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TRICYCLOHEXAPRENOL AND AN OCTAPRENEDIOL, TWO OF THE "PRIMITIVE" AMPHIPHILIC LIPIDS DO IMPROVE PHOSPHOLIPIDIC MEMBRANES

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Abstract - *Unilamellar vesicles were prepared from tricyclohexaprenol and dimyristoylphosphatidylcholine or a di-tertiary-octaprenediol and Halobacterium total polar lipids. Both tricyclohexaprenol and the octaprenediol were well incorporated in the membrane (up to 30mol% and 25mol% respectively). Effects of tricyclohexaprenol or the octaprenediol on the mechanical properties of the bilayer were investigated by the osmotic swelling method in a stopped-flow apparatus. These substances do not show a marked effect on the elasticity of the bilayer, but they do lower the water permeability through the membrane. Therefore, it was confirmed, at least in model systems, that these postulated phylogenetic precursors of cholesterol and carotenoids are membrane reinforcers.*

INTRODUCTION

In 1979, as part of a general theory of the molecular evolution of biomembrane constituents, we have postulated that some carotenoids might play the role of membrane reinforcers in bacteria by stabilizing both halves of the bilayer like a transmembrane "rivet".[1]

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* Dedicated to Professor Yu Wang on the occasion of his 80th birthday.

We have developped methodologies for the determination of the topology of α,ω-dihydroxylated carotenoids incorporated in vesicles,[2,3] and for the evaluation of reinforcement imparted by these carotenoids to phospholipid bilayers.[3,4] And finally, by using these methods, we have recently shown that bacterioruberins, the major carotenoids of *Halobacterium,* do reinforce the bilayer made of *Halobacterium* lipids.[5] Therefore, we have confirmed, at least in a system related to natural archaebacterial membranes, the functional equivalence of dipolar carotenoids with cholesterol.

We are now interested in studying the effects of other polyterpenoids postulated to be steps in a phylogenetic tree [6] (Fig. 1) on the mechanical properties of the bilayer in order to answer the following question : could the postulated phylogenetic precursors of cholesterol and polar carotenoids play the same role of membrane reinforcer ? This had already been demonstrated for hopanoids.[7,8]

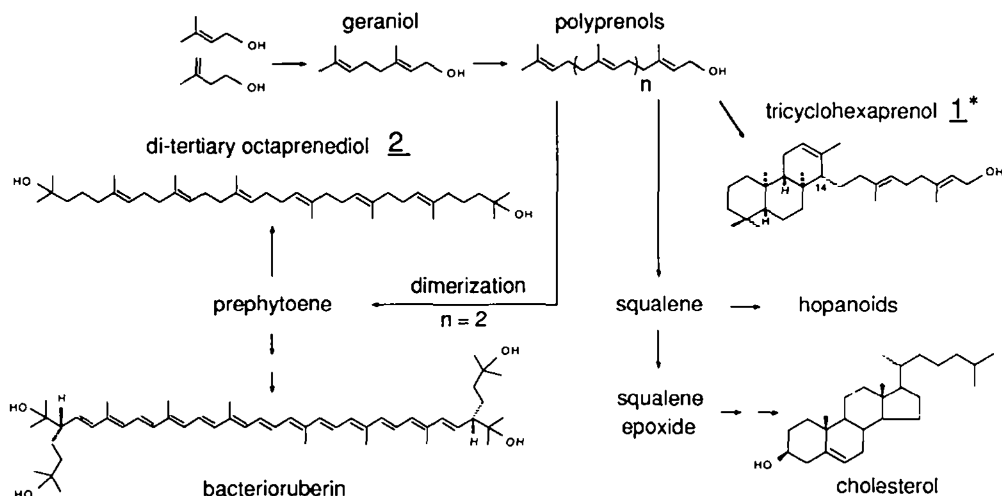

Fig. 1. Hypothetical Phylogenetic Tree of Several Cholesterol Surrogates.

* This is the stereoisomer studied. The "natural" substance, still unknown, may have a different stereochemistry at C-14 and in the side-chain.

