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Comparison of the effects of inserted C_{40}- and C_{50}-terminally dihydroxylated carotenoids on the mechanical properties of various phospholipid vesicles

Tarik Lazrak [a], Alain Milon [a], Geneviève Wolff [a], Anne-Marie Albrecht [b], Monique Miehé [c], Guy Ourisson [a] and Yoichi Nakatani [a]

[a] *Laboratoire de Chimie Organique des Substances Naturelles, UA 31 CNRS, Université Louis Pasteur, Centre de Neurochimie,* [b] *Laboratoire de Chimie Physique et d'Electroanalyse, UA 405 CNRS, E.H.I.C.S., and* [c] *Centre de Neurochimie CNRS, Strasbourg (France)*

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Key words: Phospholipid vesicle; Carotenoid; Light scattering; Stopped flow; Electron microscopy

We have measured the extent of incorporation of zeaxanthin (C_{40}) and decaprenozeaxanthin (C_{50}) in unilamellar vesicles of dimyristoylphosphatidylcholine (*n*-C_{14}) and dipalmitoylphosphatidylcholine (*n*-C_{16}). The incorporation is larger when the molecular length of the carotenoid corresponds to the thickness of the phospholipid bilayer. Stereochemically pure 2,3-di-*O*-phytanyl-*sn*-glycero-1-phosphocholine was prepared by modification of the polar heads of the phospholipids of *Halobacterium halobium*. Vesicles of this branched-chain ether phospholipid incorporate poorly the carotenoids, whereas egg lecithin vesicles incorporate them better. Osmotic swelling and water permeability of vesicles, with or without carotenoids, were measured in a stopped-flow, light-scattering system. The reinforcing effect (lower permeability and higher rigidity) of carotenoids at 1.5 mol% incorporation into diphytanylphosphatidylcholine vesicles is comparable to that of 5 mol% cholesterol; however, carotenoids have no measurable effect on the egg lecithin vesicles. These results imply that the reinforcement of the membrane depends on a subtle adjustment of the phospholipid-carotenoid system.

Introduction

Carotenoids are well known to play several important roles in living cells: as light-gathering pigments, as photoprotecting agents, as electron conductors across membranes, etc. [1–4]. As part of a general theory of the molecular evolution of biomembrane constituents [5], we have postulated that carotenoids often play another important rôle in bacteria, namely that of membrane reinforcers. As shown schematically in Fig. 1, the terminally polar carotenoids typical of many bacteria [6] could act as trans-membrane 'rivets', stabilizing both halves of the bilayer like cholesterol does for each half separately, in eukaryotes.

This rôle of carotenoids has been studied with model membranes [7] and in vivo [8]. We have recently shown that several α,ω-dipolar carotenoids can indeed be incorporated into the lipid phase of DMPC vesicles [9], and that they reinforce, as expected, the phospholipid bilayer [10].

Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPhPC, 2,3-di-*O*-phytanyl-*sn*-glycero-1-phosphocholine; egg PC, egg phosphatidylcholine; LUV, large unilamellar vesicles; MS, mass spectrum; NMR, nuclear magnetic resonance; SUV, small unilamellar vesicles; TLC, thin-layer chromatography.

Correspondence: Y. Nakatani, Laboratoire de Chimie Organique des Substances Naturelles, UA 31 CNRS, Université Louis Pasteur, Centre de Neurochimie, 5 rue Blaise Pascal, 67084 Strasbourg, France.

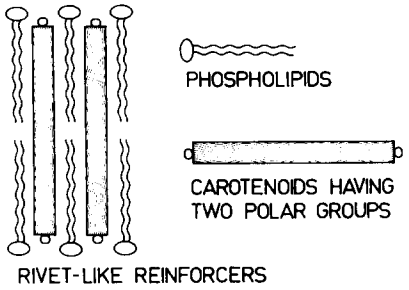


Fig. 1. Mechanism of reinforcement of lipid bilayers by α,ω-dihydroxycarotenoids.

We have also observed that carotenoids are only incorporated in unilamellar DMPC vesicles up to a critical saturation value [9]. We have postulated that this critical value is related to the dimensional fit between the carotenoid and the phospholipid constituents.

In the present paper, we describe first the correlation between the structure of two dipolar carotenoids, (3*R*,3′*R*)-zeaxanthin (C_{40}) 1 and its C_{50} synthetic homologue 2 (cf. Fig. 5), and their incorporation in different phospholipid unilamellar vesicles: DMPC, DPPC, DPhPC and egg PC. DPhPC was chosen as a model of archaebacterial lipids, with their structural peculiarities of branched chains and of ether linkages.

