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Effects of polar carotenoids on dimyristoylphosphatidylcholine membranes: a spin-label study

Witold K. Subczynski [a,b], Elzbieta Markowska [a], Wieslaw I. Gruszecki [c] and Jan Sielewiesiuk [c]

[a] *Biophysics Department, Institute of Molecular Biology, Jagiellonian University, 31-120 Krakow (Poland),* [b] *National Biomedical ESR Center, Medical College of Wisconsin, Milwaukee, WI (USA) and* [c] *Institute of Physics, Maria Curie-Sklodowska University, Lublin (Poland)*

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Spin labeling methods were used to study the structure and dynamic properties of dimyristoylphosphatidylcholine (DMPC) membranes as a function of temperature and the mole fraction of polar carotenoids. The results in fluid phase membranes are as follows: (1) Dihydroxycarotenoids, zeaxanthin and violaxanthin, increase order, decrease motional freedom and decrease the flexibility gradient of alkyl chains of lipids, as was shown with stearic acid spin labels. The activation energy of rotational diffusion of the 16-doxylstearic acid spin label is about 35% less in the presence of 10 mol% of zeaxanthin. (2) Carotenoids increase the mobility of the polar headgroups of DMPC and increase water accessibility in that region of membrane, as was shown with tempocholine phosphatidic acid ester. (3) Rigid and highly anisotropic molecules dissolved in the DMPC membrane exhibit a bigger order of motion in the presence of polar carotenoids as was shown with cholestane spin label (CSL) and androstane spin label (ASL). Carotenoids decrease the rate of reorientational motion of CSL and do not influence the rate of ASL, probably due to the lack of the isooctyl side chain. The abrupt changes of spin label motion observed at the main phase transition of the DMPC bilayer are broadened and disappear at the presence of 10 mol% of carotenoids. In gel phase membranes, polar carotenoids increase motional freedom of most of the spin labels employed showing a regulatory effect of carotenoids on membrane fluidity. Our results support the hypothesis of Rohmer, M., Bouvier, P. and Ourisson, G. (1979) Proc. Natl. Acad. Sci. USA 76, 847–851, that carotenoids regulate the membrane fluidity in *Procaryota* as cholesterol does in *Eucaryota*. A model is proposed to explain these results in which intercalation of the rigid rod-like polar carotenoid molecules into the membrane enhances extended *trans*-conformation of the alkyl chains, decreases free space in the bilayer center, separate the phosphatidylcholine headgroups and decreases interaction between them.

Introduction

Both cholesterol and carotenoids belong to terpenoids and have common first stages of biosynthesis [1]. In later stages, the biosynthesis of cholesterol requires the presence of molecular oxygen (for epoxidation of squalene) [2]. *Procaryota*, which appeared in the early stages of biological evolution, when oxygen was not present in the earth's atmosphere, in contrast to *Eucaryota*, do not contain cholesterol in their membranes. Instead, they contain carotenoids or other terpenoids. It is already accepted that cholesterol is a major lipid component which regulates membrane fluidity in *Eucaryota* [3]. The hypothesis that carotenoids are cholesterol surrogates in some *Procaryota* was postulated by Rohmer et al. in 1979 [4] and even earlier by Nes in 1974 [5].

A few papers describe the interaction of carotenoids with model and biological membranes. They could be divided into two groups: (1) investigating localization and orientation of carotenoids in the lipid bilayer [6–12]; and (2) measuring the influence of carotenoids on membrane properties such as fluidity, phase transi-

Abbreviations: ASL, androstane spin label; CSL, cholestane spin label; CuKTSM$_2$, (3-ethoxy-2-oxobutyraldehyde bis (N^4,N^4-dimethylthiosemicarbazonato)copper(II); DMPC, L-α-dimyristoylphosphatidylcholine; EPR, electron paramagnetic resonance; SASL, stearic acid spin label; 5-SASL, 5-doxylstearic acid spin label; 9-SASL, 9-doxylstearic acid spin label; 12-SASL, 12-doxylstearic acid spin label; 16-SASL, 16-doxylstearic acid spin label; T-PC, tempocholine dipalmitoylphosphatidic acid ester.

Correspondence: W.K. Subczynski, National Biomedical EPR Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA.

tions, and transport of small molecules [8,13–18]. Most authors agree that polar dihydroxycarotenoids are located in the hydrocarbon core of the lipid bilayer with the long axis perpendicular [7,10] (or almost perpendicular [9,12]) to the membrane surface and with two polar groups anchored in the head group region on both sides of the membrane. They decrease fluidity of model [7,14] as well as biological membranes [13]. Also, the permeability of water [15] and oxygen [17,18] is strongly diminished in the presence of carotenoids.

Previously, we have systematically studied phosphatidylcholine–cholesterol interaction by using electron paramagnetic resonance (EPR) spin labeling technique. We paid special attention to the effect of cholesterol on: (1) *gauche-trans* isomerization of the alkyl chains [19,20], (2) rotational and wobbling motion of small rigid molecules analogues of cholesterol [21], (3) oxygen permeability [22,23] and (4) translational diffusion of a small hydrophobic probe a copper square-planar complex; 3-ethoxy-2-oxobutyraldehyde bis(N^4,N^4-dimethylthiosemicarbazonato)copper(II) called $CuKTSM_2$ [24]. These works indicated that cholesterol can influence all types of motions studied in the lipid bilayer.

