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# Spin-label studies on phosphatidylcholine-polar carotenoid membranes: effects of alkyl-chain length and unsaturation

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Spin-labeling methods were used to study the structure and dynamic properties of phosphatidylcholine (PC)-dihydroxycarotenoid membranes as a function of phospholipid alkyl chain length, alkyl chain saturation, temperature and mol fraction of carotenoids. (1) Dihydroxycarotenoids, zeaxanthin and violaxanthin increase order and decrease motional freedom of the lipid alkyl chains in fluid-phase PC membranes. The effect of carotenoids decreases as the alkyl chain length of saturated PC increases. (2) The abrupt changes of spin-label motion observed at the main-phase transition of the saturated PC membranes are broadened and shifted to lower temperatures. At a carotenoid concentration of 10 mol%, they disappear for short-chain PC membranes (12–14 carbons), but are still observed for long-chain PC membranes (18–22 carbons). (3) In fluid-phase PC membranes possessing short alkyl chains (12–14 carbons), the activation energy of the rotational diffusion of 16-doxylstearic acid spin label (16-SASL) is significantly lower at a carotenoid concentration of 10 mol%. The difference decreases as the alkyl-chain length increases. (4) The presence of unsaturated alkyl chains greatly reduces the effects of carotenoids on the mobility of the polar headgroups as observed with tempocholine dipalmitoylphosphatidic acid ester and on the order of alkyl chains near the polar headgroup region as observed with 5-doxylstearic acid spin label (5-SASL). The effect of unsaturation is, however, moderate in the membrane center as shown with 16-SASL. Also, the effect of carotenoids on the order and motion of the rigid and highly anisotropic molecules dissolved in the PC membranes is significantly greater in saturated PC membranes.

## Introduction

Carotenoids constitute a large class of substances which is wide-spread in living organisms. Although they are synthesized by Prokaryota and plants, they occur also in many animals including vertebrates [1]. In the human body carotenoids are present in ocular retina [2,3] and in blood [4,5]. It has been shown in many cases that carotenoids are effective protectors against the destructive action of light and oxygen [6–8]. This biological function of carotenoids is due to their photophysical and photochemical properties. The other biological function of carotenoids is related to their rigid molecular structure. Biological membranes usually contain a substance which regulates their thermodynamical and mechanical properties. In Eukaryota this role is played by cholesterol or its derivatives. It has been postulated that carotenoids and other terpenoids play a similar role in membranes which do not contain cholesterol [9,10]. Evidence has also appeared that carotenoids influence the fluidity of cell membranes of *Acholeplasma laidlavii* [11]. There are papers dealing with modification of the mechanical, physicochemical properties, as well as dynamics of artificial membranes containing carotenoids [12–18]. Our previous works considered orientation of polar carotenoids in dimyristoylphosphatidylcholine (DMPC) [19] and digalactosyldiacylglycerol [20] multibilayers, effect of violaxanthin and zeaxanthin on the oxygen diffusion-concentration product in DMPC liposomes [21,22] and effect of violaxanthin and zeaxanthin on the structure and dynamics of DMPC membranes [23].

In this paper, we investigated the interactions of violaxanthin (zeaxanthin (5–6,5′-6′)-diepoxide) with phosphatidylcholine (PC) membranes having saturated

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Abbreviations: ALS, androstane spin label; CSL, cholestane spin label; DLPC, L-α-dilauroylphosphatidylcholine; DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; DSPC, L-α-distearoylphosphatidylcholine; DBPC, L-α-dibehenoylphosphatidylcholine; EYPC, egg-yolk phosphatidylcholine; EPR, electron paramagnetic resonance; SASL, stearic acid spin label; 5-SASL, 5-doxylstearic acid spin label; 16-SASL, 16-doxylstearic acid spin label; T-PC, tempocholine dipalmitoylphosphatidic acid ester.

alkyl chains of different length and the interaction of zeaxanthin with egg-yolk phosphatidylcholine (EYPC) membranes having unsaturated chains with *cis* double bonds. In photosynthetic membranes, these carotenoids are interconverted one into another by the reactions of the so called violaxanthin cycle. The aim of the study is to elucidate the role of alkyl-chain length and unsaturation in the carotenoid-membrane interaction. We will present the investigation of PC-dihydroxycarotenoid interaction with particular attention to: (1) mobility of the hydrocarbon chains of PC using stearic-acid spin labels (SASL); (2) wobbling and rotation of highly anisotropic rigid molecules dissolved in the membrane using cholestane spin label (CSL) and androstane spin label (ASL); (3) motion of the polar headgroups using tempocholine dipalmitoylphosphatidic acid ester (T-PC). Also, special attention will be paid to the match in hydrophobic lengths between phospholipid alkyl chains and a conjugated hydrocarbon chain of carotenoid and to the steric nonconformability between the 30° bend of the *cis* double bond of the unsaturated alkyl chains and the rigid rod-like structure of carotenoid.

