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A DIRECT STUDY OF THE COHESION OF LECITHIN BILAYERS: THE EFFECT OF HOPANOIDS AND α,ω-DIHYDROXYCAROTENOIDS

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The swelling of phospholipid unilamellar vesicles under an osmotic shock can be followed by stopped-flow transmittance measurements, which make it possible to evaluate "directly" the mechanical properties of the membrane. The double-layer membrane is reinforced by rigid "pegs" (cholesterol, hopanoids), or rigid "rivets" (distally hydrophilic carotenoids).

Cholesterol 1 is an universal constituent of eukaryotic biomembranes, in which it improves the mechanical properties. Though a variety of physico-chemical techniques have been used to study the phospholipid bilayers (1), most of them are inadequate to evaluate the mechanical effect of cholesterol. A "direct" confirmation of this effect on the oriented lecithin monolayers is available by isothermal or isobaric surface tension measurements (2). This method has also been used (3) to demonstrate the similar effect of one type of hopanoids, which we had postulated to be surrogates, as well as phylogenetic precursors, of cholesterol in many prokaryotes (4). Fig. 1A shows schematically how sterols and hopanoids play the role of an oriented rigid "peg", which condenses the fluid mosaic structure by cooperative van der Waals forces (5). The other major type of biomembrane reinforcement, which we had postulated to occur in prokaryotes, implies rigid "rivets" instead of "pegs" (Fig. 1B). This is the role tentatively assigned (4) to the α,ω-dihydroxylated C_{40}-C_{50} carotenoids frequently found in bacteria (6). This postulate is so far supported only by indirect experiments (7). The surface tension method mentioned above can obviously be applied only to single-layered assemblies, and no analogous method is available for bilayers.

We describe here the principle of a "direct" method for the study of the elastic properties of large unilamellar vesicles, the size of which is made to

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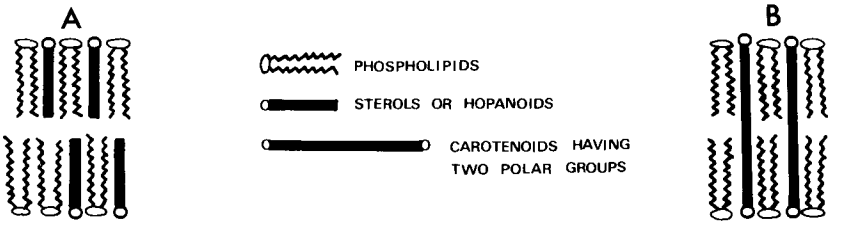


Fig. 1 Hypothetical Reinforcement Mechanism of Lipid Bilayers in Membranes.

change by osmotic shock. It had been shown 15 years ago that multilamellar vesicles (liposomes) behave as almost perfect osmometers (8).

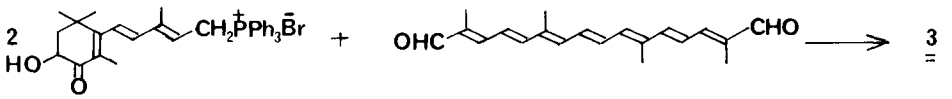
MATERIALS AND METHODS

Chemicals. DMPC[+] (Sigma, USA) was used as purchased. This chemical migrated as a single spot on silica plates with chloroform-methanol-water (65:25:4 V/V/V) as solvent. Cholesterol 1 (Sigma, USA) was recrystallized from ethanol. n-Hexadecanol 4 (Fluka, Switzerland) was used as purchased. Bacteriohopanetetrol 2 and androst-4-en-17β-ol 5 were gifts from Prof.M. Rohmer (Mulhouse) and Dr.St.A.Szpilfogel (N.V.Organon, Oss) respectively. α,ω-Dihydroxy C_{50} carotenoid 3 was prepared analogously to the synthesis of astaxanthin (9) by the condensation of Scheme 1. The starting materials were gifts from Hoffmann-La Roche (Basle).

Physico-chemical properties of 3 : mp 195-197°C; λ max ($CHCl_3$) : 530nm (ε 126,400), (cf. astaxanthin : λ_{max} ($CHCl_3$) : 488nm (ε 125,000) (10)); MS[+] : m/e 728 ($M^+ = C_{50}H_{64}O_4$, base peak); NMR ($CDCl_3$) :δ 1.22 (6H, s, 2 x CH_3) 1.34 (6H, s, 2 x CH_3), 2.00 (24H, s, (broad), 8 x CH_3-C=C-), 4.26 (2H, dd, J = 13Hz, J' = 6Hz, H-COH), 6.2-6.8ppm (20 H, m, -CH=C-); IR ($CHCl_3$) : 3480 (νOH), 1650 (ν C=O), 1600-1550 (ν C=C), 1385, 1366 (δ C-H CH_3 gem), 1076, 1030 (δ C-O), 960cm^{-1} (δC-H trans double-bond).

