2. Organization of Carotenoid-Phospholipid Bilayer Systems

Incorporation of Zeaxanthin, Astaxanthin, and their C_{50} Homologues into Dimyristoylphosphatidylcholine Vesicles

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Four carotenoids (zeaxanthin **1**, astaxanthin **2**, and their C_{50} synthetic isoprene-homologues **3** und **4**) have been incorporated into bilayer unilamellar vesicles of dimyristoylphospatidylcholine as proved by coelution on a *Sepharose* column. The incorporation into the bilayer proper has been proved by the sensitivity to phase transition of UV/VIS spectra and circular dichroism. The UV/VIS absorptions of the carotenoids are typical of a lipidic environment. At the temperature of phase transition occur both intermolecular phenomena (aggregation of carotenoid molecules) and intramolecular changes (conformational change). The relative solubilities of the various carotenoids in this phospholipid membrane can be fitted with molecular parameters (length of the lipophilic carotenoid segment *vs.* lipid bilayer thickness).

Introduction. – Carotenoids play an important role in living cells. Their occurrence and functions have been extensively studied [1–3]; they are generally accepted as light-gathering pigments in photosynthesis [4], photo-protecting agents against molecular oxygen [5], and electron conductors across membranes [6].

We have postulated as part of a general theory of the molecular evolution of biomembrane constituents [7] that carotenoids might play another important, though less sophisticated role in bacteria as membrane stabilizers. As schematically shown in *Fig. 1*, the

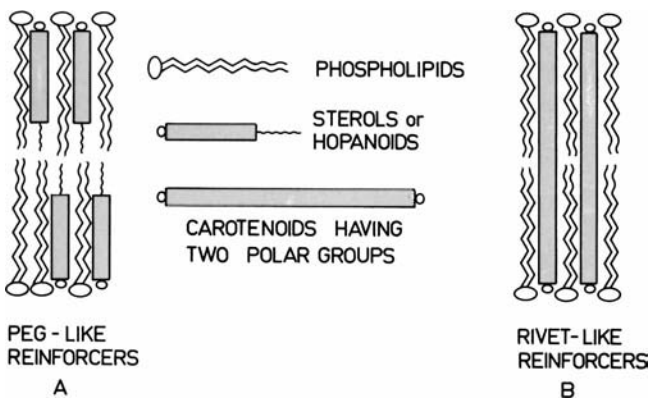


Fig. 1. *Hypothetical reinforcement of lipid bilayers by sterols or hopanoids (A) or α,ω-dihydroxylated carotenoids (B)*

terminally hydroxylated carotenoids typical of so many bacteria could act as trans-membrane rivets, stabilizing both halves of the bilayer. This mechanism of stabilization would be contrasting with the one-layer stabilization brought about by cholesterol in Eucaryotes [8] and by hopanoids in many bacteria [9].

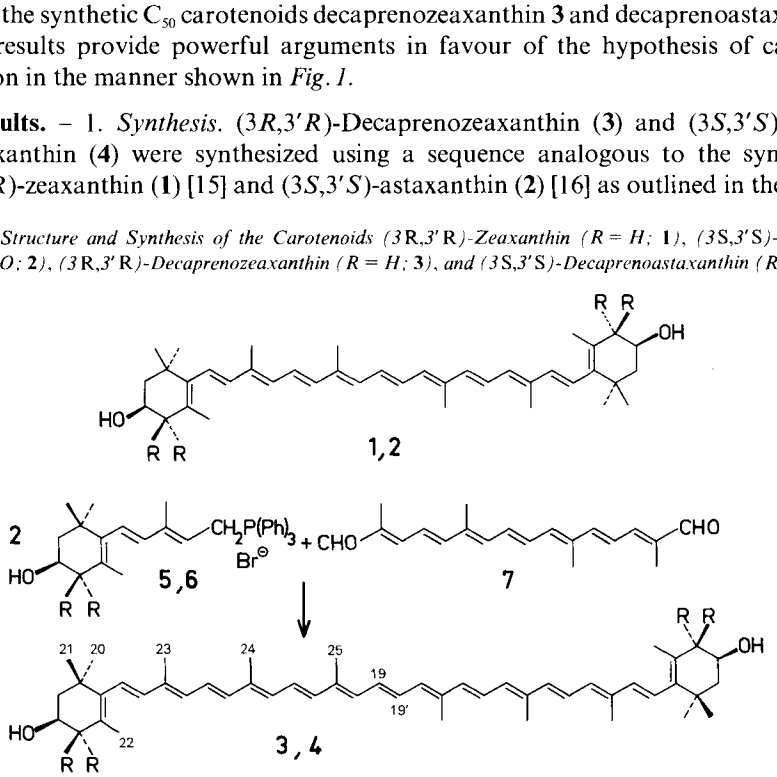
Previously, it had already been postulated that carotenoids could be mechanical reinforcers of membranes, but on the basis of the structurally unacceptable assumption of inclusion into one half of the bilayer, which implied non-existent (*E*)-configurations either for all the double bonds [10] or for the central one [11]. (In the present work, we have checked that all the carotenoids used show UV/VIS spectra without (*E*)-bands.)

That bacterial carotenoids do play a significant role in membranes has been demonstrated by several studies, both on model membranes [12] and *in vivo* [13]. We have recently shown that a synthetic terminally hydroxylated C_{50} carotenoid can act, like hopanoids and cholesterol, as reinforcer of lipid unilamellar vesicles [14]. In spite of this accumulation of results compatible with the function suggested for carotenoids, we felt it was necessary to study their topology once they are incorporated in membranes.