## Materials and Methods

Dilauroylphosphatdiylcholine (DLPC) containing alkyl chains of twelve carbon atoms (12C), DMPC (14C), dipalmitoylphosphatidylcholine (DPPC) (16C), distearoylphosphatidylcholine (DSPC) (18C) and dibehenoylphosphatidylcholine (DBPC) (22C) were purchased from Sigma (St. Louis, MO, USA). EYPC, which consisted of 70% 1-palmitoyl-2-oleoyl-PC [24] was also purchased from Sigma. Spin labels were purchased from Molecular Probes (Eugene, OR, USA). T-PC was a generous gift from Dr. Ohnishi at Kyoto University. The carotenoids zeaxanthin and violaxanthin were extracted from fresh nettle leaves. The extract was saponified by the 'cold' method [25]. Carotenoids were separated by thin-layer chromatography on activated Kiselgel plates (Merck, FRG) using the solvent system: benzene/ethyl acetate/methanol (75 : 20 : 5 (v/v)). The purity of carotenoids was checked by HPLC. It appeared that violaxanthin contained traces (less than 1%) of lutein or zeaxanthin and a non-polar carotenoid. Zeaxanthin contained a few percent of lutein and violaxanthin and traces of a non-polar carotenoid. The visible absorption spectra of both carotenoids agreed with those known from the literature and did not show any features of the *cis*-isomerization. The concentration of carotenoids was determined spectrophotometrically using the extinction coefficients given in Ref. 25.

The membranes used in this work were multilamellar dispersions of investigated PC containing various amounts of carotenoids in the range of miscibility [26] (0–10 mol%) and 1 mol% of spin label. Membranes were prepared in the following method [27]: Chloroform solutions of the lipids, carotenoids and spin labels were mixed (containing $10^{-5}$ mol of total lipids) and the chloroform was evaporated with a stream of nitrogen gas and then under a reduced pressure (0.1 mmHg) for at least 12 h. A buffer solution (0.1 ml) was added to the dried lipids at about 20°C above the phase transition temperature of the phospholipid membranes and vortexed vigorously. The buffer used for the study was 0.1 M borate at pH 9.5. A rather high pH was chosen to ensure that all SASL probe carboxyl groups were ionized in the PC membranes [28–30]. The structures of phosphatidylcholine membranes are not altered at this pH [27,30,31]. In some cases, to obtain better signal-to-noise ratio in the electron paramagnetic resonance (EPR) spectra, the lipid dispersion was centrifuged briefly at $12\,000 \times g$ for 15 min at 4°C, and the loose pellet (about 20% lipid (w/w)) was used as the sample. It is highly probable that in our preparation part of the carotenoids forms small aggregates or microprecipitates in the water phase of the suspension [12,13]. However, carotenoid aggregates remain outside of the lipid bilayer, and should not influence the EPR spectra, which come from lipid-soluble spin labels. Moreover, the EPR signals are one component, typical of spin labels in a lipid bilayer. The centrifugation to some degree allows the separation of the vesicles from external carotenoid aggregates, which settle down at the conic end of the microcentrifuge tube. About 30 $\mu$l of the sample (dispersion or top portion of the loose pellet) was transferred to a gas-permeable capillary (0.7 mm i.d. and 0.3 mm wall thickness) made of a methylpentene polymer called TPX [32]. This plastic is permeable to nitrogen, oxygen and other gases and is substantially impermeable to water. The TPX sample tube was placed inside the EPR dewar insert and equilibrated with nitrogen gas that was used for temperature control. The sample was thoroughly deoxygenated to prevent EPR line broadening and prevent possible oxidation of the sample. EPR spectra were obtained with a Varian E-3 or Varian E-109 X-band spectrometer using Varian temperature control accessories. All preparations and measurements were performed in darkness or dim light.

The chemical structures of dihydroxycarotenoids and spin labels and also their approximate localization in the membrane are shown in Ref. 23. This reference also contains the typical EPR spectra with indicated spectral parameters used in the present work.

## Results and Discussion

### *Saturated phosphatidylcholines*

The central line heights ($h_0$) in the EPR spectra of the 5-doxylstearic acid spin label (5-SASL) in liposome membranes composed of saturated PC with an addi-

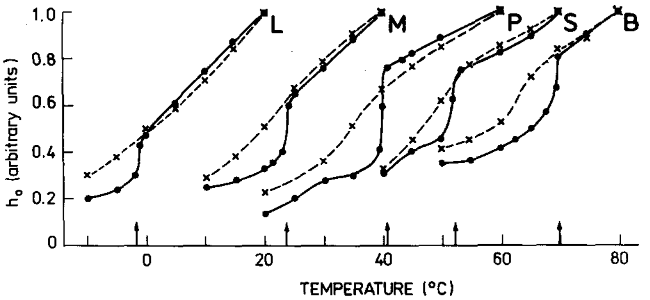

Fig. 1. Temperature profiles of peak height ($h_0$) of the central line of 5-SASL EPR spectra in PC membranes without additions (●——●) and containing 10 mol% violaxanthin (×- -×). L, M, P, S and B represent DLPC (saturated alkyl chains with 12 carbons), DMPC (14 carbons), DPPC (16 carbons), DSPC (18 carbons) and DBPC (22 carbons), respectively. Arrows indicate the main phase-transition temperatures of pure PC membranes measured using spin-labeling methods.

tion of 10 mol% of violaxanthin or in membranes composed of the PC without any additions are shown in Fig. 1 as functions of temperature. For each of investigated PC the range of temperature includes the main phase transition that is visible as a relatively abrupt drop of $h_0$-value. In the presence of violaxanthin, the phase transition becomes broadened and shifted to lower temperatures (DPPC, DSPC and DBPC) or disappears completely (DLPC and DMPC). As can be seen, the effect of carotenoids on $h_0$ is much bigger in gel phase than in fluid-phase membranes. Similar results were obtained with 16-doxylstearic-acid spin label (16-SASL).