In a second part, we compare the reinforcing effect of these carotenoids on vesicles formed from these phospholipids using the stopped-flow light scattering method previously introduced [10]. We show the importance of the adjustment between the molecular length of the α,ω-dipolar carotenoid and the thickness of the membrane in which they are incorporated; we also show that these carotenoids, when incorporated in DPhPC vesicles, do reinforce them, whereas they have no noticeable effect on egg PC vesicles.

Very recently, the interaction of another C_{40} α,ω-dihydroxycarotenoid, lutein, with multilamellar vesicles of DPPC has been studied for completely different reasons and unaware of our earlier work; it confirms, mostly by differential calorimetry, the incorporation of the carotenoid into the lipid bilayer [11].

Experimental procedure

Materials. (3*R*,3*R*′)-Decaprenozeaxanthin was prepared as described elsewhere [9]. Zeaxanthin was kindly provided by F. Hoffmann-La Roche (Basle). They were kept under argon at −20 °C, and when necessary were recrystallized twice before use; their purity was checked by the value of their extinction coefficient and the non-appearance of the '*cis* band' in their ultraviolet-visible spectrum (both are all-*trans*). Cholesterol, from Fluka AG (Buchs), was recrystallized twice from ethanol. DMPC and DPPC, from Avanti Polar Lipids Inc. (Birmingham, were kept at −20 °C in chloroform solution in sealed tubes. Egg PC, prepared as described by Nielsen [12], was kept under the same conditions. The purity of the lipids was checked by TLC on F_{254} silica gel plates (0.25 mm thick) from Merck, Darmstadt (eluent: chloroform/methanol/conc. ammonia, 65 : 25 : 4, v/v).

Synthesis of DPhPC (see Scheme I)

General. Solvents were distilled over calcium hydride under argon just before use. TLC: F_{254} silica gel plates, 0.25 mm thick (Merck, Darmstadt). Column chromatography: silica gel 60 (60–200 μm, Merck). Infrared: Pye-Unicam-SPS 300 S (Philips) spectrophotometer. ^{1}H-NMR: Bruker SY 200 MHz spectrometer; solvent: C^2HCl_3, tetramethylsilane as internal standard. Mass spectra: double focussing Thomson THN-208 spectrometer; NH_3 chemical ionization, fast heating probe, 70 eV. Microanalyses are due to Mrs. M. François, Institut de Chimie, Strasbourg. The starting material (acetone-insoluble lipid fraction of *Halobacterium halobium*) was provided by Dr. A. Escaut and Mr. D. Marie, ICSN, Gif-sur-Yvette, by a procedure adapted from that described in Ref. 13.

2,3-Di-O-((3′R,7′R,11′R)-3′,7′,11′,15′-tetramethylhexadecyl)-sn-glycerol(2,3-di-O-phytanyl-sn-glycerol) 3

The acetone-insoluble lipid fraction (4.4 g) of *H. halobium* was heated for 18 h under reflux, in an argon atmosphere, with 0.7 N HCl-methanol (1.2 l). Once cooled to room temperature, the solvent was removed under reduced pressure, water was added, and the mixture was extracted with diethylether. The diethylether phase was washed three times with small volumes of saturated NaCl, dried over anhydrous magnesium sulfate, and filtered. The solvent was removed in vacuo and

the residue was chromatographed on a column of silica gel (80 g, diameter 4 cm), using a gradient of diethylether in methylene chloride (0–5%). The colorless oil obtained (1.57 g) was shown to be 2,3-di-*O*-phytanyl-*sn*-glycerol 3 by its infrared and ^{1}H-NMR spectra, by its microanalysis and by its rotatory power: $[\alpha]_D + 8°$ (3.8 g/100 ml $CHCl_3$) (literature, +8.5° [14,15]).

2,3-Di-O-((3'R,7'R,11'R)-3',7',11',15'-tetramethylhexadecyl)-sn-glyceryl 2-bromoethyl phosphate 4

To a stirred solution of 2-bromoethyl phosphorodichloridate (579 mg, 2.4 mmol) in dry diethylether (12 ml) at 0 °C and under argon, was added dropwise, over 1 h, a solution of the diphytanyl glyceryl ether 3 (850 mg, 1.3 mmol) in freshly distilled triethylamine (2.5 ml)/dry diethylether (12 ml). After 24 h, triethylamine (2 ml) and water (2 ml) were added to decompose the excess reagent, and the mixture was heated under reflux for 4 h, then cooled to room temperature, and acidified to pH 2 by addition of 1 N HCl. The organic phase was separated and the aqueous phase was extracted three times with diethylether. The combined diethylether phases were washed three times with water, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was chromatographed on a column of silica gel (60 g, diameter 3 cm), using a gradient of methanol in methylene chloride (0–10%). The colorless oil obtained (760 mg) was shown by its spectral properties to be the expected product 4. Its reactivity made it impossible to prepare an analytical sample.