Here, we describe results obtained by optical methods (UV/VIS and CD spectra) on unilamellar DMPC (dimyristoylphosphatidylcholine) vesicles, both below and above the phase-transition temperature, having incorporated one of the following four polar carotenoids (= carotenols or xanthophylls): the naturally occurring C_{40} carotenoids (3*R*,3′*R*)-zeaxanthin (= (3*R*,3′*R*)-β,β-carotene-3,3′-diol; **1**) and (3*S*,3′*S*)-astaxanthin (= (3*S*,3′*S*)-3,3′-dihydroxy-β,β-carotene-4,4′-dione; **2**) and their higher isoprene-homologues, the synthetic C_{50} carotenoids decaprenozeaxanthin **3** and decaprenoastaxanthin **4**. These results provide powerful arguments in favour of the hypothesis of carotenoid inclusion in the manner shown in *Fig. 1*.

Results. – 1. *Synthesis.* (3*R*,3′*R*)-Decaprenozeaxanthin (**3**) and (3*S*,3′*S*)-decaprenoastaxanthin (**4**) were synthesized using a sequence analogous to the syntheses of (3*R*,3′*R*)-zeaxanthin (**1**) [15] and (3*S*,3′*S*)-astaxanthin (**2**) [16] as outlined in the *Scheme*.

Scheme. *Structure and Synthesis of the Carotenoids (3*R,3′R*)-Zeaxanthin (R = H; **1**), (3*S,3′S*)-Astaxanthin (R,R = O; **2**), (3*R,3′R*)-Decaprenozeaxanthin (R = H; **3**), and (3*S,3′S*)-Decaprenoastaxanthin (R,R = O; **4**)*



2. *Aggregation of Carotenoids in H_2O/EtOH Solutions.* Addition of H_2O to a solution of each carotenoid in pure EtOH or acetone gives rise to a second band (large blue shift) in the VIS spectra, which has been assigned to the so-called 'card-packed' or 'infinite' aggregate structure [17–20]. *Fig. 2* shows the spectra observed with decaprenoastaxanthin **4** in EtOH/MeOH/H_2O mixtures: the spectrum of *Run 1* with a maximum at 510 nm corresponds to the monomer and the one of *Run 7* with a maximum at 420 nm to the almost pure 'infinite' aggregate in solution.

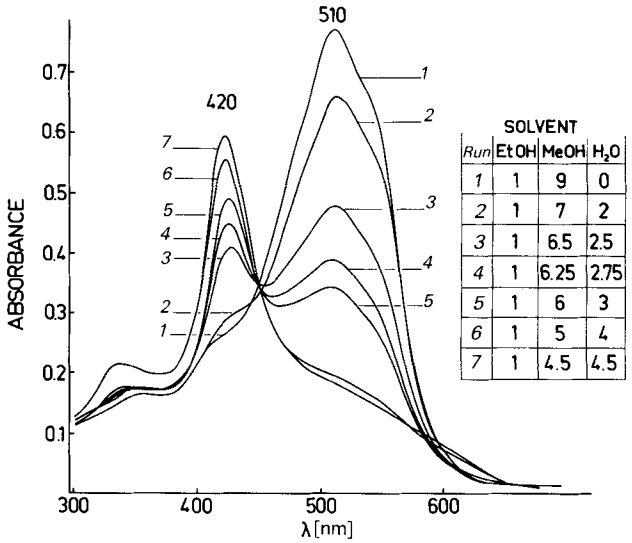


Fig. 2. *VIS spectra of (3S,3'S)-decaprenoastaxanthin* **4** *in EtOH/MeOH/H_2O mixtures of different composition*

The UV/VIS spectra of the C_{50} carotenoids **3** and **4** display an isosbestic point showing that there are only two species in solution: the monomer and the 'infinite' aggregate. In the case of the C_{40} carotenoids zeaxanthin **1** and astaxanthin **2**, the spectral changes are more complex suggesting the coexistence of dimers, trimers, *etc.* and, maybe, also of 'head-to-tail' aggregates [18].

It is interesting to note that the UV/VIS spectra of these carotenoids in the solid state are comparable to those of the monomers in solution *(Table 1)*. In solution, at a H_2O concentration higher than that of *Run 7* (see *Fig. 2*), a maximum reappears at 510 nm which corresponds probably to the formation of microcrystals.

Table 1. *UV/VIS Absorption Maxima of Carotenoids*

Carotenoid	EtOH solution λ_{max} [nm] (ε^a)	Solid state λ_{max} [nm]	Aggregate λ_{max} [nm]
1	461 (130000)	435	382
2	486 (135000)	490	402
3	518 (180000)	530	413
4	527 (190000)	525	420

[a]) In $M^{-1} \cdot cm^{-1}$, ±5%.

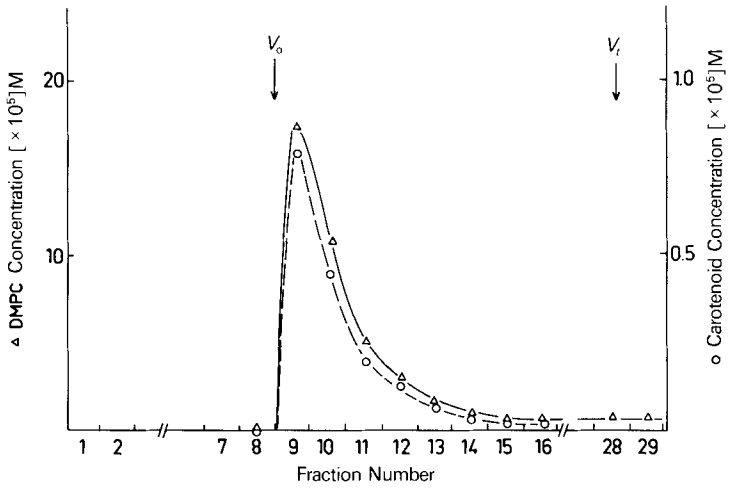


Fig. 3. *Elution profile of a vesicle suspension (DMPC/decaprenoastaxanthin* **4***).* Flow rate: 40 ml/h, fraction volume: 80 ml, void volume: 30 ml. Proportion of carotenoid: before filtration 10%, after filtration 4%.