In Fig. 2, the temperature dependence of the order parameter ($S$) of 5- and 16-SASL in the investigated membranes is displayed. The order parameter in fluid-phase membranes composed of PC without any additions, when compared just above the temperature of the main phase transition ($T_m$), diminishes with the length of the alkyl chains of PC. The same parameter, when compared at the same temperature demonstrates a very small dependence on chain length. However, in the case of 5-SASL, one can easily notice a small increase in the value of $S$ with the length of the alkyl chains of PC (Fig. 2A).

In general, the order parameter $S$ grows when violaxanthin is introduced into the membrane. This effect of violaxanthin diminishes with increasing temperature and alkyl chain length. The influence of carotenoids on the spin probe movement as monitored by the increase in the value of $S$ can be related to the lowering of temperature ($\Delta T$), which causes the same increase in $S$-value as introducing 10 mol% of carotenoids into the membrane. The values of $\Delta T$ obtained from Fig. 2 are shown in Table I. The values given in Table I show the following: (1) the effect of introducing violaxanthin is greater in the interior of the membrane (16-SASL position) than near the polar headgroup region (5-SASL position); (2) the shorter the length of the PC alkyl chains, the greater is the ordering effect of carotenoids; (3) the effect of the carotenoids in the region near the polar headgroups (5-SASL) is minimal (or there is no effect) on DPPC and DSPC membranes having acyl chains consisting of 16- and 18-carbon atoms, respectively.

The presence of carotenoids in the membranes changes the values of rotational correlation time of the 16-SASL free radical moiety. As a rule, the admixture

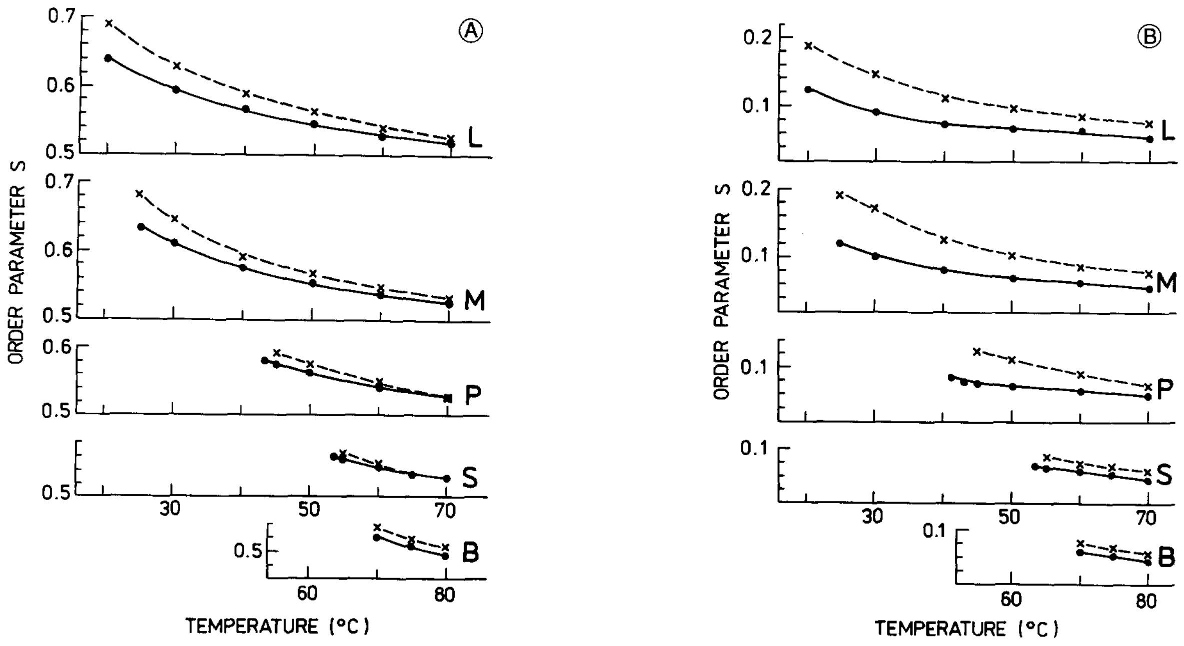

Fig. 2. Order parameter $S$ of 5-SASL (A) and 16-SASL (B) as a function of temperature in fluid-phase PC membranes with 0 mol% (●——●) and 10 mol% (×- -×) violaxanthin. Symbols are the same as in the previous figure. Notice the shift of temperatures for the lowest graph.