^{1}H-NMR spectrum (60 MHz): δ 0.8–0.9 (30 H, CH_3), 1.0–1.4 (40 H, CH_2), 1.5–1.7 (8H, CH), 3.4–3.75 (9H, CH_2O, CHO), 3.9–4.4 (4H, CH_2OP). MS: *m/e* 842 $(M+1)^+$, 776 $(M - Br + NH_3)^+$, 759 $(M - Br)^+$.

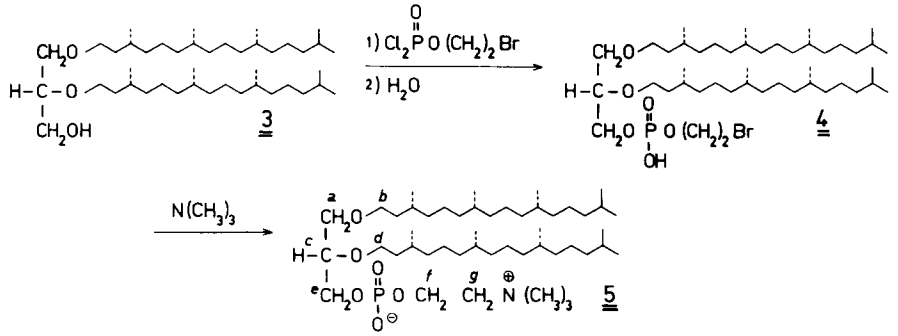
2,3-Di-O-((3'R,7'R,11'R)-3',7',11',15'-tetramethylhexadecyl)-sn-glycero-1-phosphocholine (2,3-di-O-phytanyl-sn-glycero-1-phosphocholine) 5

To a stirred solution of the bromoethyl phosphate 4 (1.0 g) in dry chloroform (25 ml) at 0 °C was added gradually over 15 min trimethylamine (5 ml), dried over KOH pellets, and the mixture was refluxed for 2 h. The same procedure was repeated twice, and finally the mixture was refluxed overnight. Solvents were removed in vacuo, and the residue (1.25 g) was chromatographed on a silica gel column (85 g, diameter 3 cm), eluting with a gradient of methanol in methylene chloride (15–30%). The colorless oil obtained (998 mg, 87%) was shown by its spectral properties to be the expected product 5.

^{1}H-NMR spectrum (200 MHz): δ 0.82–0.88 (30 H, 10 × dd, $J = 6.5$ Hz, CH_3) 0.94–1.40 (40 H, m, CH_2), 1.46–1.61 (8H, m, CH), 3.37 (9H, s, NCH_3), 3.42–3.59 (7H, m, a,b,c,d), 3.80–3.85 (4H, m, e,g), 4.31 (2H, m,f) (see Scheme I). MS: *m/e* 818 $(M+1)^+$, 804, 776, 759, 747, 670, 653, 634. $[\alpha]_D + 1°$ (3.3 g/100 ml $CHCl_3$).

Preparation of vesicles

Small unilamellar vesicles (SUV) were prepared as described elsewhere [10], except that the sonication time was 30 min at 25 °C. Large unilamellar vesicles (LUV) were prepared by the ether injection method [10] or by the reverse-phase evapora-



Scheme I. Synthesis of 2,3-di-*O*-phytanyl-*sn*-glycero-1-phosphocholine.

tion method [16], slightly modified as follows for the incorporation of carotenoids. The phospholipid (10 mg) and the carotenoid (variable) were dissolved in tetrahydrofuran and the solvent was evaporated in vacuo; the residue was dissolved in 1.6 ml of diethylether, 0.2 ml of ethanol and 0.2 ml of tetrahydrofuran. An aqueous buffer solution was added (680 μl; 350 mM NaCl/1 mM $EDTANa_2$/5 mM NaN_3/10 mM Tris-HCl (pH 8) and the mixture was sonicated under argon in a Bronsonic 221 bath. The time of sonication is not critical as long as the suspension is stable for at least 30 min after its rmeoval from the sonication bath; a typical duration was 5 min at 25 °C. The solvents were removed slowly in a rotatory evaporator, the vacuum being maintained at 350–550 torr. The resulting gel was broken up by vortex-mixing two or three times during the evaporation. The remaining solvents were removed under a higher vacuum (150–250 torr) until an opalescent red solution was obtained, to which was added more of the pH 8 Tris buffer (4.32 ml). This was then dialyzed overnight against the same buffer (2 l) at 4 °C in the dark, to remove the remaining solvents (dialysis tubing Spectrapor 2, Spectrum Medical Industris, Los Angeles).