3. *Incorporation of Carotenoids in Vesicles.* We have prepared small unilamellar vesicles of L-α-dimyristoylphosphatidylcholine (DMPC) by sonication [21] and large unilamellar vesicles by the ether-injection method [22]. The size of the vesicles was calibrated by sequential filtration through polycarbonate membranes [23]. *Fig. 3* shows the elution profile of a vesicle 'solution' (DMPC/**4**) on a *Sepharose 4BCL* gel. This gel filtration separates the particles according to their size [24]; furthermore, in the case of binary systems, it allows the separation of the vesicles from the other particles initially present in the sample, for instance microprecipitates, micelles, aggregates or monomer in solution.

We have prepared vesicles with varying concentrations of carotenoid in the phospholipid; in the present paper, we characterize the proportion of carotenoid in the phospholipid-vesicle suspension by the % molar ratio, *i.e.* carotenoid concentration/(carotenoid concentration + phospholipid concentration); this ratio varied from 0.5 to 50% in the samples prepared.

In most cases, the incorporation was not complete and, after gel filtration, the concentration of carotenoid was lower than the initial value due to the elimination of microprecipitates and aggregates on the gel. This separation was also obtained by filtration of a vesicle suspension through polycarbonate filters; *Fig. 4* shows the VIS spectrum of the same sample before (*a*) and after (*b*) filtration through 0.1 μm filters. The shoulder at 420 nm in the spectrum (*a*) is characteristic of the 'infinite' aggregates (outside the vesicles).

We have also characterized the vesicle sample after gel filtration by electron microscopy. *Fig. 5* shows that the vesicles are of fairly homogeneous size (*R ca.* 120 nm).

By the filtration method, we have measured the 'solubility' of each carotenoid in a DMPC bilayer, *i.e.* the maximum concentration of carotenoid which can be incorporated even in the presence of a large excess of carotenoid before filtration: this maximum concentration is, therefore, indeed comparable to a solubility. *Table 2* gives the results for the four carotenoids studied. We shall discuss later these results, but it may be noted at

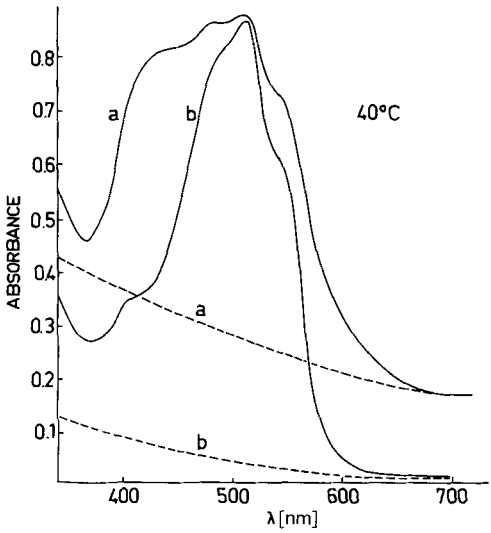


Fig. 4. *VIS spectra of two vesicle suspensions.* (a) *Vesicle suspension (DMPC/5% decaprenozeaxanthin* **3**) *after filtration through 0.8 μm polycarbonate filters.* (b) *Same suspension after filtration through 0.4, 0.2 and 0.1 μm polycarbonate filters, successively* (measured concentration of carotenoid: 2.5%). Dotted lines indicate the absorbance due to light scattering by vesicles.

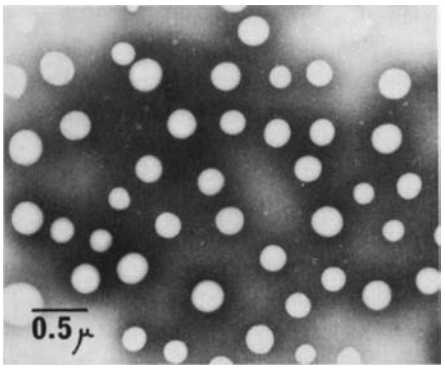


Fig. 5. *Electron microscopy of a vesicle suspension of DMPC/5% astaxanthin.* Negative staining by ammonium molybdate.

Table 2. *Maximum 'Solubility' of Carotenoids in DMPC Vesicles*

Carotenoid	**1**	**2**	**3**	**4**
Proportion [%]	8.5	15	2.5	10

once that saturation in carotenoids is attained at molar ratios much lower than those observed in natural membranes with cholesterol (for which %-molar ratios as high as 100 are apparently possible).

Fig. 3 shows that there is a coelution of the carotenoid and the phospholipid: the maximum of the absorbance in the UV/VIS coincides with the peak of phosphorus content. This is an indication of good incorporation, but does not differentiate between incorporation *into* the vesicle bilayer, incorporation *inside* the vesicle, or superficial binding *onto* the vesicle exterior. The results of two studies have resolved this ambiguity: the study of the influence of solvent and that of the temperature dependence of the UV/VIS spectrum of the vesicle suspension.