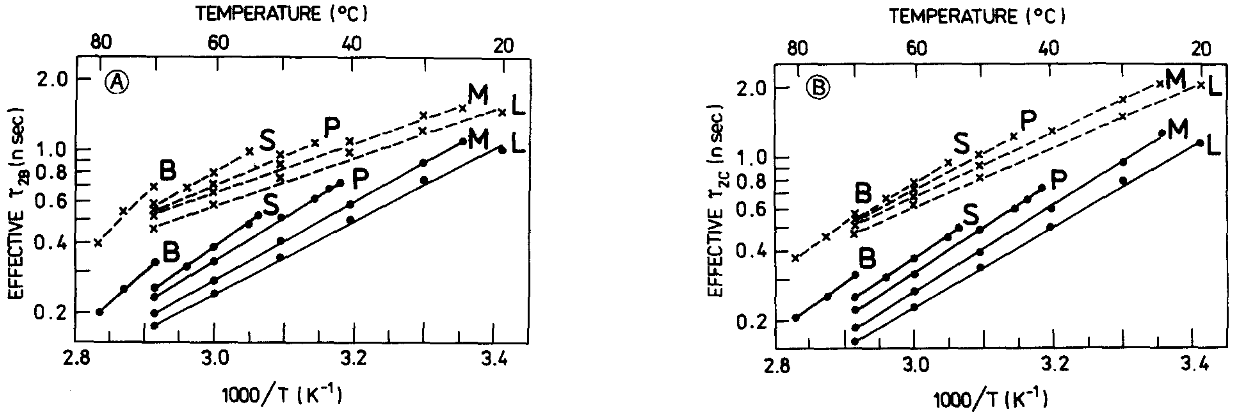

Fig. 3. Effective rotational correlation time $\tau_{2B}$ (A) and $\tau_{2C}$ (B) of 16-SASL in PC membranes plotted as a function of reciprocal temperature with 0 mol% (•——•) and 10 mol% (×– –×) violaxanthin. Symbols are the same as in Fig. 1.

of 10 mol% of violaxanthin increases the correlation time and suppresses its abrupt increase at the fluid to gel-phase transition (data not shown). Fig. 3 presents correlation times calculated from the linear term of the linewidth parameter ($\tau_{2B}$) (Fig. 3A) and with the quadratic term ($\tau_{2C}$) (Fig. 3B) as a function of the reciprocal of the temperature. The anisotropy of the spin label movement is manifested as a difference between $\tau_{2B}$ and $\tau_{2C}$. Only in DLPC and DMPC membranes without any additions at temperatures close to the main phase transition $\tau_{2B}$ differs significantly from $\tau_{2C}$. The anisotropy is not as distinct in other membranes studied. Addition of violaxanthin decreases motional freedom of the 16-SASL free radical moiety, which is indicated by a large increase in correlation time (Fig. 3). At temperatures near the phase transition of all of the studied lipids, the presence of carotenoids strongly increases the difference between $\tau_{2B}$ and $\tau_{2C}$, indicating the onset of anisotropic rotational diffusion. Carotenoids significantly increase the anisotropy of the 16-SASL motion in DLPC and DMPC, but less so in DPPC. A weak effect is observed in DSPC, and, again, a fairly strong effect is observed in DBPC membranes. The presence of violaxanthin in membranes not only strengthens the anisotropy of motion of 16-SASL (Fig. 3), but also lowers the activation energy of this motion (Fig. 4). This decreasing of the activation barrier is distinct in membranes composed of PC with short hydrocarbon chains and disappears in membranes composed of PC with long hydrocarbon chains (see Fig. 4). A lower activation barrier should result in a smaller correlation time of rotational motion, but we observed an increase in the correlation time in the presence of violaxanthin. Hence, violaxanthin exerts some spatial constraints on the rotational motion of the spin label, that is decreases the length of the diffusional step. The decrease of diffusional step length by carotenoid seems to be a cause for increasing the correlation time. The effect is especially significant in membranes made of short chain PC. In spite of the fact that the spatial constraints exerted by the carotenoids are, in this case, partly compensated for by

TABLE I

*Decrease of temperature (ΔT) causing the same increase of the order parameter S as introducing 10 mol% of violaxanthin into the membrane (in °C)*

| Membrane | $T$ slightly higher than $T_m$ | | $T$ = 70°C | |
|---|---|---|---|---|
| | 5-SASL | 16-SASL | 5-SASL | 16-SASL |
| DLPC | 8 | 15.4 | 5 | 30 |
| DMPC | 6 | 17.2 | 5 | 26 |
| DPPC | 5 | 21 | 0 | 17 |
| DSPC | 2.4 | 11 | 0 | 12 |
| DBPC | 4 | 10 | 4 | 10 |

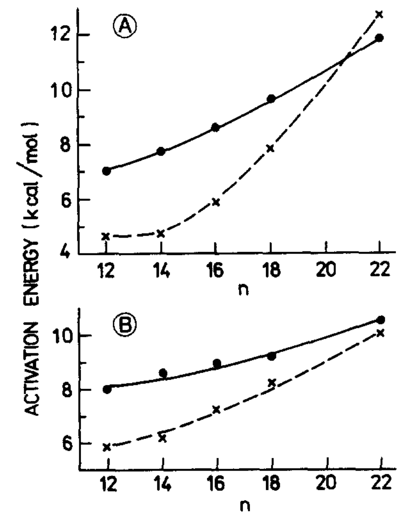

Fig. 4. Activation energy for rotational diffusion of 16-SASL in fluid-phase PC membranes plotted as a function of the length (number of carbons, $n$) of alkyl chains of the host lipid for membranes with 0 mol% (•——•) and 10 mol% (×– –×) violaxanthin. The activation energy was calculated from an Arrhenius display (log $\tau$ vs. $1/T$) presented in Fig. 3 from $\tau_{2B}$ (A) and from $\tau_{2C}$ (B).

lowering of the activation energy, the increase of the correlation time in the short chain PC is greater than in the long chain PC. For example, the presence of violaxanthin in DLPC membranes increases $\tau_{2B}$ by a factor of 2.7 and in DBPC membranes by a factor of 2.0.