The vesicle suspension was filtered through polycarbonate (Nuclepore, Pleasanton): prefilters P 80, with 0.8-, 0.4-, 0.2- and 0.1-μm filters – twice through each, and/or the vesicle suspension was concentrated to 1 ml in an ultrafiltration cell (Amicon stirred cell) with a PM10 Amicon filter; immediately after concentration, the suspension was filtered on a Sepharose 4BCL column (Pharmacia France, Bois d'Arcy), previously saturated with another sample of the same lipid (elution conditions: void volume, 44 ml; total volume, 90 ml; flow rate, 40 ml/h; elution by 4-ml fractions, 4 °C). Both of these procedures allow the elimination of external carotenoid aggregates and give vesicles of reasonable homogeneity, as shown by electron microscopy (Fig. 2). The dissymmetry of light scattering by the vesicles ($Z = I_{45°}/I_{135°}$) is determined in a FICA 4200 photogoniodiffusiometer equipped with a 5 mW He-Ne vertically polarized laser (SA Optilas, France).

The concentration of the phospholipid and of the carotenoid in vesicles was measured, respectively, by phopshorus determination [17] and by ultraviolet-visible spectroscopy (Uvikon 820 Kontron spectrophotometer).

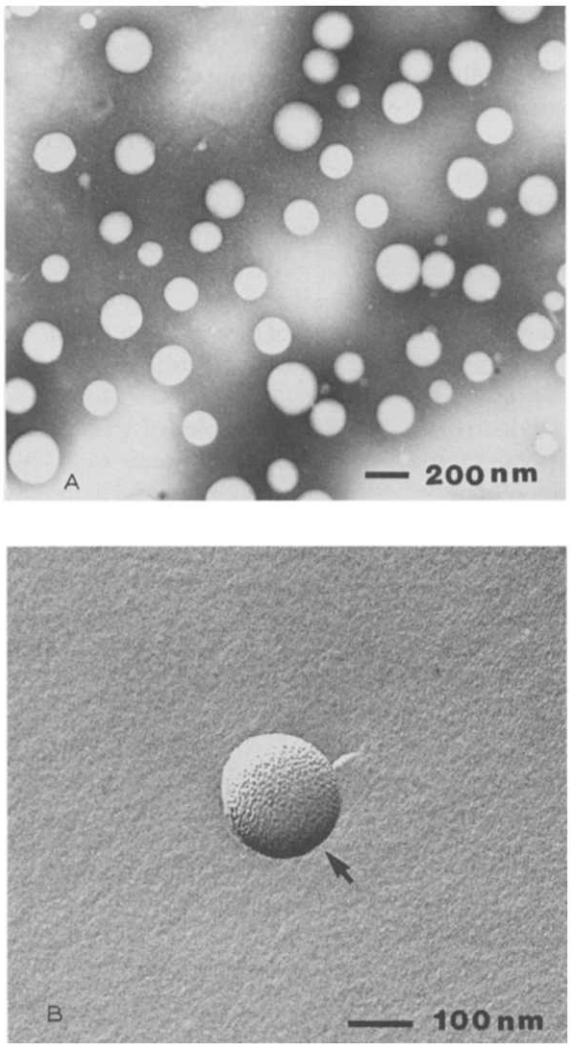


Fig. 2. (A) Negative staining by ammonium molybdate on DPhPC vesicles prepared by sonication method (SUV) and filtered through 0.8-, 0.4-, 0.2- and 0.1-μm polycarbonate membranes (twice each). The concentration of the lipid is 1 mg/ml. As shown, the size is homogeneous and the average value of the radius measured on 54 vesicles is 84 nm. (B) Freeze fracture on DPhPC vesicles prepared by ether injection method (LUV) (concentration of lipid, 1 mg/ml). The vesicles are unilamellar. The radius is about 93 nm. The arrow shows the direction of the black platinum shadow.

Electron microscopy

The vesicle samples were filtered again through 0.1-μm polycarbonate filters just before the electron microscopy experiments.

Negative staining. To a copper grid coated with a Formvar carbon film was applied, and blotted dry, a drop of a 0.1 mg/ml solution of bacitracin (Sigma). Vesicles, at a concentration of 4 mg lipid/ml, were then applied to the grid for 1 min and drawn off with filter paper. A drop of 2% ammonium molybdate solution was immediately added for 1 min, drawn off with filter paper, and allowed to dry.