4. *Solvent Effects on the UV/VIS Spectra of Carotenoids.* According to *Bayliss* [25], the absorption maximum depends on the refractive index of the solvent, and a shift of the absorption ($\Delta\tilde{\nu}$) to lower frequencies (in cm^{-1}), going from vacuum to a solvent of refractive index n is given by *Eqn. 1*, where f is the oscillator strength, a the radius of the spherical solute molecule, and $\tilde{\nu}$ the absorption frequency in vacuum.

$$\Delta\tilde{\nu} = \text{const.}\ (f/\tilde{\nu}\, a^3)\ (n^2 - 1)/(2n^2+1) \qquad (1)$$

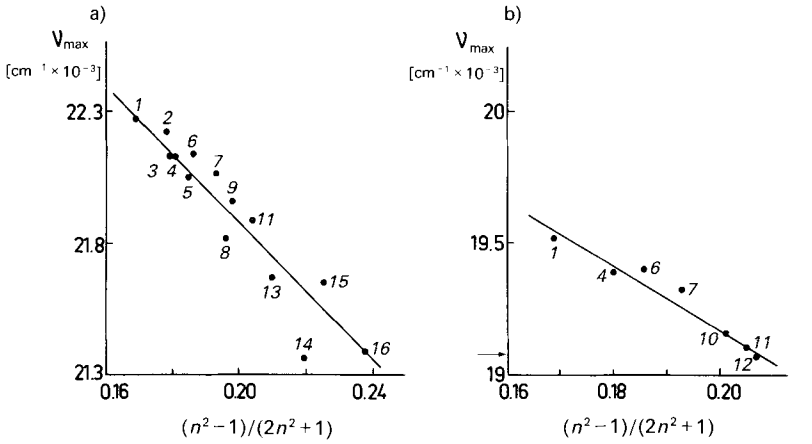


Fig. 6. *Plot of the absorption frequency* vs. $(n^2\text{-}1)/(2n^2n^2+1)$ *for several solvents of refractive index* n: a) ***zeaxanthin* 1**, b) ***decaprenoastaxanthin* 4**. The arrow on plot *b)* indicates the absorption frequency of the carotenoid when incorporated into vesicles, and above the phase transition temperature. See *Table 3* for the list of solvents (numbers 1–16).

Table 3. *Absorption Maximum of Zeaxanthin* **1** *and Decaprenoastaxanthin* **4** *in Several Organic Solvents (25°C)*

Solvent	Number[a])	Refractive index (25°C)	$\tilde{\nu}_{max} \cdot 10^{-3}$ [cm^{-1}] **1**	$\tilde{\nu}_{max} \cdot 10^{-3}$ [cm^{-1}] **4**
MeOH	*1*	1.326	22.27	19.52
Et_2O	*2*	1.352	22.25	
Acetone	*3*	1.357	22.13	
EtOH	*4*	1.359	22.13	19.39
AcOEt	*5*	1.370	22.05	
2-Propanol	*6*	1.375	22.14	19.40
2-Butanol	*7*	1.395	22.06	19.32
Tetrahydrofuran	*8*	1.404	21.82	
1-Pentanol	*9*	1.408	21.96	
1-Hexanol	*10*	1.416		19.16
1-Octanol	*11*	1.427	21.89	19.10
1-Decanol	*12*	1.437		19.07
$CHCl_3$	*13*	1.444	21.67	
Dimethyl sulfoxide	*14*	1.476	21.37	
Toluene	*15*	1.494	21.65	
Benzyl alcohol	*16*	1.538	21.39	

[a]) This number refers to *Fig. 6.*

Fig. 6 and *Table 3* give the corresponding data for zeaxanthin **1** (*a*) and decaprenoastaxanthin **4** (*b*). *Buchwald* and *Jencks* [17] have already obtained similar results for astaxanthin **2**. Taking $n = 1.44$ for the phospholipid bilayer [26] and $n = 1.33$ for H_2O, we get the following absorption maxima for **4**: $\lambda_{max} = 525$ nm for a solution in the phospholipid, and $\lambda_{max} = 512$ nm for a solution in H_2O. The former value corresponds well with the observed value of 524 nm (above T_c), confirming that the carotenoid molecules must be present in the phospholipid bilayer and not in the interior of the vesicles (which would of course give the same coelution).

5. *Aggregation of Carotenoids in DMPC Vesicles at the Phase Transition.* Several authors have reported important changes in the absorption spectrum of xanthophylls incorporated in vesicles when the temperature fell below the phase-transition temperature. This was either attributed to *cis/trans* isomerization of double bonds in the carotenoid [11] or to the aggregation of carotenoids in the vesicles [27]. Aggregation of amphiphiles in bilayers below the transition temperature is a quite general phenomenon and is not limited to carotenoids [28] [29].

We have carefully studied the temperature dependence for the four carotenoids at different concentrations in order to be able to distinguish between processes operating at the molecular and at the intermolecular level.

We first established that changing temperature around the phase-transition temperature produces little change in the VIS spectrum of true solutions of carotenoid in octanol; the same holds for the aggregate suspensions in H_2O/EtOH mixtures. The presence of phospholipid vesicles is necessary to induce changes in the VIS spectra. *Fig. 7* shows the VIS spectrum of decaprenozeaxanthin **3** incorporated in vesicles, at several temperatures, and *Table 4* summarizes the results for the four carotenoids. Above the phase-transition temperature, only the spectrum of the monomer was observed in all cases. When the temperature was progressively decreased, no appreciable change was observed until the phase transition was reached, when the absorption maximum shifted to a much shorter wave-length (*Table 4, Fig. 8*).