The investigated carotenoids modify the membranes composed of short-chain PCs to a higher degree than those composed of long-chain PCs. The effect of the carotenoids is bigger in the interior of the membrane than near the polar headgroup region (see $S$ for 5- and 16-SASL). The admixture of carotenoids increases the rotational correlation time of 16-SASL, and in membranes that are composed of short chain (12–16C) PCs, decreases the activation energy of rotational diffusion.

We believe that differences in the effect of carotenoids on bilayers composed of PCs with alkyl chains of differing length is due to the interrelation between the thickness of hydrophobic core of the bilayer and the length of carotenoid molecule. It is most likely that when dihydroxycarotenoid molecules intercalate into the membrane, their polar hydroxyl groups are positioned near the middle of the glycerol backbone regions of the PC molecule on opposite halves of the lipid bilayer. Therefore, we think that the thickness of the glycerohydrocarbon region of the membrane is important. Unfortunately, we do not have at disposal sufficient information for the estimation of the bilayer thickness of PCs investigated over a wide temperature range. Because of that, we used an approximation derived by Cornell and Separovic [33] to calculate the thickness of the glycerohydrocarbon regions ($H$) of a fully hydrated membrane at temperatures well above their respective phase transitions:

$$H = (18.7 + 0.66n)\ \text{Å} \tag{1}$$

$n$ is the number of alkyl-chain carbons in the series of PCs, ranging from 12 to 22. The thicknesses obtained for DLPC, DMPC, DPPC, DSPC and DBPC are: 26.6 Å, 27.9 Å, 29.3 Å, 30.6 Å and 33.2 Å, respectively. It can be seen that the thickness of the hydrophobic core of DLPC and DMPC bilayers is sufficiently smaller than the distance between hydroxyls in zeaxanthin and violaxanthin molecules, which is equal to 30.2 Å [12]. The thickness of the hydrophobic core of the DBPC bilayer is essentially bigger than 30.2 Å. The X-ray diffraction data on DMPC multibilayers [19] has shown that an admixture of 1 mol% of violaxanthin or zeaxanthin increases the thickness of the bilayer hydrophobic core by about 2 Å. No published data exists for the other phosphatidylcholines, but we can expect that dihydroxycarotenoids thicken any lipid bilayer whose undisturbed thickness is smaller than the length of carotenoid molecule. In such a situation, the carotenoid molecules have a tendency to orient themselves closely to the normal to the plane of the bilayer and impose rather *trans*- than *gauche*-conformation on the neighbor alkyl chains. Both of these effects result in an increased thickness of the lipid bilayer. Cholesterol also increases the thickness of thin PC bilayers such as DMPC and DPPC, while it reduces the thickness of DSPC membranes [34]. The effect, like that in DSPC, can be expected in a DBPC bilayer where the carotenoid molecule is too short to span the undisturbed membrane.

We will now discuss how these considerations help us to understand our results. Carotenoids will influence the EPR spectra of 5-SASL if they disturb the near polar headgroup region of the membrane; this is the case in DLPC and DMPC membranes. Because of the small thickness of the bilayer, the rigid carotenoid molecules must be oriented at quite a wide angle with respect to the normal to the bilayer plane. So, carotenoid molecules differ substantially in their orientation from the alkyl chains that reside in the $L_\alpha$-state perpendicular to the plane of the bilayer [19]. In DPPC and DSPC bilayers the order parameter of 5-SASL is almost uninfluenced by carotenoids, suggesting that the thickness of the hydrophobic core and the length of carotenoid molecules are very close to each other. Carotenoid molecules, like the alkyl chains of DPPC and DSPC, are perpendicular to the plane of the bilayer and do not disturb significantly the environment of the spin moiety of 5-SASL. DBPC bilayer has the thickness of hydrophobic core bigger than the length of carotenoid molecule and at least one of the two rings of carotenoid molecule must be located near the polar headgroup region and must disturb the movement of the spin moiety of 5-SASL. In effect, we observe measurable increase in the order parameter of 5-SASL in DBPC membranes. The most fluid central part of membrane is ordered by carotenoids independent of the length of the alkyl chains (see Fig. 2B).

### *Egg-yolk phosphatidylcholine*

In our previous investigations [19,21], we did not observe any essential differences between violaxanthin and zeaxanthin influence on the DMPC membranes. However, in the case of unsaturated lipid digalactosyl diacylglycerol the ordering effect of zeaxanthin was distinctly bigger than that of violaxanthin [20]. This is why we have decided to use zeaxanthin with unsaturated EYPC membranes.