In the present work, we would like to test the hypothesis that carotenoids could regulate membrane fluidity in a way similar to that of cholesterol. To compare the effect of both modifiers, we used the same model membranes and the same spin labeling technique that had been used previously to study the effect of cholesterol. Fig. 1 illustrates the chemical structures of dihydroxycarotenoids, cholesterol and spin labels, and also their approximate localization in the dimyristoylphosphatidylcholine (DMPC) bilayer. We will present the investigation of the DMPC-dihydroxycarotenoids interaction with particular attention to: (1)

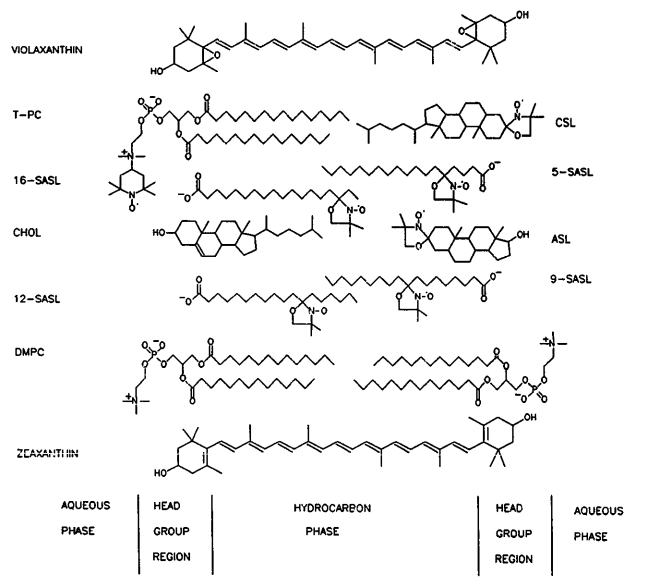


Fig. 1. Cross-sectional drawing of DMPC bilayer including polar carotenoids (zeaxanthin and violaxanthin), cholesterol (CHOL) and spin labels (T-PC, CSL, ASL, 5-, 9-, 12-, and 16-SASL).

mobility of the hydrocarbon chains of DMPC using stearic acid spin labels (SASL); (2) wobbling and rotation of small rigid molecules dissolved in the membrane using cholestane spin label (CSL) and androstane spin label (ASL); and (3) motion of the polar headgroups using tempocholine dipalmitoylphosphatidic acid ester (T-PC).

Materials and Methods

DMPC was purchased from Sigma (St. Louis, MO) and spin labels from Molecular Probes (Eugene, OR). T-PC was a generous gift from Dr. S. Ohnishi at Kyoto University. The carotenoids, zeaxanthin and violaxanthin were extracted from fresh nettle leaves. The extract was saponified with KOH by the 'cold' method [25]. Carotenoids were separated by thin-layer chromatography on activated Kiselgel plates (Merck, FRG) with the solvent system: benzene/ethyl acetate/methanol (75:20:5, v/v). The narrow strips of the center of zeaxanthin and violaxanthin zones were taken off the TLC plates. 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 has been determined spectrophotometrically using the extinction coefficient given in Ref. 25.

The membranes used in this work were multilamellar dispersions of DMPC containing various amounts of carotenoids in the range of miscibility [26] (from 0 to 10 mol%) and 1 mol% of spin label. Briefly, membranes were prepared in the following method [19]: chloroform solutions of the lipids, carotenoids and spin labels were mixed (containing 10^{-5} moles of total lipids) and 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 45°C and vortexed vigorously. The buffer used for the study was 0.1 M borate at pH 9.5. To ensure that all SASL probe carboxyl groups are ionized in phosphatidylcholine membranes, a rather high pH was chosen [27–29]. The structures of phosphatidylcholine membranes are not altered at this pH [19,29,30]. In some cases, to obtain better signal-to-noise ratio in the EPR spectra, the lipid dispersion was centrifuged briefly at $12000 \times g$ for 15 min at 4°C, and the loose pellet ($\approx 20\%$ lipid w/w) was used as the sample. It is highly probable that in our preparation, part of the carotenoid forms infinite aggregates or micorprecipitates in the water phase of the suspension [10,14]. Also, carotenoid aggregates remain outside of the lipid bilayer, and should not influence the ESR spectra, which come from lipid-soluble spin labels. Moreover, the EPR signals are one component, and, typical of spin labels, in the lipid bilayer. The centrifugation to some degree allows the separation of the vesicles from external carotenoid aggregates, which settles down at the conic end of the microcentrifuge tube. About 30 μl 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