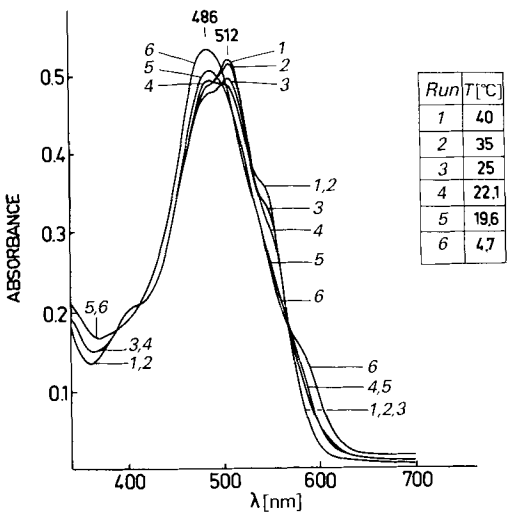


Fig. 7. *VIS spectrum of a vesicle suspension (DMPC/2.5% decaprenozeaxanthin* **3***) at several temperatures*

Table 4. *Absorption Maximum of Carotenoids in DMPC Vesicles above and below the Phase-Transition Temperature (T_c = 23°C)*

Carotenoids	Carotenoid proportion [%]		λmax [nm] $T > T_c$	λmax [nm] $T < T_c$ [a])
	Initial	Final		
1	30	9.5	452	430
	15	8.5	452	430
2	50	15	460	442
	10	5.0	466	450
3	30	2.5	510	485
	15	2.5	510	485
4	20	9.0	514	461
	10	8.0	514	462
	10	6.6	515	467
	5	3.4	519	472
	1	1	523	501

[a]) λ_{max} at 5–10°C.

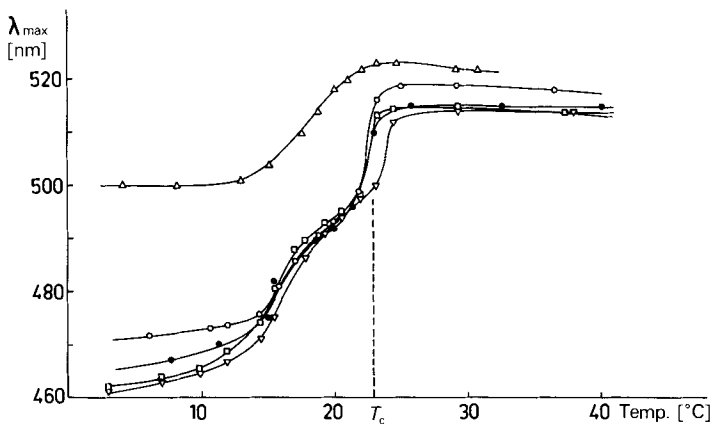


Fig. 8. *Variation of the absorption maximum with temperature for several proportions of incorporated carotenoid.* Vesicles were prepared from DMPC and decaprenoastaxanthin **4** at the following proportions: △ 1%, ○ 3.4%, ● 6.6%, □ 8.0%, and ▽ 9.0%; T_c: phase-transition temperature.

Fig. 8 shows that the amplitude of this shift clearly depends on the carotenoid/DMPC ratio: as more carotenoid is incorporated, the amplitude of the shift becomes larger; this apparently suggests that the shift is somehow linked with aggregation. However, it is highly probable that part of the observed phenomenon is also occurring at the monomolecular level. Indeed, even at the lowest concentration we tried (molar ratio 0.5%), the shift still existed. Furthermore, the shift observed is much too small to be explained by aggregation, according to the 'exciton model' of spectral shifts [19] [29].

According to the 'exciton model' the spectral shift $\Delta\tilde{\nu}$ produced by aggregation from the monomer is given by *Eqn. 2*

$$\Delta\tilde{\nu} = 2\,(N\text{-}1)\,\mu^2\,(1\text{-}3\cos^2\theta)/hcNr^3 \qquad (2)$$

where h is *Planck*'s constant, c the light velocity, N the number of molecules aggregated, μ the transition dipole moment, r the separation of the molecular centers, and θ the tilt angle between the line of centers and the direction of the transition moment. Applying *Eqn.* (*2*) to (3*R*,3'*R*)-decaprenozeaxanthin **3** gives $\lambda_{max} = 413$ nm for $N = \infty$ and $\lambda_{max} = 457$ nm for $N = 2$. Thus, even for as small an aggregate as the dimer, the spectral shift expected would be much larger than the one observed (to 485 nm at a molar ratio of 2.5%). The conclusion that a molecular phenomenon is at play is reinforced by the study of the circular dichroism.

6. *Circular Dichroism of Incorporated Carotenoids in Vesicles.* CD has proved to be a powerful tool in the study of conformational changes and molecular association in carotenoids [30–32]. We have studied CD spectra of our four carotenoids in solution, in aggregates, and in vesicles as a function of temperature around the phase transition. The results are summarized in *Fig. 9*.

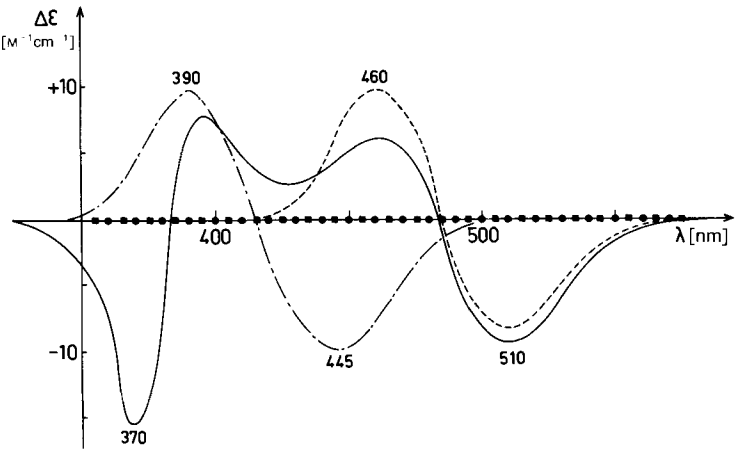


Fig. 9. *CD of decaprenozeaxanthin* **3** *in several systems.* ● Solution in THF; $6.5 \cdot 10^{-6}$M. ■ Vesicle suspension; DMPC/1.5% **3**; carotenoid concentration $1.2 \cdot 10^{-5}$M, temp. > 23°C (23, 26, 31°C). —·— Aggregate of **3** in acetone/H_2O 1:1; carotenoid concentration $8.7 \cdot 10^{-6}$M. ····· Vesicle suspension; DMPC/0.8% **3**; carotenoid concentration 10^{-5}M, temp. 17.8°C. ——— Vesicle suspension, DMPC/1.5% **3**; carotenoid concentration $1.2 \cdot 10^{-5}$M, temp. 6.5°C.