Freeze-fracturing. Small samples of the vesicles suspension were sandwiched between two low-mass copper platelets. The assembly was frozen by dipping into pasty nitrogen at 63 K (−210 °C) and stored in liquid nitrogen no longer than 5 min. The cryofixed samples were freeze-fractured in a Balzers BAF 301 apparatus at 168 K (−105 °C) at $4 \cdot 10^6$ torr. The fractured samples were immediately replicated with Pt/C at a 45 °C angle, followed by C at a 90 ° angle. Replicas were floated on water, cleaned with sodium hypochlorite solution, rinsed in distilled water and picked up on 300-mesh uncoated grids.

Negative stainings and replicas were examined with a Philips EM 420 electron microscope.

Stopped-flow measurements

The stopped-flow measurements were performed on a Durrum-Gibson spectrophotometer, modified to measure the light-scattering intensity at 90 °C. The light beam is a 6 mW He-Ne vertically polarized laser of high-amplitude stability (Model 120S SA, Spectra-Physics, Les Ulis, France). All the parameters were adjusted as described previously [10]. The vesicle suspensions were kept at 30 °C, degassed under vacuum and filtered through 0.2-μm polycarbonate filters just before the experiments. The concentration of the phospholipid was approx. 10^{-4} M and the experiments were run at 25 ± 0.1 °C.

Molecular mechanics

The dimensions of molecules were determined by molecular mechanics with a MPX 32 graphic computer using the Sybyl software (Tripos Associates, St. Louis).

Results and Discussion

Characterization of diphytanylphosphatidylcholine vesicles

The vesicle suspensions were characterized by different electron microscopic techniques. Those of *n*-acylphosphatidylcholines have often been described; those of diphytanylphosphatidylcholine appear to have been prepared for the first time, and therefore deserve comment.

The negative staining by ammonium molybdate (Fig. 2A) shows a suspension of vesicles prepared by the sonication method; they are SUV of rather homogeneous size (average radius estimated for 54 vesicles in a random field: 84 ± 11 nm). The result of freeze-fracture performed on vesicles prepared by the diethylether evaporation method is shown in Fig. 2B (average radius, 93 ± 11 nm). The size was also measured on the suspension by light scattering, using a simulation programme [10] and by the Zimm-plot method, leading to values in reasonable agreement with those measured by electron microscopy.

Incorporation of carotenoids in DMPC, DPPC and egg PC vesicles

As in a previous study of ours [9], using DMPC, we have confirmed that we can obtain, with the phospholipids studied here, the incorporation of the carotenoids in the lipophilic part of the vesicles. Fig. 3 presents the profile of elution from a Sep-

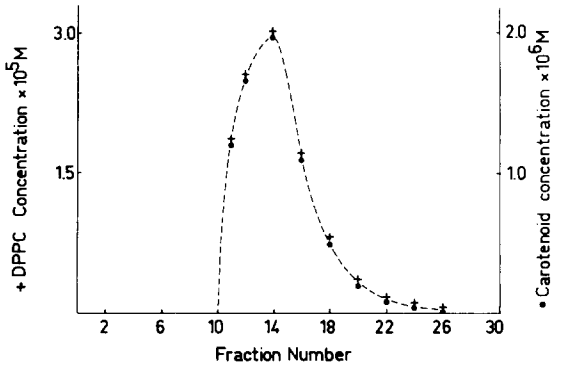


Fig. 3. Elution profile of DPPC-decaprenozeaxanthin vesicles. Sepharose 4BCL column; flow rate, 40 ml/h; elution, 4 ml fractions; void volume, 44 ml; total volume, 80 ml; carotenoid content (●) by ultraviolet-visible spectrum, phospholipid content (+) by phosphorus determination (see Experimental procedure).

harose 4BCL column of DPPC-decaprenozeaxanthin large unilamellar vesicles: the carotenoid and the phospholipid are co-eluted. Fig. 4 shows the ultraviolet-visible spectrum of egg lecithin-zeaxanthin small unilamellar vesicles after successive filtrations through 0.8, 0.4, 0.2 and 0.1 μm polycarbonate filters to eliminate aggregates and homogenize the size. The absence of an absorption peak at 382 nm shows the absence of aggregates of zeaxanthin molecules; the position of the absorption maximum, at 462 nm, corresponds to the monomeric carotenoid, with its chromophore placed in a lipidic environment such as the membrane interior [9].

