the orientation of carotenoids, organization of alkyl chains and the distance between lipid headgroups discussed in the text.

carotenoids on the polar headgroup region may be analysed in Fig. 6 where proton resonance in choline group in pure and pigmented membranes are compared. In that case, the effect of Zea and Car are opposite. The inclusion of nonpolar carotene to the membrane increases motional freedom of polar headgroups, presumably via generation of a free space in that portion of the bilayer. A split of that resonance maximum brought about by the addition of Pr^{3+} is also indicative of a free space in the headgroup region of the phosphatidylcholine membrane. As may be seen from Fig. 8, Car is much more effective than Zea in reducing a penetration barrier of Pr^{3+} to the headgroup region of the membrane, in accordance with the conclusion drawn above.

The influence of Zea on mechanical properties of EYPC membranes is strongly pronounced while forming small unilamellar vesicles out of the large multilamellar liposomes by a sonication procedure. A mean size and a type of liposomes are well characterised by the intensity ratio of the shift-reagent-split maxima of $-N^+(CH_3)_3$ resonance of polar heads exposed to the extravesicular compartment and covered inside liposomes [26–29] (I_{out}/I_{in}, see Fig. 9). Such a ratio being typically as high as 1.4 for small unilamellar vesicles was not possible to be reached during the prolonged sonication of the carotenoid-containing samples and remained lower than 1 (typically for a dispersion of multilamellar vesicles) at the Zea concentrations above 2 mol% (see Fig. 9). This effect also expresses reinforcement of the membrane structure by the xanthophyll pigment.

The very sharp differences in the effect of β-carotene and its polar derivative on structural and dynamic properties of EYPC membranes, reported above, may explain the differences in the efficacy of carotenes and xanthophylls in protection of lipid molecules against free-radical-induced damage [32]. Zeaxanthin appeared to be much more effective than β-carotene in protection of EYPC membrane, however, there were no differences observed in such a protection in the organic solvents [32]. As it follows from the results presented above, β-carotene is not only effective in reinforcement of the membrane structure by rigidifying of the hydrophobic core but even makes the membrane less compact in its polar region. This latter effect of β-carotene may have a direct effect on a decrease of the penetration barrier of not only positively charged particles, as demonstrated above, but also small molecules including active oxygen species responsible for initiation of the lipid peroxidation process. In contrast, polar carotenoid pigments like zeaxanthin seem to be well suited to stabilise the biomembrane structure and provide protection against oxidative damage.

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