Vesicle preparation. Suspensions of large unilamellar vesicles of DMPC have been prepared in a 350mM LiCl solution by the method of Deamer and Bangham (11), with or without cholesterol or other additives at various concentrations.

Stopped-flow measurements. The transmittance change at 450 or 700nm wavelength was monitored in a 2cm cuvette of a Gibson-Durrum stopped-flow spectrophotometer at 33°C (above the phase transition temperature of DMPC). The photomultiplier signal was recorded on a Tektronix oscillograph, transferred via a special interface to an Olivetti ICU 600 computer with graphical display, and processed.



Scheme 1 Synthesis of the C_{50}-carotenoid.

RESULTS AND DISCUSSION

Rapid mixing (3ms) in a stopped-flow spectrophotometer (12) of the above suspensions of vesicles with an equal volume of a 50mM LiCl solution resulted in an increase of transmittance. This phenomenon was postulated to

[+] ABBREVIATIONS: DMPC : L-α-dimyristoylphosphatidylcholine ; MS : mass spectrometry.

be due to the swelling of the vesicles, which was confirmed by the measurement of the average diameter of the vesicles by the quasi-elastic light scattering method (13). With pure DMPC vesicles, the increase of transmittance was completed in approximately 1s (exponential region); with cholesterol or equivalents, the rate of swelling was slower (e.g. 2-5 s). Furthermore, the amplitude of the transmittance change (that is, the amplitude of the optical density change) is lowered by the presence of cholesterol. The effect of the various additives can be compared by the rate and amplitude of transmittance change. The "direct" linkage between the rate and amplitude of vesicle volume change and the mechanical properties of the double layer is intuitive (cf. the analogy with a rubber balloon), as well as theoretically grounded (14). We limit the present report to a description of the qualitative evaluation of amplitude changes.

In brief, our theory shows that the vesicle radius after an instantaneous osmotic shock changes exponentially with time (14).

$$R_t - R_e = \Delta R \{1 - \exp(-kt)\} \quad (1)$$

where R_e : initial equilibrium vesicle radius, R_t : vesicle radius at time t, k : rate constant of the phenomenon, ΔR : amplitude of vesicle radius change, which can be written as equation **(2)** , when R_e is sufficiently large.

$$\Delta R = 4 \pi R_e^2 \Pi / E \quad (2)$$

where Π : osmotic pressure difference across the skin, E : elastic stretching modulus of the vesicle skin.

On the other hand, equation **(3)** is derived easily from Debye theory (15).

$$D = 128 \pi^5 n_o^4 LN \alpha^2 / 6.9 \lambda^4 \quad (3)$$

where D : optical density, n_o : refractive index of the solution, L : length of cell, N : number of vesicles per unit volume, α : polarizability of the vesicle particle, λ :wavelength of light in the solution. Assuming that α^2 depends only on vesicle radius, we can lead the equation **(3)** to **(4)** .

$$D_t - D_o = \Delta D \{1 - \exp(-kt)\} \quad (4)$$

where D_o : initial optical density, D_t : optical density at time t, ΔD : amplitude of optical density change, which can be written as follows.

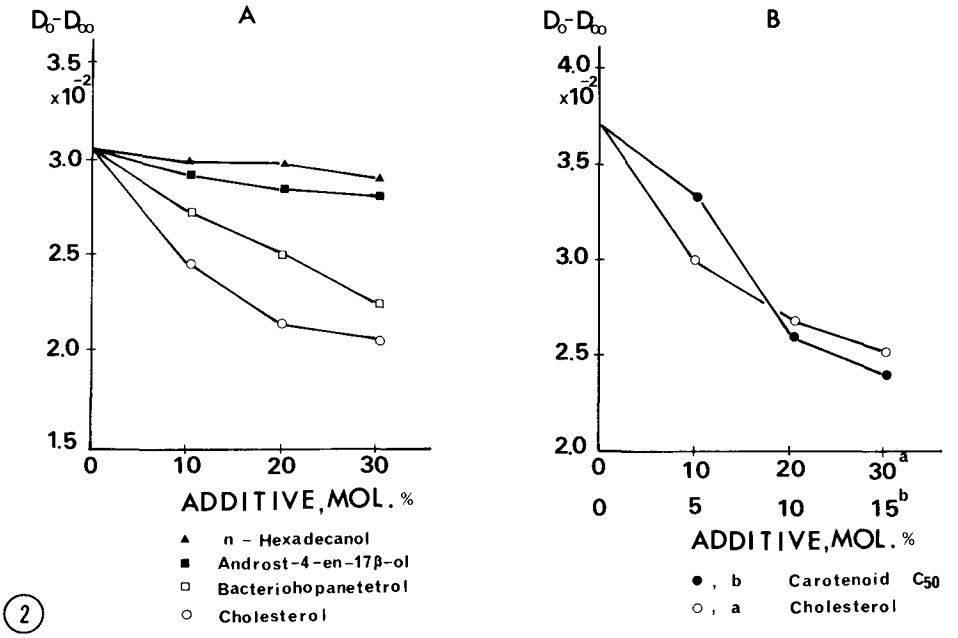
$$\Delta D = 128 \pi^5 n_o^4 LN (\partial \alpha^2 / \partial R)_{Re} \Delta R / 6.9 \lambda^4 \quad (5)$$