In Fig. 5, the order parameter $S$ of 5- and 16-SASL in EYPC membranes with and without any additions of zeaxanthin are shown as a function of temperature. As in saturated PCs, the effect of carotenoids is significantly greater in the central part of the membrane than near the polar headgroup region. However, in contrast to the saturated PCs, the effect does not depend on temperature. The presence of carotenoids in EYPC

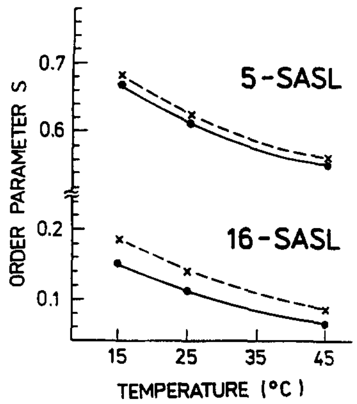

Fig. 5. Order parameter $S$ of 5-SASL and 16-SASL as a function of temperature in EYPC membranes with 0 mol% (●———●) and 10 mol% (× – – ×) zeaxanthin.

membranes increases the rotational correlation times $\tau_{2B}$ and $\tau_{2C}$ of 16-SASL (Fig. 6). However, the effect is less than that in saturated PC membranes. The rotational diffusion of 16-SASL in pure EYPC membranes is anisotropic at temperatures below 30°C. The presence of carotenoids in the membrane strengthens this anisotropy as shown by an increase in the difference between $\tau_{2B}$ and $\tau_{2C}$. The curves presented in Fig. 6 for EYPC are close to those presented in Fig. 3 for DMPC and DPPC, suggesting some similarities between carotenoid interaction with EYPC membranes and short chain saturated PC membranes. The plots of log $\tau$ vs. reciprocal temperature shown in Fig. 6 are not straight lines, and it is impossible to characterize rotational diffusion of the 16-SASL free-radical moiety in EYPC membranes by a single activation energy. But, at any temperature, the curves referring to the membranes containing zeaxanthin have less slope than those referring to the EYPC membranes without any addition. This means that carotenoids decrease the activation barrier for rotational diffusion of 16-SASL. Similar results were obtained with DOPC membranes (data not shown). Indeed, the average thickness of the glycerohydrocarbon region of the EYPC bilayer calculated by Cornell and Separovic [33] on the basis of previous measurements [35–39] is about 29 Å. In any case, this value is smaller than the distance between hydroxyl groups in zeaxanthin and violaxanthin molecules, especially if we take into account that these groups are located near the middle of the glycerol backbone of the PC molecules.

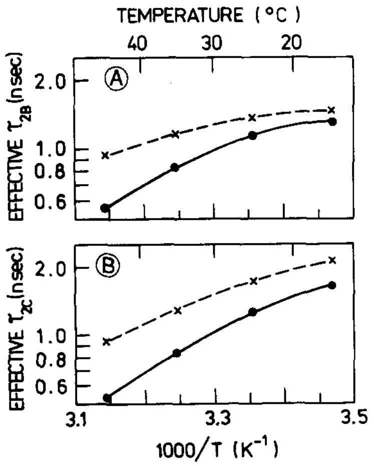

Fig. 6. Effective rotational correlation time $\tau_{2B}$ (A) and $\tau_{2C}$ (B) of 16-SASL in EYPC membranes plotted as a function of reciprocal temperature for membranes with 0 mol% (●———●) and 10 mol% (× – – ×) zeaxanthin.

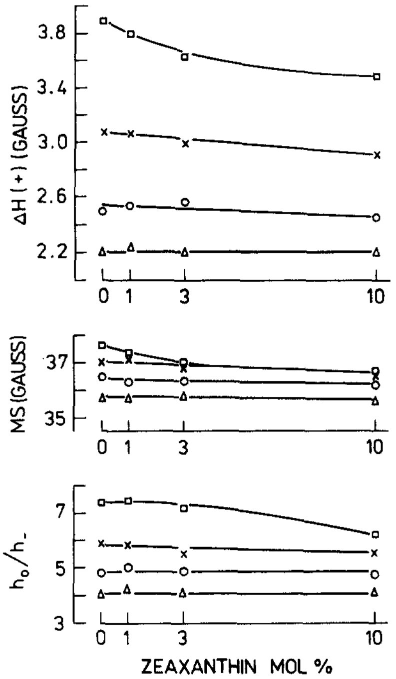

Fig. 7. EPR spectral parameters of T-PC in EYPC membranes plotted as a function of mol fraction of zeaxanthin at 15°C (□), 25° (×), 35°C (○) and 45°C (△). $\Delta H(+)$, peak-to-peak width of the low ($M_I = +1$) field line; MS, maximum splitting and $h_0/h_-$, ratio of the central ($M_I = 0$) to the high ($M_I = -1$) field-peak heights.