In a THF solution of decaprenozeaxanthin **3**, there is no detectable CD band between 400 and 650 nm, in agreement with the results obtained for zeaxanthin in acetone by *Mendelsohn* and *van Holten* [27].

In H_2O/acetone 1:1, the same carotenoid **3** gives, according to its UV/VIS spectrum, 'infinite' aggregates; it then shows two CD bands ($\Delta\varepsilon^{390} = 10$ $M^{-1} \cdot cm^{-1}$, $\Delta\varepsilon^{445} = -10$ $M^{-1} \cdot cm^{-1}$) and no CD band at the wave-length of the main absorption of the monomer (above 500 nm). At higher concentrations of H_2O in acetone (1% acetone), the appearance of a CD band above 500 nm is probably due to the existence of a precipitate in suspension [32].

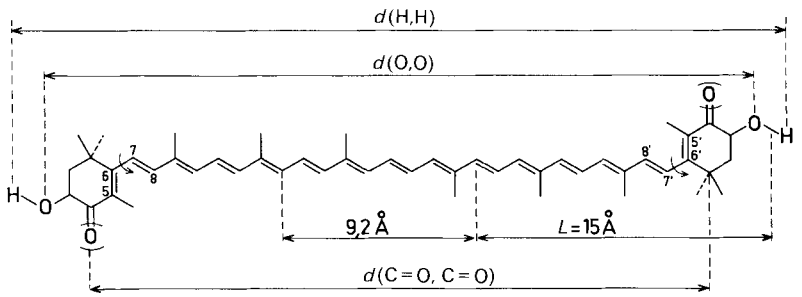
When the carotenoid is incorporated in vesicles, three distinct phenomena occur (see *Fig. 9*): *1*) *Above* the phase-transition temperature, there is no detectable CD absorption band between 400 and 650 nm, like in a pure solvent. *2*) *Below* the phase-transition temperature, at a low concentration of carotenoid in the vesicles (0.8%) and at a tempera-

ture close to T_c (17.8 °C), a two-wave dichroism signal appears, centered near the absorption of the monomer, and free of any signal close to the absorption of the aggregate. *3*) At a higher concentration of carotenoid (1.5%) and at a lower temperature (6.5 °C), the CD curve shows signals as well around the absorption maximum of the monomer as around that of the aggregate.

Discussion. – The results presented in this paper establish several important points in carotenoid-phospholipid incorporation experiments. We have employed a procedure giving pure preparations of rather homogeneous vesicles and characterized them by electron microscopy. We have obtained several strong indications proving the incorporation of the carotenoids studied into the vesicle, and more precisely into the phospholipid bilayer. The coelution of the carotenoids with DMPC in the gel filtration shows directly the first point. The UV/VIS spectral changes at the phase-transition temperature, and the position of the absorption maximum above T_c, which is typical of a lipidic environment, are strong arguments in favour of the second point.

We have seen that the spectral changes at T_c can be assigned to two causes: the onset of aggregation and an intramolecular change. The latter could be a change of solvation (the phospholipid solvent undergoing itself a major change) or a conformational change. The first effect appears to be responsible for the reversible alteration observed around T_c in the UV spectrum of the isolated olefinic bonds present in the phospholipid molecules themselves [33]; we believe our CD results to be in favour of a conformational alteration.

One possible interpretation would be a change in conformation about the s-*cis* diene at C(5) to C(8) (or C(5′) to C(8′)) (*Fig. 10*): above T_c the lipid is fluid, and in the carotenoid, rotation around the single bond C(6), C(7) or C(6′), C(7′) is unimpeded. By contrast, below T_c, the rigid state of the lipid chains could restrict rotation and their compact arrangement could permit one of the C(6), C(7) conformers to predominate. This would also explain the loss of fine structure in UV/VIS spectra measured below T_c [30] [34] (see *Fig. 7*).



	Zeaxanthin 1	Decaprenozeaxanthin 3	Astaxanthin 2	Decaprenoastaxanthin 4
d(H,H)[Å]	31.7	40.9	31.7	40.9
d(O,O)[Å]	30.2	39.4	30.2	39.4
d(C=O, C=O)[Å]			25.0	34.2

Fig. 10. *Intramolecular dimensions of the carotenoids as calculated by molecular mechanics.* L = 15 Å corresponds to the length of the phospholipid lipophilic segment, *i.e.* the average distance between the carbonyl group and the methyl group of DMPC, above T_c. This agrees much better with the half-length of the C_{40} carotenoids **1** and **2** than with that of the C_{50} carotenoids **3** and **4**.