Among a variety of candidates (Fig. 1), we first chose two substances : tricyclohexaprenol **1** [8-(12-isocopalen-15-yl)-3,7-dimethyl-(2E,6E)-2,6-octadienol] and the di-tertiary octaprenediol **2** [6E,10E,14E,18E,22E,26E]-2,6,10,14,19,23,27,31- octamethyl - 6,10,14,18,22,26 - dotriacontahexaene-2,31-diol]. Tricyclohexaprenol could be a surrogate of cholesterol with its molecular dimensions, amphiphilic nature and partial rigidity (Fig. 2),[6] and could be a product of a simple, even of an abiotic, e.g. clay-catalyzed cyclization of hexaprenol (Fig. 1). The structural differences between tricyclohexaprenol and cholesterol are that : (i) the OH group in the former is attached to the flexible part of the molecule whereas that in the latter is attached to the rigid part, (ii) the volume of the rigid part in the former is smaller than that of cholesterol.The octaprenediol **2** could be an α,ω-dihydroxylated

carotenoid surrogate with its molecular dimensions and amphiphilic nature (Fig. 2), and could be the product of an acid-catalyzed hydration of prephytoene, the tail-to-tail dimerization product of geranylgeraniol (Fig. 1). However, octaprenediol has not a strongly rigid structure, contrary to carotenoids. How much rigidity in an inserted molecule is necessary to modify the mechanical properties of the bilayer ? Did the biosynthesis of polyterpenoids evolve towards increasing the rigidity of the molecule ?

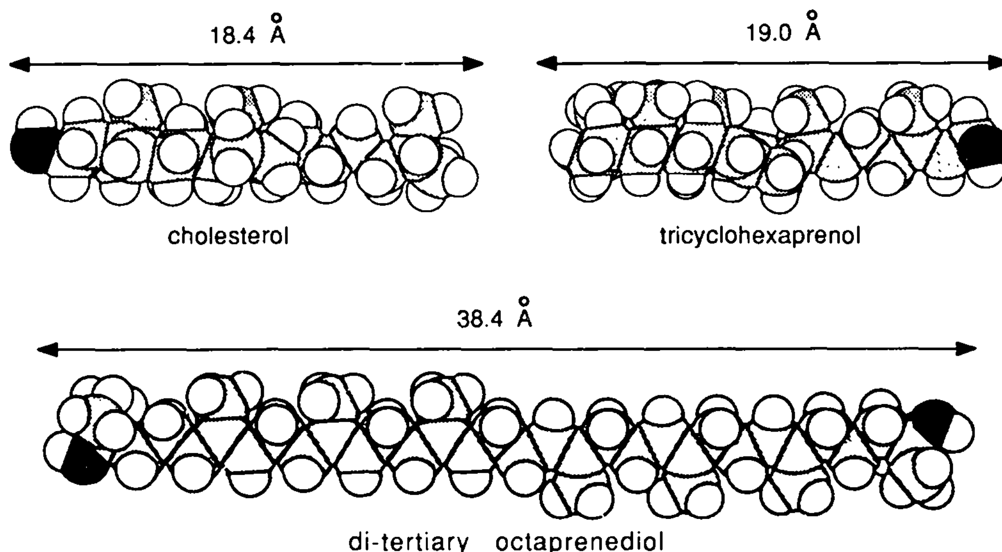

Fig. 2. Molecular Shape of the Molecules.