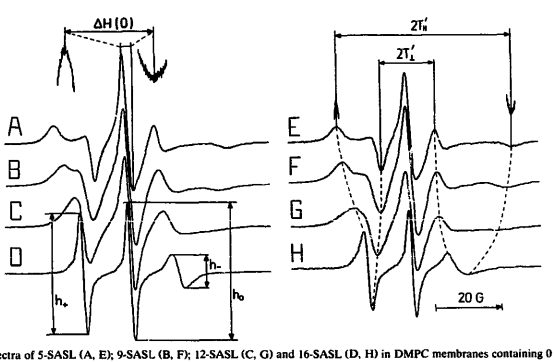


Fig. 2. EPR spectra of 5-SASL (A, E); 9-SASL (B, F); 12-SASL (C, G) and 16-SASL (D, H) in DMPC membranes containing 0 mol% (left) and 10 mol% (right) zeaxanthin at 25°C. The measured values are indicated. The outer wings were also magnified by recording at 10-times higher receiver gain. Peak-to-peak central line widths were recorded with expended abscissa (magnetic field scan range by a factor of 10).

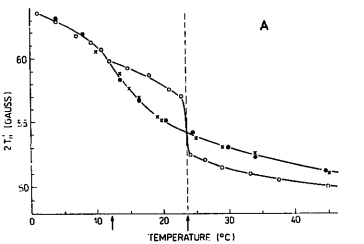
polymer called TPX [31]. This plastic is permeable to nitrogen, oxygen and other gases and is substantially impermeable to water. The TPX sample tube was placed inside the EPR dewar insert and equilibrated with nitrogen gas that was used for temperature control. The sample was thoroughly deoxygenated yielding correct EPR line shape and preventing 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.

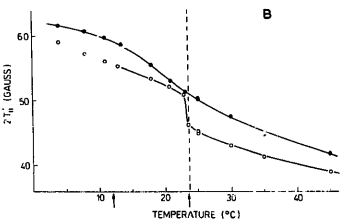
Results and Discussion

Effects of carotenoids on the motion of alkyl chains

Fig. 2 shows typical EPR spectra of 5-, 9-, 12-, and 16-doxylstearic acid spin labels (5-, 9-, 12- and 16-SASL, respectively) in fluid phase DMPC membranes. Maximum splitting ($2T_{\parallel}$) values have been used as a convenient parameter to monitor motional freedom of the nitroxide radical group of these probes. $2T'_{\parallel}$ value decreases as motional freedom increases. Because of structural similarities, it seems apparent, as noted previously, that SASL is a good probe of the alkyl chain mobility of phospholipids. From spectra presented in Fig. 2, it can be seen that the effect of 10 mol% of zeaxanthin on the alkyl chain motion is very big at all depths in the membrane.

Fig. 3 shows the temperature profiles (cooling experiments) of maximum splitting of SASL in DMPC membranes for 0 and 10 mol% zeaxanthin or violaxanthin. There are three remarkable features in these figures. (1) In fluid phase membranes, the presence of carotenoids decreases the mobility of all SASL spin labels. (2) Phase transition, indicated by an abrupt changing of the maximum splitting, shifts to lower temperatures in the presence of 1 and 3 mol% carotenoids (data not shown), and disappears at 10 mol% of carotenoids. (3) In gel phase membranes (below main phase transition temperature), zeaxanthin and violaxanthin increase alkyl chain motion of 5-SASL (Fig. 3A) and 16-SASL (profile of $2T'_{\parallel}$ is not presented), but decrease alkyl chain motion of 9- and 12-SASL (Fig. 3B and 3C). Observation (1) is consistent with previous results showing rigidifying effect of polar carotenoids on model and biological membranes [13,14]. Observation (2) confirms results obtained by Kolev et at. [32] for dipalmitoylphophatidylcholine membranes using differential scanning calorimetry measurements. Observation (3) needs further discussion. Carotenoids not only restrict the rotational motion of SASL molecules as a whole, but also the segmental motion of the alkyl chains. This can be seen as an increase of the anisotropy and decrease of the rate of rotational motion above the main phase transi-





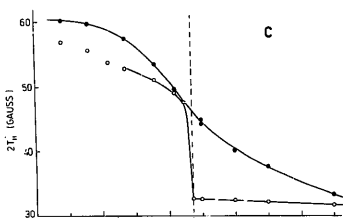


Fig. 3. $2T'_{\parallel}$ values of 5-SASL (A), 9-SASL (B) and 12-SASL (C) in DMPC membranes plotted as a function of temperature without (○) and in the presence of 10 mol% zeaxanthin (●) or 10 mol% violaxanthin (×). Arrows indicate the main phase transition and pretransition temperatures of pure DMPC membranes. Below the pretransition temperature, pure DMPC membrane reaches equilibrium after about 10 h [48] and to indicate that fact points in Figs. 2B and C are not connected in that temperature region. Measurements with 5-SASL were performed for equilibrated membrane. Cholesterol 0.03 mol% [48] and zeaxanthin 1 mol% (data not shown) increase the rate of approach to equilibrium to few minutes.