Finally, we want to discuss the significance of our results for the elaboration of the hypothesis which led us initially to undertake this study [7], that of the functional equivalence of bacterial carotenoids and cholesterol. With a molecular-dynamics programme, we determined several structural parameters of the carotenoids *(Fig. 10)* and compared them with those of the lipid bilayer. By analogy with the well studied geometry of inclusion of cholesterol in bilayers [35], we can assume the existence of a H–bond between the OH groups of zeaxanthin and the ester carbonyl groups of DMPC. For this fit to be achieved at both ends of the zeaxanthin molecule which are 3.17 Å apart *(Fig. 10)*, the total thickness of the lipid bilayer should approach this value. For the all-*trans* conformer of the myristoyl chain, which is the one obtained below T_c, we measure on the model a length (from the carbonyl to the terminal methyl) of 17.2 Å; the total thickness should then be about 35 Å. In the fluid state, there is an average of 2 gtg kinks per chain, each gtg kink shortening the chain by as much as 1.4 Å [36]. For the DMPC bilayer above T_c, the total thickness should, therefore, be about $2L = 30$ Å *(Fig. 10)*. These values correspond well with the length of zeaxanthin **1** (C_{40}), but not with that of decaprenozeaxanthin **3** (C_{50}) *(Fig. 10)*. This may be the reason for the much poorer incorporation of the C_{50} homologue **3**, compared to zeaxanthin **1**, into DMPC vesicles (see *Table 2*). For astaxanthin **2** and its C_{50} homologue **4**, the comparison is more complex, as there are two possible sites of H-bonding.

In conclusion, we would like to point out that the results just discussed apply strictly only to the model systems we have chosen. Indeed, to our knowledge, DMPC is not the major phospholipid of bacteria which would contain zeaxanthin as their major carotenoid. Nevertheless, we have chosen to study the incorporation into DMPC because its phase transition at 23°C makes it convenient for laboratory studies. Eubacterial phospholipids are varied, often branched or cyclized, anyway usually with chains corresponding approximately in length to C_{16}–C_{18}; archaebacterial ones are exemplified in their simplest form by diphytanylglyceryl ethers, *i.e.* with a linear extension of C_{16}. Their bilayer thickness would be about 40 Å in the fluid state and 45 Å in the gel state, and would, therefore, fit better with C_{50} carotenoids similar to (but not necessarily identical with) our synthetic **3** and **4**; their cross-section would also fit better with the cross-section of carotenoids than the straight chains of DMPC. These points have already been alluded to previously [7]; the present study of simple models makes it particularly desirable that it be followed by similar experiments combining real procaryotic lipids with real procaryotic carotenoids. We can safely predict that these experiments will give results even clearer than the present ones.

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Experimental Part

General. Solvents used for synthesis were distilled on CaH_2 in an inert atmosphere just before use. Anal. TLC: silica gel *F254* plates (0.25 mm thick; *Merck*, Darmstadt). Column chromatography: silica gel *60* (40–60 μm; *Merck*). *Reichert* hot-stage microscope. UV/VIS: *Uvikon 820 (Kontron)* instrument for solns., *Beckman Acta CIII* for the solid state in Prof. *J. Dehand*'s laboratory; λ_{max} [nm] (ε). CD: *Jobin-Yvon Dichrographe, Mark III*, in Prof. *M. Daune*'s laboratory. IR: *Pye-Unicam-SP3-300S (Philips)* spectrometer; in cm^{-1}. ^1H-NMR: *Bruker SY* spectro-

meter (200 MHz); in $CDCl_3$ with TMS as internal standard. MS: historical double-focusing *Thompson-CSF THN-208 B* spectrometer; 70 eV; direct introduction with fast heating [37]. Electron microscopy: *Philips 420 instrument*. Microanalyses are due to Mrs. *M. François*, Institut de Chimie, Strasbourg.

(3R,3'R)-Decaprenozeaxanthin(= (1R,1'R)-4,4'-(3,7,11,16,20,24-Hexamethyl-1,3,5,7,9,11,13,15,17,19,21,23,25-hexacosatridecaene-1,26-diyl)-bis[3,5,5-trimethyl-3-cyclohexen-1-ol]; **3**). A soln. of crocetin-dialdehyde (**7**; 603 mg, 2.03 mmol) and the phosphonium salt **5** (2.149 g, 4.15 mmol) in 1,2-epoxybutane (30 ml) was heated under reflux under Ar for 24 h. After evaporation of the solvent and filtration of the larger part of triphenylphosphine oxide, the mixture was chromatographed on silica gel with CH_2Cl_2 MeOH. The crude product was twice crystallized from THF/MeOH to give 400 mg (28%) of pure **3**, m.p. 185 185.5°. UV/VIS (THF): 327 (46200), 472 (131000), 511 (181000), 564 (154000). IR ($CHCl_3$): 3700–3600, 1385, 1365, 1030, 965. ^{1}H-NMR[1]): 1.08 (*s*, 12 H, CH_3 (20,20'), CH_3 (21,21')); 1.38 (*d*, J = 4.8, 2 OH); 1.49 (*dd*, J_{gem} = 11, J(2a,3a) = 11.5, 2 H, H_a-C(2,2')); 1.75 (br. *s*, 6 H, CH_3(22,22')); 1.79 (*dd*, J_{gem} = 11, J(2e,3a) = 3,2 H, H_e-C(2,2')); 1.98–2.00 (br. *s*, 18 H, CH_3(23,23'), CH_3(24,24'), CH_3(25,25')); 2.05 (*dd*, J_{gem} = 17, J(4a,3a) = 10, 2 H, H_a-C(4,4')); 2.40 (*dd*, J_{gem} = 17, J(4e,3a) = 6.0, 2 H, H_e-C(4,4')); 4.02 (*m*, 2 H, H_a-C(3,3')); 6.13–6.64 (*m*, vinyl H). MS (70 eV): 700 (100, M^+), 682 (8), 608 (5), 594 (13), 542 (4). Anal. calc. for $C_{50}H_{68}O_2$ (700.54): C 85.66, H 9.78; found: C 85.4, H 9.9.