Both octaprenediol and tricyclohexaprenol are still unknown in living organisms. Thus, the choice of the phospholipids with which one associates them for physico-chemical studies is largely arbitrary. We decided to choose *Halobacterium* lipids as a model system for the study of octaprenediol, by analogy to the case of bacterioruberins. DMPC had been previously used to study other structural equivalences, so we used it once again in order to compare the effects of tricyclohexaprenol with compounds already studied.[4]

EXPERIMENTAL

Materials

Tricyclohexaprenol was prepared as described elsewhere,[9] and was also a gift of Prof. E.J. Corey.[10] The octaprenediol was prepared as described elsewhere.[11] Dimyristoylphosphatidylcholine (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, DMPC) was from Avanti Polar Lipids Inc. (Birmingham) and diphytanylphosphatidylcholine (2,3-di-O-phytanyl-*sn*-glycero-1-phosphocholine, DPhPC) was obtained by the procedure described previously.[3] The total polar lipids of *Halobacterium* were obtained from lipid extracts of either *Halobacterium cutirubrum* or *Halobacterium halobium*, as described in ref. 12 and 13. These micro-

organisms were shown to have approximately the same lipid composition [14] and our lipid samples presented identical ^{1}H and ^{13}C NMR spectra. The lipids were kept at -20°C in chloroform solution in sealed tubes. Their purity was checked by TLC on F_{254} silica gel plates (0.25mm thick from Merck, Darmstadt; eluent : chloroform/methanol/conc. ammonia, 65/25/4, v/v).

Molecular mechanics : The molecules were built and their length was determined (Fig. 2) using the Sybyl software (Tripos Associates Inc., St. Louis) . Atomic coordinates were created, and used by the MolDraw program [15] to generate the space-filling models of Fig. 2.

Preparation of vesicles

Large unilamellar vesicles were prepared either by the ether injection method as described in ref. 4 or by the reverse-phase evaporation method as described in ref. 3 with the following modifications :

Ether injection : an ether/ethanol solution (4/1, v/v, unless otherwise stated) of DMPC and tricyclohexaprenol (5-15mg lipid/ml), or an ether/ethanol/ tetrahydrofuran solution (8/1/1, v/v) of lipids and octaprenediol, was injected (injection rate 0.2ml/min) into 5ml of buffer (350mM NaCl/1mM EDTA-Na_2/5mM NaN_3/10mM Tris-HCl, pH 8, "Ultrapure" water from Millipore) maintained at 60°C.

Reverse-phase evaporation : the phospholipid and the additive were dissolved in 0.1ml of ethanol and 0.5ml of ether (20μmol lipid/ml); 0.2ml of the aqueous buffer was added and the mixture was sonicated in a Bransonic 221 bath for 5min at 30°C. The organic solvents were then slowly removed from the emulsion in a rotatory evaporator at 30°C. The resulting gel was broken up by vortex-mixing, and evaporated with a water pump. The resulting opalescent solution was diluted with 4.8ml of buffer.

Purification of vesicles

The vesicle suspensions were dialyzed twice against 800ml of buffer to remove the remaining organic solvent (dialysis tubing Spectrapor 2, Spectrum Medical Industries, Los Angeles). The samples were then filtered through polycarbonate filters (Nucleopore, DMF, France) ; 0.8μm, 0.4μm and 0.2μm filters , twice through each. A further step of purification was achieved by concentrating the vesicles (from 10ml to 2ml) in a ultrafiltration cell (Amicon, Paris) on a 0.08μm polycarbonate filter, thus separating vesicles from possible smaller particles.

Gel filtration was performed on a Sepharose 4B CL column (Pharmacia France SA, Bois d'Arcy) previously saturated with another sample of the same lipid composition (elution conditions : total column volume 90ml, flow rate 40ml/h, temperature 4°C).

The size of the vesicles (dissymmetry $Z = I(45°)/I(135°)$) was determined by light scattering with a photogoniodiffusiometer Fica 4200 equipped with a 5 mW He-Ne laser vertically polarized (SA Optilas, France). The measurements were done at 25°C for the octaprenediol-*Halobacterium* lipids vesicles (which show no phase transition above 0°C) and at 33°C for the tricyclohexaprenol-DMPC vesicles (i.e. above the DMPC phase transition of 23°C), just as for the stopped-flow experiments.

The concentration of each constituent was determined before and after the purification steps as indicated below.