The influence of carotenoids on the polar headgroup region in EYPC bilayer measured with T-PC is shown in Fig. 7. To demonstrate this influence we examined the following parameters of the EPR spectra of T-PC: the peak-to-peak width of the low field line ($\Delta H(+)$), maximum splitting (MS) and the ratio of the central to the high field peak heights ($h_0/h_-$). These are the same parameters as those used in our previous paper [23] dealing with DMPC bilayers. As can be seen from Fig. 7, the addition of carotenoids affects the polar headgroup region in the same manner as an increase of temperature. In other words, the presence of zeaxanthin in EYPC bilayer increases the headgroup mobility. These results are quite similar to those obtained earlier with DMPC [23], and a similar effect of zeaxanthin on the spectral parameters of T-PC is obtained in EYPC bilayers at a lower temperature of

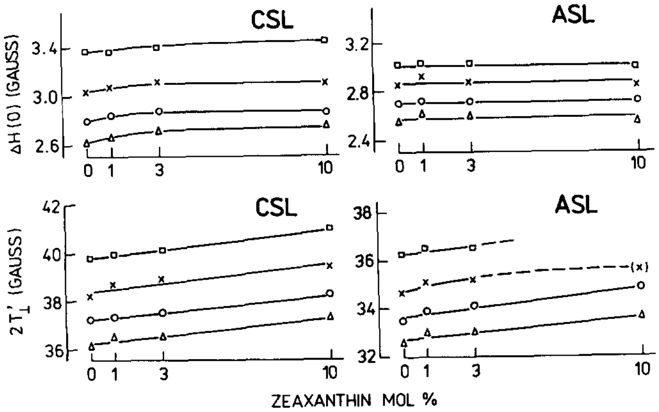

Fig. 8. Peak-to-peak central line widths ($\Delta H(0)$) (top) and $2T'_\perp$ values (bottom) of CSL and ASL in EYPC membranes as a function of mol fraction of zeaxanthin at 15°C (□), 25°C (×), 35°C (○) and 45°C (△). $T'_\perp$ values for ASL at 15°C and 25°C in the presence of 10 mol% of zeaxanthin were not determined because of the presence of a small signal from the fast-tumbling label that masked the measured peak.

about 10°C. For higher temperatures, the effect of carotenoids is much smaller and practically disappears at 35°C. This is true also in DMPC liposomes. Therefore, carotenoids modify the polar headgroup region in unsaturated EYPC bilayers in the same way as in saturated DMPC bilayers.

In order to look at the motion of rigid, highly anisotropic molecules incorporated into EYPC bilayer, we measured the peak-to-peak width of the central line ($\Delta H(0)$) and the maximum splitting ($2T'_\perp$) of the ESR spectra of CSL and ASL, which report the motion of these cholesterol-like molecules as a whole. As shown in Fig. 8 (bottom), the presence of zeaxanthin in EYPC bilayer influences $2T'_\perp$ of both CSL and ASL similarly, indicating a strong ordering effect of carotenoids on the motion of rigid, highly anisotropic molecules. According to the discussion in the previous work [23], $2T'_\perp$ reflects the wobbling motion of the rigid portion of CSL and ASL molecules. There is a correlation between an increased value $2T'_\perp$ and the narrowing of semicone angle accessible for the wobbling motion of CSL and ASL. In previous papers [40,41], EPR spectra showing anisotropic motion of CSL and ASL in various phosphatidylcholine membranes were simulated. On the basis of these data, calibration curves were constructed analogous to the curves presented by Gaffney for SASL [42], which connected $2T'_\perp$ values obtained from the EPR spectra of CSL and ASL with the order parameter and the semicone angle of spin labels. From data shown in Fig. 8 and the calibration curves, one can obtain the semicone angles of CSL and ASL. In EYPC bilayers at 45°C, introduction of 10 mol% of zeaxanthin changes the semicone angle of CSL from 54° to 48° and that of ASL from 75° to 66.5°. By comparison, the corresponding changes in DMPC bilayers for CSL are from 48.5° to 37.5° [40] and for ASL are from 69° to 49° [41]. Therefore, the effect of carotenoids on the semicone angle confining the wobbling motion of CSL and ASL is, roughly speaking, 2-times smaller in EYPC bilayer than that in DMPC bilayers.

The parameter $\Delta H(0)$ presented in Fig. 8 (top) reflects the rate of rotational motion of CSL and ASL. In EYPC bilayers, this parameter is less sensitive to the presence of carotenoids than maximum splitting. In the case of CSL, the presence of 10 mol% of zeaxanthin increases $\Delta H(0)$ by approximately 0.12 G, and, thereby, decreases the rate of reorientational motion. However, in the case of ASL, $\Delta H(0)$ is not influenced by carotenoids; that is, zeaxanthin does not inhibit the reorientational motion of ASL in EYPC bilayers. Similar results have been obtained with DMPC [23]. The results displayed in Fig. 8 suggest that zeaxanthin confines the cone of wobbling motion of both CSL and ASL and slows the rate of reorientational motion of CSL, but not ASL. This difference is probably due to the lack of an isooctyl side chain in the ASL molecule.

The presence of unsaturated alkyl chains in PC membranes greatly decreases the ordering effect of carotenoids as was observed with 5-and 16-SASL. It has to be pointed out that unsaturation alone (without carotenoids) gives only modest effects [27]. Also, the motional freedom of T-PC, as well as CSL and ASL, showed that alkyl-chain unsaturation greatly moderates the effects of carotenoids. This effect can be explained in the following way. The intercalation of the carotenoid molecule into PC membranes promotes *trans* conformation (extended structure) of single bonds in saturated PC membranes due to its rigid rod-like structure (i.e., the so-called 'ordering effect' of carotenoids). In lipids containing unsaturated chains, the structural nonconformability between the rigid rod-like molecule of carotenoid and the rigid bond of 30° at the *cis*-double bond diminishes the ordering effect. In a similar way, the presence of unsaturated chains diminishes the ordering effect of cholesterol [27,40,41].