It was then found that there is a correlation between the molecular length of the carotenoid and the extent of its incorporation into phospholipid bilayers of different thicknesses (Table I and II and Fig. 5). The extent of incorporation, which is a kind of 'two-dimensional solubility', was measured by analyzing the carotenoid and the phospholipid in the vesicles purified as indicated above, and expressed as a molar percentage, i.e., by $s = 100 \cdot c_{carot}/(c_{carot} + c_{phospholip})$. In every case, some of the carotenoid initially added is not included and is probably relegated to microprecipitates or extra-vesicular aggregates. The value of s does not depend on the method used to prepare the vesicles (sonication, ether injection or reverse-phase evaporation), within the limits of accuracy of the analyses (see footnote a of Table II). We have also checked, on DMPC-zeaxanthin vesicles (ether injection method), that the same values of s were obtained whether the dialysis of the vesicles suspension ($T_m = 24°C$) was carried out at 4 or at 30 °C. Table II and Fig. 6 show the values of s obtained for the two carotenoids studied, in different phospholipids, and shows that zeaxanthin is better incorporated in DMPC, its decapreno homologue better in DPPC vesicles.

This is correlated, as shown in Table I, with the

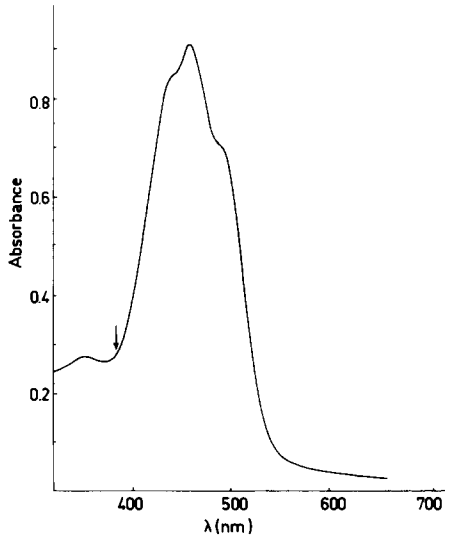


Fig. 4. Visible spectrum of egg PC-9.5 mol% zeaxanthin after filtration through 0.8-, 0.4-, 0.2- and 0.1-μm polycarbonate filters successively No observation of the absorption band at 382 nm, corresponding to aggregated zeaxanthin (arrow).

TABLE I

SELECTED INTERATOMIC DISTANCES IN THE CAROTENOIDS AND IN THE LIPID BILAYERS CALCULATED BY MOLECULAR MECHANICS

Interatomic distances were measured after conformational energy minimization, performed by the Simplex method [18], part of the Sybyl software package. In d(H,H), H is in the OH groups (carotenoids) (cf. Fig. 5). In d(O,O), O is the OH groups (carotenoids) or in the CO groups (bilayers) (cf. Fig. 5).

Carotenoids and lipid bilayers	Interatomic distances (Å)	
	d(H,H)	d(O,O)
Zeaxanthin	31.7	30.2
Decaprenozeaxanthin	40.9	39.4
DMPC bilayer		≈ 30
DPPC bilayer		≈ 40

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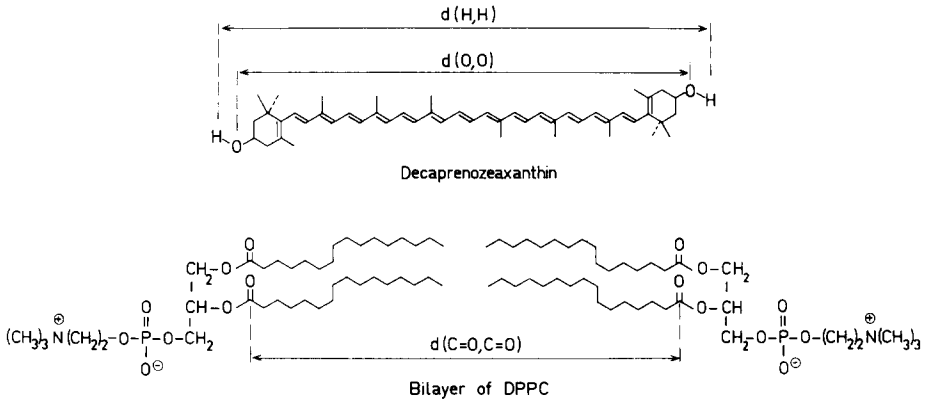

Fig. 5. Structures of decaprenozeaxanthin and DPPC.

best fit of the estimated molecular dimensions of the carotenoid and of the phopsholipid *. Such a correlation was expected, as a consequence of the inclusion of the carotenoids in a trans-membrane fashion. A closely related observation was made recently by Djerassi's group [20], who showed that liposomes made of very-long-chain *n*-acyl diunsaturated phospholipids, such as those found in sponges, do not incorporate cholesterol at all, and proposed the same arguments to explain this remarkable fact.

In egg PC, which bears mostly C_{18} unsaturated chains at C-2, and a mixture of C_{16} and C_{18} saturated chains at C-1 [21], both carotenoids are incorporated fairly well (Table II). This is obviously a consequence of the size heterogeneity of the phospholipids.