Composition of vesicles

Concentration of phospholipids : it was measured by phosphorus determination.[16] In the case of the mixed polar heads of *Halobacterium* lipids, the lipid concentration was related to the phosphorus concentration by using the known lipid composition : glycolipid sulfate 22%, phosphatidylglycerophosphate 70%, phosphadidylglycerosulfate 4%, phosphatidylglycerol 4%.[13]

Concentration of tricyclohexaprenol : it was measured by gas chromatography on a Girdel 3000 F1 apparatus (Delsi, France) equipped with a Ross injector, using a fused silica capillary column coated with OV-1701 (Spiral, France; inner diameter 0.32mm ; film thickness 0.2μm ; length 25m) ; column temperature : 200 to 285°C, 4°C/min ; carrier gas : helium ; flame ionization detector. The compound was first silylated with N-methyl N-trimethylsilyltrifluoroacetamide (MSTFA, Pierce Inc, USA) ; MSTFA/THF (anhydrous) 2:1, v/v ; 15μl/μg of compound ; 1h at room temperature. A standard curve was established from 0.05μg to 0.3μg of tricyclohexaprenol (retention time : 28.3min). Another standard curve was established from 0.02μg to 0.4μg of trimethylsilylcholesterol (retention time : 22.5min). It had a slope slightly higher than that of tricyclohexaprenol (ratio : 1.4).

The samples were prepared as follows : an aliquot of the vesicle suspension was extracted 3 times with 1ml of ether ; the organic phase was dried with anhydrous Na_2SO_4, then the ether was evaporated and cholesterol (5mg) was added as internal reference. The sample was dried for a few hours over P_2O_5 under vacuum, then 50μl of silylating agent was added under argon. Further extracts of the vesicles aliquot did not contain tricyclohexaprenol. The method led to very reproducible results, provided that the samples were carefully dried before silylation.Furthermore, the chromatograms showed no degradation of the compound after the experiment.

Concentration of the octaprenediol : it was determined by ^1H NMR from the ratio of the peak area of the octaprenediol allylic methylenes at 2.02ppm (20H) and the peak area of a known amount of tetramethylsilane as external reference (sealed glass insert put into the NMR tube). Spectra were acquired on a Bruker AM 400 FT spectrometer, operating at 400.13 MHz with a pulse angle of 30°, at room temperature. Typical conditions were a spectral width of 4.4 kHz using 32K data points. An excellent signal-to-noise ratio was achieved after averaging 514 transients. Solutions of the octaprenediol (from 2mM to 30mM) and DPhPC (20mM) were prepared in 0.3ml of $CDCl_3$ and a standard curve was obtained. It was shown that the DPhPC concentration (up to 230mM) did not influence the assay.

The octaprenediol concentration in the vesicle samples was determined after extraction with chloroform. The NMR sample contained both the octaprenediol and phospholipids in approximately the same amount as that employed in the calibration curve.

Electron microscopy : negative staining

Carbon-Formvar-coated grids were covered with a 0.1mg/ml solution of bacitracin (Sigma, St Louis, USA) and blotted dry. A drop of the vesicle suspension, at a lipid concentration of 2mg/ml, was applied to the grid and drawn off with filter paper. A drop of a 2% ammonium molybdate solution was added for 1min, drawn off with filter paper and allowed to dry. All the solutions were pre-filtered through a 0.2µm polycarbonate filter. Preparations were examined with a Philips EM 420 electron microscope.

Stopped-flow measurements

The osmotic swelling experiments were performed on a Durrum-Gibson stopped-flow spectrophotometer, modified to measure the light scattering intensity at 90°.[4] Before each experiment, the photomultiplier voltage was adjusted to get 200 mV output voltage with toluene. The scattering intensity with vesicle suspensions was typically between 2 and 5 V (lipid concentration about 10^{-4}M). The solutions were kept at 40°C, degased under vacuum, and filtered through 0.4µm polycarbonate filters, to limit noise due to dust and bubbles.Then they were equilibrated at the temperature indicated in Table III for 15 min before the osmotic shock experiment.