Assuming that the values of n_o, N and $(\partial \alpha^2 / \partial R)_{Re}$ are approximately the same in each experiment, the equations **(5)** and **(2)** can be combined as **(6)** .

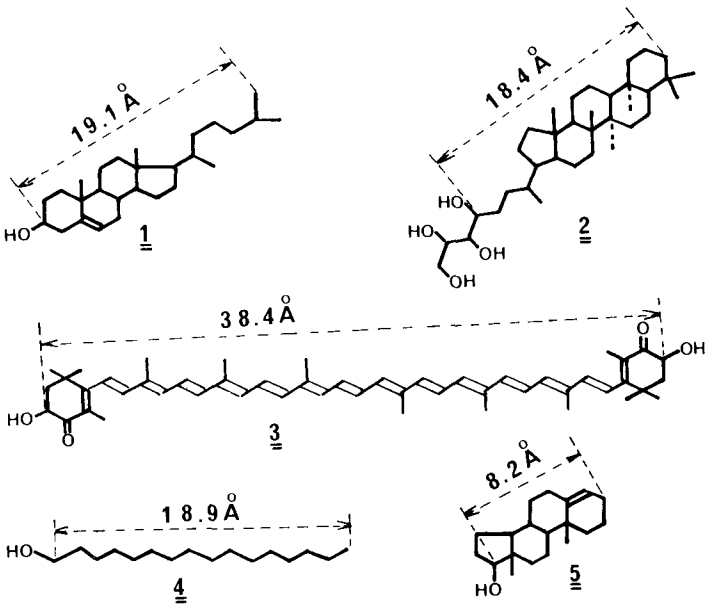
$$\Delta D = C \Delta R = C' R_e^2 / E \quad (6)$$

where $C = 128 \pi^5 n_o^4 LN (\partial \alpha^2 / \partial R)_{Re} / 6.9 \lambda^4$, $C' = 4\pi C\Pi$

Equation **(6)** shows that the amplitude of vesicle radius change (ΔR) is proportional to that of optical density change (ΔD) and that the measurement of transmittance change (i.e., optical density change, ΔD) can be linked to the elastic stretching modulus of the vesicle skin, E.



(2)



(3)

Fig. 2 (A) Amplitude of optical density change (D_0-D_∞) at 450nm as a function of molar concentration of additives, ▲ n-hexadecanol, ■ androst-4-en-17β-ol, □ bacteriohopanetetrol, ○ cholesterol; (B) Amplitude of optical density change (D_0-D_∞) at 700nm as a function of molar concentration of additives, ○ cholesterol, ● carotenoid 3, a : concentration of cholesterol, b : concentration of carotenoid 3

Fig. 3 Structure of Additives Used

Fig. 2 shows the effect of increasing concentrations of various additives on the amplitude of swelling. The more efficient is an additive (i.e., the more it increases the rigidity of the bilayer), the more marked is its effect to reduce the amplitude of swelling, i.e., $D_o - D_\infty$.

We can see that :

- a non-rigid additive like n-hexadecanol 4 has no measurable effect on the amplitude of swelling,
- a additive like androst-4-en-17 β-ol 5, rigid but not short, has no effect on the amplitude of swelling,
- cholesterol 1 has the expected marked effect to rigidify bilayer,
- bacteriohopanetetrol 2 has a similar effect, though not quite as large,
- the synthetic α,ω -dihydroxylated carotenoid 3 mimics the effect of cholesterol, of course at half the molar concentration.

All these results conform to the expectations deduced by the study of the interactions DMPC/additives with space-filling models.

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