tion temperature. The resulting effect strongly depends on the position of the segment in the hydrocarbon chain and is the largest for 9- and 12-SASL. Additionally, below the main-phase transition temperature, carotenoids prevent the hydrocarbon chains from coming together and crystallizing, and seem to induce collapse of the pre- and main-transitions into a less cooperative process of structural reorganization. This last effect probably will result in increased motion. The balance of all these effects, as observed with the $2T_{\parallel}$ spectral parameter, produces in the gel phase an increase of motion using 5- and 16-SASL and a decrease of motion using 9- and 12-SASL. All three observations are consistent with the hypothesis of Rohmer et at. [4]: carotenoids indeed decrease fluidity of DMPC membranes when the fluidity is high (fluid phase membranes), and increase it when fluidity is low (gel phase membranes). Also, broadening of the abrupt changes of membrane fluidity at the phase transition is favorable for the functioning of biological membranes [3]. Cholesterol shows similar regulation of membrane fluidity [19,33–35].

In the membrane SASL labels undergo rapid anisotropic motion about the long axis of the spin label and wobbling of the long axis within the confines of a cone imposed by the membrane environment. The anisotropic rotational motion of the spin labels give rise to the new features of the EPR spectra that can be used to calculate the order parameter (S) of SASL [36]. S was calculated from EPR spectra using the equation [37]

$$S = 0.5407(T'_{\parallel} - T'_{\perp})/a_0 \qquad (1)$$

where

$$a_0 = (T'_{\parallel} + 2T'_{\perp})/3 \qquad (2)$$

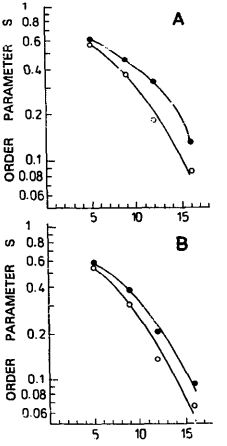


Fig. 4. Order parameter S of SASL measured as a function of nitroxide position (n) along the acyl chain in DMPC bilayers with 0 mol% (○) and 30 mol% (●) zeaxanthin at 35°C (A) and 45°C (B).

$T'_{\parallel}$ and $T'_{\perp}$ were measured directly from the EPR spectra as it is shown in Fig. 2. Results of S measurements for 35°C and 45°C are displayed in Fig. 4. The

TABLE I

Effect of inclusion of 10 mol% of zeaxanthin and 30 mol% of cholesterol in DMPC membranes on the semicone angle (degree) of SASL, CSL and ASL

Temp. (°C)	Host lipid	SASL				CSL	ASL
		5-	9-	12-	16-		
25	DMPC	43.5	53.0	65.5	80.0	38.0	52.0
	DMPC + Zx [a]	40.0	45.0	52.0	74.5	26.5	30.0
	DMPC + Chol [b]	32.0 [d]	37.0	45.0	61.0	n.d. [g]	n.d.
35	DMPC	46.0	60.0	73.5	82.0	47.0 (48.0 [f])	63.0 (54.0 [e])
	DMPC + Zx	43.5	53.5	62.5	77.5	32.0	42.5
	DMPC + Chol	39.0 [d]	47.0 [e]	54.0 [e]	64.0 [e]	n.d.	32.0 [e]
45	DMPC	48.5	64.5	77.5	83.5	48.5	69.0 (70.0 [e])
	DMPC + Zx	47.0	59.5	72.0	81.0	37.5	49.0
	DMPC + Chol	40.0 [d]	n.d.	n.d.	n.d.	n.d.	38.0 [e]

[a] Zx = 10 mol% zeaxanthin, [b] Chol = 30 mol% cholesterol, [c] Measured at 37°C, [d] from [19], [e] from [21], [f] from [38], [g] n.d. = not determined.

order parameter, can be related to the semicone angle (θ_c) according to the equation [38]

$$S = \cos\theta_c(1 + \cos\theta_c)/2 \qquad (3)$$

The changes of the semicone angle are also shown in Table I. It has to be pointed out that in the case of SASL spin labels, S reflects the segmental order parameter of the segment to which the nitroxide fragment is attached. It can be seen that 10 mol% of zeaxanthin significantly increases the order parameter of the hydrocarbon chains of DMPC. The increase of the apparent order parameter is bigger in the center of the bilayer (at 16-SASL position) than close to the polar headgroups (5-SASL position); however, the effect is greatest in the middle of the alkyl chains (at 9-, 12-SASL position). The ordering effect of zeaxanthin decreases when temperature increases. The results are indicators of the dependence of the alkyl chain flexibility gradient [36,39–41] on the presence of rigid molecules such as carotenoids or cholesterol. These molecules significantly decrease the flexibility gradient from the membrane surface to a depth of the 12th carbon.