RESULTS AND DISCUSSION

Formation of vesicles

In a previous study,[5] it has been shown that, in the presence of bacterioruberins, major carotenoids of *Halobacterium* membranes, DPhPC gives a poor yield of vesicle formation (10 to 25% yield depending on the method) whereas the *Halobacterium* total polar lipids give a 70 to 80% yield. This was confirmed by the present experiments as mixtures of DPhPC and the octaprenediol led to a yield of vesicles (either by the ether injection method or by the reverse phase evaporation method) of less than 10% after polycarbonate filtration. Such a low yield made further studies meaningless. On the contrary, the yield with *Halobacterium* total polar lipids was high (between 80 and 90% after polycarbonate filtration). This may be explained by the shape of the lipid molecules : according to the cone shape model proposed by Israelachvili et al.,[17] the large hydrophobic volume of the DPhPC branched chains would not favour the formation of a lipid bilayer, but rather a hexagonal H_{II} phase. The lipids of *Halobacterium* are composed of phytanyl chains with different anionic polar heads : phosphatidylglycerol, phosphatidylglycerophosphate, phosphatidylglycerosulfate and glycolipid sulfate. These large head-groups give the molecules a more cylindrical shape and thus would favour the formation of bilayers.

In the case of tricyclohexaprenol-DMPC vesicles, the yield of vesicle formation was high (70-80%), as expected for this phospholipid (the missing 20-30% are due to losses during the purification steps).The vesicle formation was confirmed by electron microscopy, both for tricyclohexaprenol-DMPC and for octaprenediol-*Halobacterium* lipids vesicles.

Solubility of tricyclohexaprenol and of the octaprenediol in the vesicles

The solubility of these additives in the vesicles is expressed as a molar percentage (i.e. 100 x [concentration of additive] / [concentration of additive + concentration of lipid)]. Table I shows the incorporation ratio of tricyclohexaprenol in DMPC vesicles. The first ether injection was carried out with an ether-ethanol solution 3:7 (v/v) ; tricyclohexaprenol was not completely soluble in this mixture, as shown by the concentration measured before polycarbonate filtration (14% instead of the initial 30%). The fact that this ratio was not modified by the polycarbonate filtration indicates that the product was fully incorporated in the vesicles. The following experiments were run using an ether-ethanol solution 4:1 (v/v) which totally solubilized the starting materials. The final ratio of tricyclohexaprenol measured was always equal to about 30mol%, even when the vesicles were prepared with equimolar mixtures of DMPC and tricyclohexaprenol. A gel filtration step did not modify significantly this incorporation ratio (26% of final incorporation).

Table I. Incorporation Ratio of Tricyclohexaprenol in DMPC Vesicles

Composition before vesicle preparation	Vesicle preparation method	Incorporation mol% of tricyclohexaprenol	
		Before polycarbonate filtration	After polycarbonate filtration
DMPC + 30mol% tricyclohexaprenol	ether injection a)	14	14
	ether injection b)	31	29 c)
	ether injection b)	35	32
	ether injection b)	31	30
	reverse phase	-	33
	ether injection b)	-	35 d)
DMPC + 50mol% tricyclohexaprenol	ether injection b)	47	31
	ether injection b)	49	34

a) this injection was performed with an ether/ethanol solution (3:7, v/v) ; see text.
b) the injection was performed with an ether/ethanol solution (4:1, v/v).
c) this sample was filtered on a Sepharose 4B CL column, leading to an incorporation ratio of 26%.
d) this sample was concentrated on a 0.08μm filter to exclude the small particles ; this process did not modify the final composition of the vesicles.

Another attempt to purify the vesicles was to concentrate them in an Amicon stirred-cell on a 0.08μm polycarbonate filter, in order to separate from the vesicles the small particles that could be present in the sample after polycarbonate filtration. Indeed, it was checked that the filtrate contained small-size particles (Z=2.5 whereas for the vesicles, Z=6.5), the composition of which was identical with the vesicle composition (35% of

tricyclohexaprenol) ; they represented less than 1% of the amount of vesicles. Therefore, the sample did not contain significant amounts of small particles (micelles or small aggregates) of different lipid composition but was a homogeneous vesicle preparation.