Incorporation of carotenoids in DPhPC vesicles (preliminary study)

The above study of the incorporation of carotenoids in *n*-acyl phospholipid vesicles has little direct biological significance, insofar as the membrane reinforcement rôle of dipolar carotenoids had been postulated only for prokaryotic membranes, many of which are characterized by their branched or cyclic acyl chains [5]. As a first step towards a detailed study of the biological systems present in bacteria, we have selected one model case, that of the di-*O*-phytanylphosphatidylcholine 5, used as a model of archaebacterial lipids. To make it possible to compare results with those obtained with the *n*-acyl phospholipids, we have also used zeaxanthin and decaprenozeaxanthin; the first one, widespread in algae and higher plants [22], is a bacterial carotenoid as its mono- and dirhamnosides [23], but it has so far not been reported in archaebacteria, whereas decaprenozeaxanthin is only a synthetic substance [9]. We plan to follow these model studies with genuine bacterial and archaebacterial components. However, at this preliminary stage, we also preferred to use a homogeneous phospholipid, and chose to convert 2,3-di-*O*-phytanyl-*sn*-glycerol (obtained

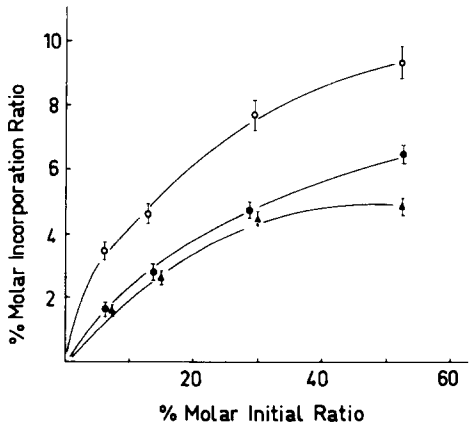

Fig. 6. Plot of incorporated % molar ratio versus initial % molar ratio: egg PC-zeaxanthin (○), egg PC-decaprenozeaxanthin (●) and DPPC-decaprenozeaxanthin (▲) vesicles.

* We are aware of the difficulties linked with the averaging of lengths of mobile molecules. The comparison made here is based on the molecular mechanics models, taking into account an average of 2 gtg kinks per chain in the fluid state [19] and comparing the lengths of hydrocarbon chains.

from total lipids of *H.* halobium [14,15]) to the chemically and stereochemically pure diphytanylphosphatidylcholine (DPhPC) 5. This required cleavage of the various phosphorylated head-groups [14], and rebuilding the phosphatidylcholine head by the procedure outlined in Scheme I. It should be noted that two previous syntheses of related polar lipids have been described: that of 1,2-diphytanoyl-3-*sn*-phosphatidylcholine (an ester lipid) by Redwood et al. [24], but as a mixture of four diastereomeric forms as the phytanic acid used was derived from phytol, and that of 1,2-diphytanyl-3*O*-β-D-glucosyl-*sn*-glycerol by Blöcheret al. [25], but with no stereochemical indication.

The diphytanyl phosphatidylcholine (DPhPC) (C_{20} chains, four methyl branchings) has the same molecular length as DPPC (C_{16} chains); however, the hydrophobic core of the bilayer is thicker for DPhPC than DPPC, as in the former methylene groups occupy the place of the carbonyl groups of the latter. This implies that C_{50} or C_{40} carotenoid, once incorporated, must have at least one of their terminal hydroxyl groups localized within a hydrocarbon-like environment. Their incorporation should therefore perturb the DPhPC bilayer structure. Indeed, the extent of incorporation of both carotenoids is very low and practically identical (1.5 mol%).

Mechanical properties of the vesicles

In a previous study [10], we have shown that the stopped-flow, light-scattering method for osmotic swelling of vesicles is well adapted to the evaluation of the mechanical strength of the bilayer and of its permeability to water. We have established an empirical relationship between $\Delta I/I_0$ (relative light-scattering intensity change) and Z (dissymmetry measured by light scattering) for DMPC vesicles: $\Delta I/I_0$ is proportional to $(Z - 1)$ [10]. We have now confirmed that this relationship is also valid for egg lecithin and DPhPC (Fig. 7). $-\Delta I/I_0(Z-1)$ is independent of the vesicle size and can be taken as an evaluation of the membrane strength.