16-SASL exhibits so much motion that a different analysis can also be used. The effective correlation time, assuming isotropic rotational diffusion of 16-SASL, can be calculated from the linear term of the line width parameter;

$$\tau_{2B} = 6.51 \cdot 10^{-10}\Delta H_0\left[(h_0/h_-)^{1/2} - (h_0/h_+)^{1/2}\right]s \qquad (4)$$

and with the quadratic term

$$\tau_{2C} = 6.51 \cdot 10^{-10}\Delta H_0\left[(h_0/h_-)^{1/2} + (h_0/h_+)^{1/2} - 2\right]s \qquad (5)$$

ΔH_0 is the peak-to-peak width of the central ($M_1 = 0$) line in gauss and h_+, h_0 and h_- are heights of the low ($M_1 = +1$), central ($M_1 = 0$) and high ($M_1 = -1$) field peaks, respectively (see Fig. 2) [42]. When τ_{2B} and τ_{2C} are similar, it is argued that the motional model is fairly good and motion is isotropic. Addition of zeaxanthin decreases motional freedom of 16-SASL free radical moiety which is monitored by a large increase in correlation times (Fig. 5). Well above the main phase transition temperature (45°C) calculated, τ_{2B} and τ_{2C} are very similar and it is an indication that carotenoids decrease the rate of motion, but do not influence its isotropy. The motion (at 45°C) is isotropic. At lower temperatures, close to the phase transition, presence of carotenoids (10 mol%), strongly increase the difference between τ_{2B} and τ_{2C}, indicating the onset of anisotropic

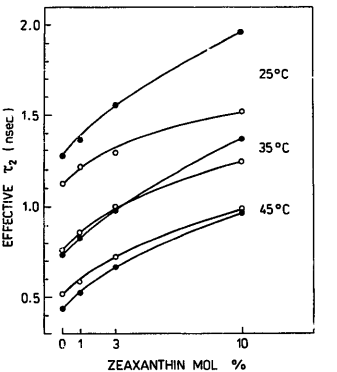


Fig. 5. Effective rotational correlation time of 16-SASL in DMPC membrane plotted as a function of mole fraction of zeaxanthin at different temperatures. ○, τ_{2B}, ●, τ_{2C}.

rotational diffusion. At these conditions (low temperature, high carotenoids concentration), 16-SASL motion is going to the slow tumbling regime, where no convenient parameterization has been established *. Also, the temperature profiles (cooling experiment) of peak-to-peak width ($\Delta H(0)$) and peak height (h_0) of the central peak of 16-SASL in DMPC and DMPC-carotenoids (10 mol%) membranes show large changes on the main phase transition in the absence of carotenoids and no abrupt changes in the presence of carotenoids (data not shown).

From an Arrhenius display of these data (log τ versus $1/T$), the activation energy of rotational motion

* In principle, we have to distinguish the rotational motion of 16-SASL molecule as a whole, which is anisotropic, from segmental motion, which comes from *gauche-trans* isomerization of the alkyl chain. Segmental motion depends on the position of a carbon in the hydrocarbon chain. For carbon atoms near the terminal methyl group (16-SASL position), it is assumed that the segmental motion is not restricted, and as a result, motion of the nitroxide fragment is approximately isotropic. Additon of polar carotenoids to the membrane decreases the motion of 16-SASL molecule as a whole and also decreases the free space for segmental motion of last carbons in the alkyl chain, causing the motion of 16-SASL to become more anisotropic. At higher temperature ($\geq$ 45°C) the motion of all molecules becomes so great that segmental motion dominates. The resulting motion of 16-SASL is almost isotropic, but slower. At lower temperatures ($\leq$ 25°C), the segmental motion is diminished and the motion of 16-SASL becomes more highly anisotropic.

TABLE II

Activation energy for rotational diffusion of 16-SASL in fluid phase DMPC membranes

Zeaxanthin (mol%)	ΔE (kcal/mol)	
	from τ_{2B}	from τ_{2C}
0	7.0	9.3
10	4.2	6.4

of the nitroxide moiety of 16-SASL was calculated for DMPC membranes containing 0 and 10 mol% of zeaxanthin. The results are collected in Table II showing decrease of activation energy in the presence of carotenoids. The results are somewhat surprising because carotenoids decrease the rate of motion (Fig. 5) and increase the order parameter (Fig. 4 and Table I) of 16-SASL. In similar experiments, the cholesterol (50 mol%) decreases the activation energy for wobbling diffusion of the CSL in dioleoylphosphatidylcholine membranes by a factor of 3 and decreases CSL reorientation rate by a factor of about 3–6 [38]. Also a large increase in the CSL order parameter was monitored. The earlier data show that cholesterol diminishes the temperature dependence of the dynamic properties of membranes [41,43]. These results are probably due to a complex interplay of changed jump distance and changed jump rate of labels induced by the presence of carotenoids and cholesterol in the membranes.