From all these results, it appears that the solubility of tricyclohexaprenol in DMPC vesicles at 4°C (temperature of dialysis) is about 30-35%, i.e. 1 molecule of tricyclohexaprenol for 2 molecules of DMPC.

Table II shows that, after polycarbonate filtration, the incorporation ratio of the octaprenediol is about 18% for an initial ratio of 25%, and about 25% for an initial ratio of 30%. Such an incorporation ratio is remarkably high considering that one molecule of octaprenediol is "equivalent" to two molecules of cholesterol, and it differs strongly from what had been observed for carotenoids (15% for astaxanthin, 8.5% for zeaxanthin in DMPC vesicles [2] and 9% for bacterioruberins in *Halobacterium* lipids vesicles [5]). Several attempts were made to measure the octaprenediol concentration in vesicles after gel filtration on a Sepharose 4B CL column. They did not lead to reproducible results.

Table II. Incorporation Ratio of the Octaprenediol in *Halobacterium* Lipids Vesicles

Composition before vesicle preparation	Vesicle preparation method	Incorporation mol% of octaprenediol after polycarbonate filtration
Halobacterium cutirubrum	ether injection	17
total polar lipids	ether injection	19
+ 25mol% octaprenediol	ether injection	18
Halobacterium halobium		
total polar lipids	ether injection	25
+ 30mol% octaprenediol		

Influence of tricyclohexaprenol and of the octaprenediol on the mechanical properties and water permeability of the vesicles.

In a previous study,[4] we have described a stopped-flow light scattering method that allows us to evaluate both the mechanical properties of vesicles and their permeability to water. We have shown that a rapid mixing of vesicles prepared in a concentrated salt solution (350mM NaCl) with a diluted buffer (50mM NaCl) leads to an exponential decrease in scattered light corresponding to the inflow of water into vesicles and to their swelling.
On the one hand, it has been shown that the half-time $t_{1/2}$ is the same whether it is measured by the osmotic shock method or by the Lawaczeck D_2O diffusion one.[18] This means that the water permeability through the membrane is the limiting factor of the kinetics. On the other hand, an empirical relationship has been found between the relative light scattering

intensity change ($\Delta I/Io$) and the size of the vesicles [as estimated by Z, the dissymmetry measured by light scattering] : $-\Delta I/Io$ is proportional to (Z-1). The expression $-\Delta I/(Io.(Z-1))$ does not depend on the vesicle size and can thus be taken as a measure of membrane elasticity.

Table III represents the results obtained for the two systems studied : *Halobacterium* total polar lipids + 25mol% octaprenediol, and DMPC + 30mol% tricyclohexaprenol.

Table III. Elasticity ($-\Delta I/Io(Z-1)$) and Water Permeability ($t_{1/2}$) of Vesicles of Different Composition and Dissymmetry (Z)

Composition	Temp. (°C)	Z	$-\Delta I/Io$ (%)	$-\Delta I/Io$ (Z-1) (%)	$t_{1/2}$ (ms)
Halobacterium cutirubrum total polar lipids [a]	25	-	-	2.9	50
Halobacterium cutirubrum total polar lipids + 9mol% bacterioruberins [a]	25	-	-	1.8	110
Halobacterium cutirubrum total polar lipids + 25mol% octaprenediol	25	5.6	9.5	2.1	80 [c]
	25	6.4	12	2.2	80
	25	6.6	16	2.9	80 [c]
	25	7.7	17	2.5	80
DMPC [b]	33	3.7	10	3.7	20
DMPC + 30mol% cholesterol [b]	33	4.2	5.5	1.7	70
	33	7	10	1.7	90
	33	9	12	1.4	115
	33	15	20	1.4	260
DMPC + 30mol% tricyclohexaprenol	33	4.7	13	3.5	55
	33	7	15	2.6	80
	33	10	16	1.8	110

a) taken from ref. 5.
b) taken from ref. 4.
c) $t_{1/2}$ was obtained by both osmotic shock and D_2O methods ; the values of both methods were the same within the experimental errors.