(3S,3'S)-Decaprenoastaxanthin(= (6S,6'S)-3,3'-(3,7,11,16,20,24-Hexamethyl-1,3,5,7,9,11,13,15,17,19,21,23,25-hexacosatridecaene-1,26-diyl)-bis[6-hydroxy-2,4,4-trimethyl-2-cyclohexen-1-one]; **4**). With the same procedure as for **3**, the *Wittig* reaction of **7** (236 mg, 0.80 mmol) and the phosphonium salt **6** (1079 g, 1.88 mmol) gave crystalline **4** (360 mg, 61%). The latter was very sensitive to air and to heat. It was recrystallized twice from $CHCl_3$/MeOH, m.p. 190–190.5°, and used immediately for the physico-chemical studies. UV/VIS (THF): 352 (39000), 527 (190000). IR ($CHCl_3$): 3480, 1650, 1600, 1385, 1365, 1270, 1120, 1070, 1030, 960. ^{1}H-NMR[1]): 1.22, 1.32 (2*s*, 12 H, CH_3 (20,20'), CH_3 (21,21')); 1.82, (*dd*, J_{gem} = 12, J(2a,3a) = 13, 2 H, H–C(2,2')); 1.95, 1.99–2.00 (2 br. *s*, 24 H, CH_3(22,22'), CH_3(23,23'), CH_3(24,24'), CH_3(25,25')); 2.15 (*dd*, J_{gem} = 12, J(2e,3a) = 6, 2 H, H_e–C(2,2')); 3.70 (*d*, J = 2, 2 OH); 4.26–4.32 (*m*, 2 H, H_a–C (3,3')); 6.16–6.71 (*m*, 20 vinyl H). MS (70 eV): 728 (M^+).

Preparation of the Vesicles. L-α-Dimyristoylphosphatidylcholine (DMPC) from *Avanti Polar Lipids, Inc.*, Birmingham, Alabama, was kept at −20° in $CHCl_3$ soln. in sealed tubes. Carotenoids **1**–**4** were kept under Ar at −20° and, when necessary, were recrystallized twice before use. The purity of the lipid was checked by TLC ($CHCl_3$/MeOH/conc. NH_3 65:25:4 *(v/v)*) and the one of the carotenoids in the UV/VIS by the ε and the non-appearance of the *cis* band.

Small unilamellar vesicles (SUV) were prepared by sonication (*Branson Sonifier B-30*, power setting 5). A soln. of the lipid and one of the carotenoid in $CHCl_3$ was evaporated to dryness *in vacuo* without heating. The desired amount of buffer ('Ultra-pure water' from *Millipore*, *Tris*-HCl (10 mM), $EDTANa_2$ (1 mM), NaN_3 (5 mM)) was added, and Ar was bubbled through for 2 h. The sample was sonicated slightly above 23° *(T_c)* under Ar for 2 h.

Large unilamellar vesicles (LUV) were prepared by Et_2O injection. An Et_2O/MeOH soln. (9:1; 5 ml) of the lipid (15 mg) and the desired amount of the carotenoid were injected within 10 min in the dark and with an Ar stream into 5 ml of buffer maintained at 60°. The resulting soln. was dialyzed against the same buffer twice (3 h, then overnight, dialysis tubing *Spectrapor 2, Spectrum Medical Industries*, Los Angeles, California) to remove the remaining solvent. The sample was then filtered through polycarbonate filters (*Nucleopore, DMF*, Paris). The filtration was performed twice through 0.8, 0.4, 0.2, and 0.1 μm filters. The soln. was concentrated to 1 ml in an ultrafiltration cell (*Amicon* stirred cell) with an *XM-50-Amicon* membrane. Immediately after concentration, gel filtration was run on a *Sepharose-4BCL* (*Pharmacia France SA*, Bois d'Arcy) column previously saturated with another sample (elution conditions: void volume 30 ml, total volume 80 ml, flow rate 40 ml/h, elution 4-ml fractions, temp. 4°). The separated fractions were kept at 4° in the dark and used within 2 days for UV/VIS and CD studies.

The concentration of the phospholipid in vesicles was measured by phosphorus determination [38]. The concentration of carotenoids in vesicles was measured as follows: to 4 ml of vesicle suspension was added 4 ml of $CHCl_3$; after thorough mixing and centrifugation, the org. phase was dried with anh. Na_2SO_4, and the UV/VIS absorbance was measured.

UV/VIS Absorption and Circular Dichroism Studies. UV/VIS spectra were recorded using the option-peak detection with a scan speed of 10 nm/min. The sample was thermostated to ±0.1° and kept at least for 15 min at the desired temp. for equilibration when above T_c and for *ca.* 1 h when below T_c. A series of spectra at several temp. were taken first in cooling runs followed by heating runs. Solid UV/VIS spectra were recorded on KBr disks.

CD spectra were typically run on solns. *ca.* $8 \cdot 10^{-4}$M in phospholipid (we have checked that even solns. $1.5 \cdot 10^{-3}$M in phospholipid alone give no CD signal) and 10^{-5}M in carotenoids. For each sample, a series of spectra

[1]) Extension of carotenoid-numbering; see the *Scheme*.

were measured first in cooling runs followed by heating runs at 0.1 nm/sec. For the previously known carotenoid **1**, the CD spectrum in solns. was comparable in the range 200–600 nm with the published ones [30].

Electron Microscopy (by Mrs. *M. Miehé*). *Carbon-Formvar*-coated grids were covered with a 0.1 mg/ml soln. of cytochrome C (*Sigma*, St Louis, MO) and blotted dry. A drop of vesicle suspension at a lipid concentration of $2 \cdot 10^{-3}$M was applied to the grid and drawn off with filter paper. A drop of 2% ammonium molybdate tetrahydrate, prefiltered through a 0.2 μm polycarbonate filter, was immediately applied to the grid, drawn off with a piece of filter paper, and allowed to dry. Preparations were examined at 80 kV.

Molecular Mechanics. The dimensions of molecules were determined by molecular mechanics with an *MPX 32* graphic computer using the *Sybyl* programme (*Tripos Associates, Inc.*, St Louis, MO).

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