Changes in maximum splitting or order parameter are often for brevity related to the changes in spin label mobility even though they are in principle static parameters. A better display, related to reorientational motion of free radical moiety, is the peak-to-peak width of the central line of EPR spectrum (ΔH_0). Fig. 6 shows the ΔH_0 of 5-, 9-, 12- and 16-SASL as a function of the mole fractions of zeaxanthin and temperature for fluid phase DMPC membranes. The effect of carotenoids is strong for all spin labels indicating that they not only decrease the cone angle of the cone inside of which wobbling motion occurs by introducing the 'rigid walls', but also decrease the rate of reorientation inside that cone for flexible hydrocarbon chains. The effect of carotenoids can be compared with the effect of cholesterol on the motion of 16-SASL within the DMPC membrane [19]. The effect of 10 mol% of zeaxanthin is bigger than the effect of 10 mol% of

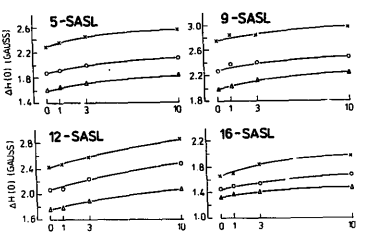


Fig. 6. Peak-to-peak central line widths of SASL (ΔH_0) in DMPC membranes as a function of mole fraction of zeaxanthin at 25°C (×); 35°C (○) and 45°C (△).

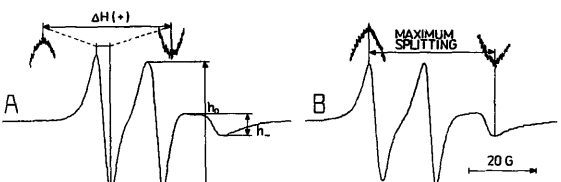


Fig. 7. EPR spectra of T-PC in DMPC membranes containing 0 mol% (left) and 10 mol% (right) zeaxanthin at 25°C. The measured values are indicated.

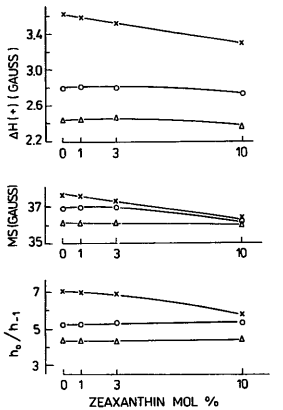


Fig. 8. Spectral parameters of T-PC in DMPC membranes plotted as a function of mole fraction of zeaxanthin at 25°C (×); 35°C (○) and 45°C (△). $\Delta H(+)$ peak-to-peak width of the low ($M_I = +1$) field line; MS maximum splitting and h_0/h_- ratio of the central ($M_I = 0$) to the high ($M_I = -1$) field peak heights.

cholesterol when they are compared at 25°C, but smaller when they are compared at 45°C.

Effect of carotenoids on the motion of polar headgroups

In our opinion, T-PC is a good probe to monitor changes that occur in the polar headgroup region of the membrane. Previously, we used that probe to monitor the influence of cholesterol on water accessibility [19], oxygen diffusion [22,23], and $CuKTSM_2$ diffusion [24] in the headgroup region. Fig. 7 shows changes in EPR spectrum of T-PC induced by 10 mol% of zeaxanthin in fluid phase DMPC membrane. Large changes in the spectrum indicate large changes of motion (mode and rate). We chose a few spectral parameters of T-PC which vary significantly in response to temperature changes. They are: maximum splitting, peak-to-peak width of the low-field line ($\Delta H(+)$) and ratio of the height of central h_0 to low h_- field peaks (h_0/h_-) and are indicated in Fig. 7. Addition of zeaxanthin induces a decrease of all these spectral parameters in fluid phase DMPC membrane. Increased temperature changes these parameters in the same direction as the addition of carotenoids (Fig. 8). Addition of 10 mol% of zeaxanthin at 25°C gives the same effect as increasing the temperature to 28°C ($\Delta H(+)$ display), to 32°C (h_0/h_- display) or even to 40°C (maximum splitting display). For higher temperatures, the effect of carotenoids is much smaller – and practically disappears at 45°C. On the basis of these observations, we infer that zeaxanthin increases the motional freedom of polar headgroups of DMPC membranes. A similar effect was observed for cholesterol [22].

We employed the method of Griffith et al. [44,45] to assess water accessibility to the membrane surface by using T-PC. This method is based on the dependence of unpaired electron spin density over nitrogen nuclei on solvent polarity. Polar solvents tend to increase the spin density on the nitrogen nuclei and, therefore, increase the T_Z value (Z-component of the hyperfine tensor). T_Z data were obtained directly from X-band EPR spectra of T-PC taken at −140°C. An increase of T_Z is a characteristic effect of zeaxanthin, and obtained values for DMPC membranes without and in the presence of 10 mol% zeaxanthin are 34.1 G and 35.0 G, respectively. This is clear evidence that water accessibility to the nitroxide radical of T-PC and, therefore, to the polar headgroup region greatly in-

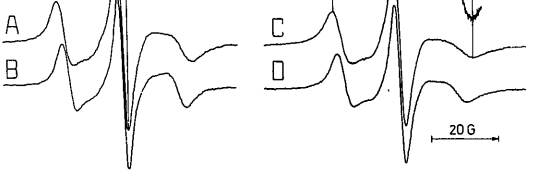


Fig. 9. EPR spectra of CSL (A, C) and ASL (B, D) in DMPC membranes containing 0 mol% (left) and 10 mol% (right) zeaxanthin at 25°C. The measured parameters are indicated.

crease in the presence of polar carotenoids. A similar effect of cholesterol was demonstrated earlier [19,38].