Halobacterium total polar lipids + 25mol% octaprenediol : The presence of the octaprenediol in the *Halobacterium* lipid vesicles decreases their water permeability, as $t_{1/2}$ rises from (50±10)ms for the lipids alone to (80±10)ms. The value of -ΔI/Io(Z-1) is more difficult to interpret : it is (2.5 ± 0.4)%, which is not markedly different from what was observed for the *Halobacterium* lipids vesicles (2.9 ± 0.2)%.[5] Thus, it appears that the octaprenediol may play a reinforcing role in a membrane, as it lowers the water permeability of the *Halobacterium* lipids bilayer ; however, although the compound is well incorporated in the lipids studied, the effects measured are relatively weak, and in particular the mechanical properties of the lipid bilayer are not significantly modified.

This can be compared with what had been observed when bacterioruberins were incorporated in *Halobacterium* lipids : 9mol% of bacterioruberins in vesicles lead to -ΔI/Io (Z-1)= (1.8 ± 0.2)% and $t_{1/2}$=110ms.[5] These values clearly indicate that the bacterial carotenoids enhance the rigidity and reduce the water permeability of the membrane more strongly than the octaprenediol does.This can be explained by the structure differences between the molecules : bacterioruberins are rigid molecules, whereas the octaprenediol is more flexible, at least intrinsically; we should however remember that insertion into the membrane should be making it taut. Also, its heads are less polar (*tert*-hydroxyl). In a previous study on DMPC vesicles, it has been shown that hexadecanol has no effect at all on the membrane properties measured by the osmotic shock method;[8] thus, it appears that this molecule, which is amphiphilic and has approximately the same length as cholesterol, cannot play any reinforcing role in the lipid bilayer because of its flexibility. We must however note that, when we had run the experiment with hexadecanol, we had not yet been aware of the necessity of measuring the incorporation ratio, which might have been low.

The octaprenediol represents an intermediate case between a rigid and a completely flexible molecule, which explains that it weakly reinforces the membrane.

DMPC + 30mol% tricyclohexaprenol : Tricyclohexaprenol decreases the water permeability of DMPC membranes : $t_{1/2}$=55ms (Z=4.7) instead of $t_{1/2}$=20ms (Z=3.7) for vesicles made of DMPC alone.[4] Different sizes of DMPC-tricyclohexaprenol vesicles were obtained, depending on the lipid concentration of the ether/ethanol solution injected during the preparation. As it has been already observed in a previous study,[4] the kinetics of swelling is affected by vesicle size and $t_{1/2}$ increases with increasing Z. It must be emphasized that tricyclohexaprenol lowers the water permeability of the DMPC membrane almost as much as cholesterol does (Table III).

However, the relative change in the scattered light measured for each preparation of DMPC-tricyclohexaprenol vesicles is not linear with respect to (Z-1), which differs from our previous studies. Thus, we can compare -ΔI/Io for various membrane compositions only for vesicles having about the same size.

It appears that tricyclohexaprenol does not significantly modify the strength of the DMPC bilayer, unlike cholesterol which markedly decreases -ΔI/Io(Z-1). These results show that the effect of tricyclohexaprenol on membranes is limited, in the case of DMPC vesicles, to a decrease of the water permeability.

In conclusion, the experimental results are those expected on the basis of our hypothesis of the biochemical evolution of polyterpenoids.[1,6] It has been shown that the octaprenediol and tricyclohexaprenol, which are still unknown in living organisms, but are likely phylogenetic precursors of carotenoids and of hopanoids and sterols, can be incorporated up to high concentrations into lipid bilayers. They lower the water permeability of phospholipid membranes, but do not affect markedly their rigidity. In that respect, they appear to be intermediate membrane effectors. Evolution may then have selected molecules having a more complete effect on membranes. These results will be followed by the study of the other phylogenetic putative precursors, such as the other octaprenediols.[11]

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