## General discussion

The results presented in this paper, as well as those in earlier works [11–14,23] suggest that the effect of polar carotenoids on the structure and dynamic properties of lipid membranes is in many respects similar to the effect of cholesterol. In particular, the investigated carotenoids remove the phase transition or make it less sharp. In fluid phase membranes, they increase the order and decrease the mobility of PC alkyl chains. Both types of modifiers increase the mobility of polar headgroups. Also, the effects of polar carotenoids and cholesterol on the movement of rigid molecules like CSL and ASL are similar. As a rule, the presence of

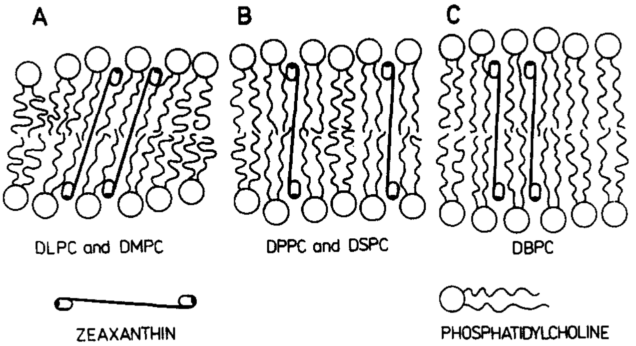

Fig. 9. Schematic drawings of PC-dihydroxycarotenoid membranes showing a different location of the carotenoids in fluid-phase membranes of various thicknesses.

unsaturated alkyl chains moderates the effect of both cholesterol and carotenoids on PC bilayers. However, there are some essential differences between the effects of cholesterol and carotenoids. As we tried to show, the main factor determining the effect of polar carotenoids on the conformation and the mobility of alkyl chains, is the thickness of PC bilayer, more strictly its relation to the length of carotenoid molecule. This is schematically displayed in Fig. 9. When compared at the same temperature, the effect of carotenoids on PC alkyl-chain order depends on the length of the alkyl chain. In the center of the membrane (16-SASL position), this ordering effect is a monotonously diminishing effect in the series from DLPC to DBPC. In the case of 5-SASL, the ordering effect is at a minimum for DPPC (16C) and DSPC (18C), where we can expect the best match of the thickness of hydrophobic core of bilayer and the length of carotenoid molecule. The effects of carotenoids in short-chain PC (DLPC and DMPC) and in long-chain PC (DBPC) have different mechanisms (see Fig. 9), but in both cases, the spin moiety of 5-SASL is influenced because there is no match between the dimensions of the membrane and the dimensions of the carotenoid molecule. In contrast to polar carotenoids, the ordering effect of cholesterol does not depend on the bilayer thickness, except for DLPC [27]. Because an alkyl chain in DLPC is shorter than cholesterol, the behavior of DLPC membranes is usually a little different than other saturated-PC membranes.

In the central region of the membrane, carotenoids exert a larger ordering effect than in the region where the spin moiety of 5-SASL is localized. On the other hand, cholesterol exerts an ordering effect mainly in the vicinity of the rigid part of its molecules, that is, between the polar headgroup layer and 10th carbon atom of the alkyl chains. The much smaller cross-section of the isooctyl portion of a cholesterol molecule than the cross-section of its rigid steroid ring produces additional possibilities for undulation and *gauche-trans*-transitions of acyl chains. These differences between the ordering effects of carotenoids and cholesterol are reflected in how both types of molecules influence the so-called oxygen transport parameter (or the product of oxygen concentration and diffusion coefficient) in PC membranes [21,43,44]. The magnitude of this parameter in DMPC bilayer is suppressed by carotenoids, mainly in the center of the bilayer [21] and by cholesterol near the polar headgroup region [43,44]. Even more, cholesterol significantly increases the oxygen transport parameter in the center of DOPC and EYPC bilayers [44].

The analogies between the effects of cholesterol and carotenoids on lipid membranes we have just discussed allow us to conclude that carotenoids can be, and most probably are, natural regulators of the fluidity in membranes that do not contain cholesterol. Such a conclusion agrees with the hypothesis of Rohmer et al. referred to in the Introduction. The different effects of cholesterol and carotenoids on oxygen transport within the membrane may also have some biological meaning. One of the main functions of carotenoids, at least in thylakoid membranes, is protection against photodynamic destruction of the photosynthetic apparatus. The decrease of the oxygen-transport parameter by carotenoids is one of the possible mechanisms of such protection [21]. On the other hand, the increase of the oxygen-transport parameter by cholesterol in unsaturated PC bilayers agrees with Skulachev's hypothesis, ascribing to eukaryotic biological membranes the role of the route for intracellular transport of oxygen and other low molecular hydrophobic substances [45]. We will not analyze in detail the relation between our results and living systems, since carotenoids, in contrast to cholesterol, occur in vivo, as a rule, in membranes that do not contain lecithins. Further experiments with carotenoids in bilayers of bacterial or thylakoid lipids are needed for a more detailed analysis of carotenoid functions in biological membranes.

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