Effect of carotenoids on the motion of CSL and ASL

To better understand the effects of carotenoids on different dynamic processes in the DMPC bilayer, other types of spin labels, CSL and ASL, were used. It has to be noted that sterol-type spin labels are fairly rigid structures and report the motion as a whole, whereas the segmental motion of the alkyl chain (*gauche-trans* isomerism) play important roles in determining the EPR spectra of SASL. When comparing ASL and CSL, one has to take into account the lack in ASL of the isooctyl side chain attached in CSL to the rigid tetracyclic fused ring. Fig. 9 shows the changes in the EPR spectra of CSL and ASL that occur after the addition of 10 mol% zeaxanthin to a DMPC bilayer. The changes are very pronounced. In Fig. 10, the maximum splitting ($2T'_{\perp}$) of CSL and ASL in the DMPC bilayer, in the absence and presence of 10 mol% polar carotenoids, is plotted as a function of temperature. The ordering effect of zeaxanthin and violaxanthin above the main phase transition temperature is demonstrated as an increase in the maximum splitting. Below the phase transition, the picture is not as clear because of the difficulty in evaluating the position of the high-field minimum in the EPR spectra of CSL and ASL. EPR spectra of CSL indicate greater membrane fluidity below the main phase transition when carotenoids are added. For CSL and ASL undergoing fast anisotropic motion around the long axis (which is the case in fluid phase membranes), the maximum splitting is related to order parameter and cone of the confine in which the wobbling motion takes place.

In previous papers [21,38], EPR spectra for the anisotropic motion of CSL and ASL in phosphatidylcholine membranes were simulated. On the basis of those data, calibration curves were constructed, analogous to that presented by Gaffney for SASL [46], 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. Using these calibration curves and the data in Fig. 10, the semicone angles for CSL and ASL in DMPC and DMPC-carotenoids (10 mol%) membranes were obtained and are summarized in Table I together with the data for DMPC-cholesterol (30 mol%) membranes [21]. For pure DMPC membrane, the semicone angle of CSL is comparable with that of 5-SASL and the semicone angle of ASL with that of 9- and 12-SASL. Incorporation of 10 mol% zeaxanthin decreases the cone angle for all spin labels. The effect is particularly large for ASL, and is comparable to the effect of 20 mol% cholesterol. The effect of carotenoids (and cholesterol) on ASL and CSL is much larger than one might expect from their effect on SASL (Table I and Refs. 19, 21 and 38). It can be explained by the fact that the intramolecular mobility of ASL and CSL is much smaller than that of SASL alkyl chain due to the rigid planar steroid ring structure. ASL and CSL must be sensitive to the sum of the changes at various 'depths' in the membrane, while SASL reflects the local change.

Changes in peak-to-peak width of the central line of the EPR spectra of CSL and ASL induced by zeaxanthin and violaxanthin are presented in Fig. 11. As it was pointed out earlier, this parameter reflects the rate of reorientational motion of spin label. There is a big difference between the effect of carotenoids on CSL and ASL in fluid phase membranes; 10 mol% of carotenoids increase ΔH_0 and thereby decrease the rate of reorientational motion for CSL, but do not change ΔH_0 for ASL. Results presented in Figs. 10

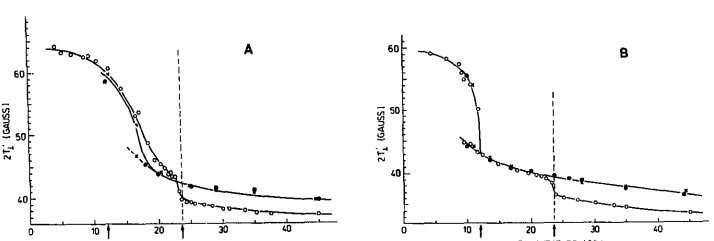


Fig. 10. $2T'_{\perp}$ values of CSL (A) and ASL (B) in DMPC membranes plotted as a function of temperature without (○) and in the presence of 10 mol% zeaxanthin (●) or 10 mol% violaxanthin (×). Arrows indicate the main phase transition and pretransition temperatures of pure DMPC membranes. Below the main phase transition temperature (CSL) and pretransition temperature (ASL), $2T'_{\perp}$ values of the major and the minor component are plotted.

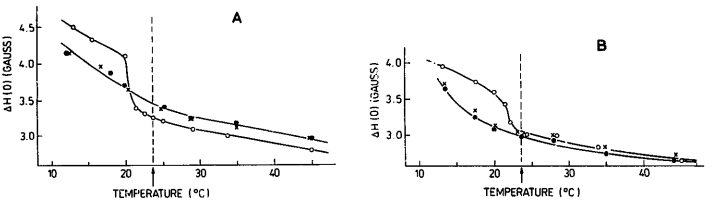


Fig. 11. Temperature profiles of peak-to-peak central line widths (ΔH_0) of CSL (A) and ASL (B) in DMPC membranes without additions (○); containing 10 mol% zeaxanthin (●) or 10 mol% violaxanthin (×). Arrow indicates the main phase transition temperature of pure DMPC membranes.

and 11 and in Table I show that polar carotenoids decrease the cone angle of the confine and decrease the rate of reorientation for CSL, but decrease the cone angle of the confine and do not change the rate of reorientation for ASL. These differences are probably due to the lack of isooctyl side chain in the ASL molecule. Fig. 11 also shows that 10 mol% of carotenoids cancels the abrupt change in ΔH_0 at the main phase transition and significantly increases the motion of CSL and ASL in gel phase membranes.