The swelling of vesicles upon an osmotic shock follows a first-order kinetics. Its characteristic half-time, $t_{1/2}$, is identical, for the various phospholipid-carotenoid vesicles studied here, to the

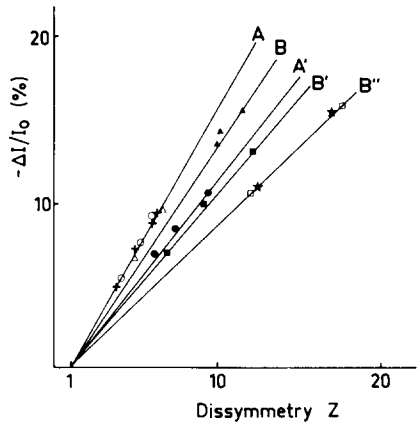

Fig. 7. Linearity between the amplitude change of light scattering ($-\Delta I/I_0$) and dissymetry Z. A: Vesicles made of egg PC (+), egg PC + 9.5 mol% zeaxanthin (○), egg PC + 6.5 mol% decaprenozeaxanthin (△); A′: egg PC + 30 mol% cholesterol (●); B: DPhPC (▲); B′: DPhPC + 1.5 mol% decaprenozeaxanthin (■); B″: DPhPC + 1.5 mol% zeaxanthin (□), DPhPC + 5 mol% cholesterol (★). All vesicles were prepared in buffer of 350 mM NaCl and rapid mixing with 50 mM NaCl. Each point is the average of at least four experiments.

half-time of permeation of water through the membrane, as measured by the ^2H_2O method (without osmotic shock) developed by Lawaczeck [26] (see Ref. 10 for experimental details). Thus, the permeability of the memrbane to water molecules is the limiting factor of the kinetics of swelling.

Influence of zeaxanthin and decaprenozeaxanthin on the mechanical properties and the water permeability of DPhPC and Egg PC vesicles

Fig. 7 and Table III report the results obtained with the different systems studied, which can be summarized as follows:

(1) The properties of egg PC vesicles, which incorporate relatively well the carotenoids studied, remain unaffected by this incorporation. This absence of any reinforcing effect is in contrast with the effect of cholesterol, which hinders appreciably the water permeability and improves the mechanical properties, as already shown by many authors and very different techniques [27].

(b) The barrier properties of DPhPC vesicles, after incorporation of the small amounts of carotenoids they can dissolve, are appreciably improved: their water permeability is reduced as efficiently by 1.5 mol% carotenoids as by 5 mol%

TABLE III

WATER PERMEABILITY ($t_{1/2}$) AND BILAYER RIGIDITY ($-\Delta I/I_0(Z-1)$) FOR VESICLES OF A GIVEN COMPOSITION AND DISSYMMETRY Z AT 25 °C

$Z = I(45\,°C)/I(135\,°)$. $t_{1/2}$ was obtained by both osmotic shock and ^2H_2O methods [25]. The values of both methods were same within the experimental error of ±5 ms.

Composition	Vesicle size		$t_{1/2}$ (ms)	$-\Delta I/I_0(Z-1)$(%)
	Z	Radius (nm)		
Egg PC	5.9	92 [a]	60	2.0±0.2
Egg PC + zeaxanthin (9.5 mol%)	4.5	85 [a]	60	2.0±0.2
Egg PC + decapreno-zeaxanthin (6.5 mol%)	5.3	90 [a]	60	2.0±0.2
Egg PC + cholesterol (30 mol%)	7.1	95 [a]	100	1.3±0.1
DPhPC	10.2	100 [a,b]	40	1.6±0.1
	10.7	102 [c]	40	1.6±0.1
DPhPC + zeaxanthin	12.0	103 [a]	100	0.8±0.1
(1.5 mol%)	17.6	109 [c]	100	0.8±0.1
DPhPC + decapreno-	5.2	90 [a]	100	1.2±0.1
zeaxanthin (1.5 mol%)	5.7	92 [c]	100	1.2±0.1
DPhPC + cholesterol (5 mol%)	12.0	103 [a]	100	0.9±0.1

[a] Values (measured by the Zimm-plot) for vesicles prepared by ether injection method.
[b] This value was checked by electron microscopy (93±11 nm). The sonication method gave a smaller size ($Z = 4.0$, $R = 82$ nm), which was confirmed by electron microscopy (84±11 nm) (see Fig. 2A and B).
[c] Values (measured by the Zimm-plot) for vesicles prepared by reverse-phase method.

cholesterol. In this comparison, one must of course remember that each carotenoid molecule can affect both layers of the membrane, so that the effective molar concentration corresponds to 3 mol%; in other words, the carotenoids studied are about as efficient as cholesterol in reinforcing DPhPC vesicles.

From the results obtained previously with DMPC [10] and the present ones, it is clear that α,ω-bipolar carotenoids do exert the expected mechanical reinforcing effect of some membranes, but that both the nature of the phospholipid and that of the reinforcing agent are important. We cannot interpret at this stage the absence of effect of the carotenoids studied on the properties of egg PC vesicles.

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