General discussion

Carotenoids are known as (1) components of photosynthetic light antennae, (2) compounds which prevent photodynamic destruction, (3) anticarcinogenic substances and (4) modifiers of membrane fluidity. The latter hypothetical function of carotenoids is less documented and is the subject of this paper. Our results clearly show that polar carotenoids decrease fluidity of membranes above the main phase transition temperature and increase it below the main phase transition temperature. Also, in the presence of carotenoids, the phase transition becomes broadened and abrupt changes of the lipid motion disappear. The effect of polar carotenoids on membrane fluidity is, in principle, similar to that of cholesterol; however, the effect of cholesterol on fluid phase membranes at low temperature (25°C) is much smaller than that of carotenoids. A quantity of 10 mol% of zeaxanthin exerts an effect similar to that of 15–20 mol% of cholesterol. Both compounds decrease the flexibility gradient along the lipid alkyl chains. It is also interesting to note that the effect of carotenoids on the membrane fluidity decreases more quickly with increases of temperature than the effect of cholesterol. Polar carotenoids as well as cholesterol increase the mobility of the polar headgroups of the membrane and increase water accessibility in that region.

The observed differences result from differences in the structure of cholesterol and polar carotenoids and from their different localization within the membrane. The cholesterol molecule contains three well defined regions: small polar hydroxyl group, rigid plate-like steroid ring (tetracyclic fused ring) and flexible alkyl chain tail (isooctyl side chain). The cholesterol molecule is located in one half of the bilayer, with its rigid plate-like portion extending to a depth of a 7–10th carbon atoms in lipid alkyl chains [47]. Dihydroxycarotenoids are rigid, rodshaped molecules with two polar groups at the ends of a hydrophobic conjugated hydrocarbon 'bar' separated by the distance of about 30 Å [10]. In contrast to cholesterol, one carotenoid molecule influences both halves of the lipid bilayer, and with two polar groups interacting with opposite hydrophilic surfaces of the membrane it can brace together the two halves of the bilayer like a tie-bar. When a polar carotenoid molecule intercalates into the membrane, its polar groups separate the phosphatidylcholine headgroups and decrease the interaction between them. Water molecules come into the free space between the separated headgroups. The ordering effect of polar carotenoids can be explained as a physical interaction of rigid rod-like molecules with saturated hydrocarbon chains that enhances the extended conformation of lipid alkyl chains. Carotenoid molecules promote the *trans*-conformation of alkyl chains from the membrane surface to the membrane center. Cholesterol molecules, however, promote the *trans*-conformation of alkyl chains from the membrane surface to a depth of 7–10 carbons [47]. The rest of the lipid alkyl chain tails stay flexible. The presence of cholesterol increases the free space in the central part of the bilayer because the cross-section of the steroid ring is larger then that of its hydrocarbon tail [24]. There is evidence (carotenoids decrease the oxygen diffusion-concentration product in the membrane center at the 16-SASL position) that polar carotenoids decrease the free space in the bilayer center [17,18].

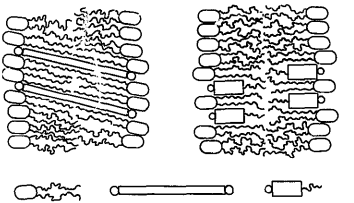


Fig. 12. Schematic drawings of DMPC-dihydroxycarotenoids and DMPC-cholesterol membranes showing a different location and a different effect of carotenoids and cholesterol on the membrane organization.

These models are displayed schematically in Fig. 12. It is possible that carotenoids induce not only extended straight conformation of the lipid alkyl chains, but also some tilt, especially at high carotenoid concentration. Support for this hypothesis comes from our previous work, which shows tilt of zeaxanthin and violaxanthin molecules in DMPC membrane and minor increase in membrane thickness [12]. Cholesterol, which 'swims' in one half of the bilayer, does not induce this kind of tilt. It has to be pointed out that at high carotenoid concentration (10 mol%), almost all hydrocarbon alkyl chains of the lipid have as a neighbor a rigid carotenoid bar.

In conclusion, we have demonstrated that carotenoids may be able to regulate the fluidity of biological membranes that do not contain cholesterol (*Procaryota*, thylakoids) like cholesterol in *Eucaryota*. It should be pointed out that in some model membranes, at least a portion of the β-carotene molecules are oriented perpendicularly to the membrane surface [9]. For this orientation, the effect of β-carotene on membrane structure and dynamics may be similar to the effect of polar carotenoids.

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