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Effects of hexagonal phase induction by dolichol on phospholipid membrane permeability and morphology

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Abstract

The effect of H_{II}-phase induction by dolichol (DOL) (C_{80-105}, 0–2 wt%) within the bilayers of multilamellar liposomes (MLV, DOPE:DOPC 2:1 or 3:1 w/w) on their permeability, lipid mixing and morphology was determined. Low-angle X-ray diffraction patterns were consistent with mixtures of bilayer and H_{II} phases, the latter increasing with increasing DOL or DOPE content and temperature. Efflux rate constants for 6-carboxyfluorescein (6-CF) from 2:1 DOPE:DOPC vesicles depended on temperature and DOPE/DOL content, increasing as much as 200-fold over DOL-free controls at 2% w/w DOL. Fluorescence resonance energy transfer assays detected lipid mixing with unlabeled target MLV. It was appreciable only when target MLV contained DOPE and increased with DOL content. Confocal scanning fluorescence microscopy was applied to study the morphological structure of fully-hydrated samples and field scanning electron microscopy the ultrastructure of cryo-stabilized samples. 3:1 DOPE/DOPC MLV, stable at pH 9.5, underwent rapid morphological changes at pH 7.4. Within minutes filaments formed and large areas of membrane surface became studded with 10–15 nm bumps and 5 nm holes, resembling in size and shape unilamellarly covered interlamellar micellar intermediates and interlamellar attachments (ILA) previously associated with H_{II}-phase transitions. The filaments, seen in MLV with and without DOL, may represent extensions of IMI into coaxial assemblages of rod micellar intermediates (RMI). These phenomena may have implications for liposomal delivery of therapeutic peptides/proteins if they can be made to trigger the convective release of liposomal contents via controlled formation of ILA between adjacent lamellae of MLV.

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Abbreviation: 6-CF, 6-carboxyfluorescein; CSFM, confocal scanning fluorescence microscopy; DOL, dolichol; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; FSEM, field-emission scanning electron microscopy; H_{II}, inverted-hexagonal; IMI, interlamellar micellar intermediate; ILA, interlamellar attachment; L_{α}, lamellar liquid crystalline phase; MLV, multilamellar vesicle; RMI, rod micellar intermediate; SEM, scanning electron microscopy; TCH, thiocarbohydrazide; TEM, transmission electron microscopy.

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1. Introduction

Drug transport across biological membranes and control over its rate and selectivity remains a challenging area of research. Optimizing rates of extravascular input, modifying distribution to tissues such as the brain and directing intracellularization of drugs are but a few examples of processes dependent on such transport.

Some membrane phospholipids (e.g., phosphatidylcholine (PC)) are 'bilayer-forming' whereas others, such as unsaturated forms of phosphatidylethanolamine (PE), are more stable in the inverted hexagonal (H_{II}) phase (Fig. 1). Gruner (1985) suggested that biological membranes may control their lipid composition homeostatically so as to remain 'perched on a critical edge of bilayer stability' with respect to bilayer-hexagonal (L_α-H_{II}) phase transitions. It has also been speculated that formation of membrane 'defects' such as local regions of perturbed phase behavior, might play an important role in dynamic cellular processes ranging from endocytosis and intracellular transport to membrane fusion (Cullis et al., 1980; Curatolo, 1987; Epand, 1989; Seddon, 1990).

A group of long-chain polyisoprenoids (hereafter referred to as 'dolichol' (DOL)) occur naturally in the membranes of nearly all eucaryotic cells as well as in the human systemic circulation (Chojnacki and Dallner, 1988). Their large size and hydrophobicity accounts for their ability to exert considerable influence on the packing and phase behavior of phospholipids by, for example, inducing formation of the H_{II} phase (Gruner, 1985; Valtersson et al., 1985; Chojnacki and Dallner, 1988). It seems reasonable to assume that drug transfer rates across membranes might be altered by DOL-induced phase changes. In the present study, the hypothesis of DOL-induced alterations in drug flux across lipid bilayers secondary to changes in phase behavior is verified by kinetic, X-ray diffraction and microscopic measurements. Pure phospholipid multilamellar vesicles (MLV) were used since they provide closely apposed bilayers and can be viewed as both 'model membranes' and drug delivery vehicles. Effects of H_{II}-phase induction by DOL on their morphology, lipid-mixing and permeability characteristics were examined. The results have important implications for the design of vesicular drug delivery systems and also provide valuable information on the modulation of drug transport rates across biological membranes.

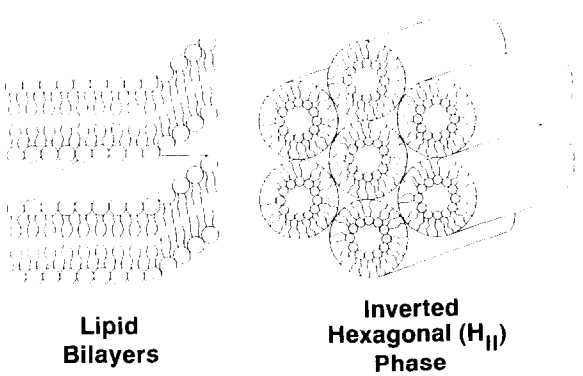


Fig. 1. Lamellar liquid crystalline (L_α) and inverted hexagonal (H_{II}) phase representations.

2. Materials and methods

2.1. Preparation / characterization of MLV

Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE) (Avanti Polar Lipids, Birmingham, AL), and porcine liver DOL (C_{80-105}, 98%, Sigma, St. Louis, MO) were used without further purification and stored desiccated in chloroform/methanol (4 : 1) at −20°C. Phospholipids were evaluated for purity by thin-layer chromatography using chloroform : methanol : 20% methylamine : water (60 : 36 : 20 : 0.3, v/v) as a one-dimensional solvent system. 6-Carboxyfluorescein (6-CF) ('isomer-free', Mol. Wt 376, $pK_a = 6$, Molecular Probes, Eugene, OR) served as a model hydrophilic anionic permeant.

MLV were prepared according to a previously published method (Szoka and Papahadjopoulous,

1980). Briefly, DOPE and DOPC (2:1 or 3:1, w/w), were mixed with various percentages of DOL (0–2 wt%) and dried under N_2 onto a clean glass surface. Vesicles formed spontaneously upon hydration of the resultant lipid film (generally 3 mg total lipid) with 10 mM Hepes buffer (500 μl, pH 9.5, 10°C) containing either 150 mM NaCl (control) or 100 mM 6-CF. MLV were then sonicated (two 5-min periods (240 W bath sonicator, Branson, Shelton, CT) separated by a 'cooldown' (5 min) between ultrasound periods), diluted with buffer at ambient temperature (about 23°C), and passed sequentially through 3 and 1 μm polycarbonate filters (Nucleopore, Pleasanton, CA). This produced a suspension consisting primarily of non-aggregated MLV less than 1 μm in diameter. Confocal scanning and scanning electron microscopies revealed a bimodal size distribution characterized by a few MLV of 1–5 μm diameter and a majority of smaller 0.5–1.0 μm vesicles. While there was evidence for the presence of small unilamellar vesicles (SUV) in the preparation, given the relatively small percentage of lipid in the SUV subpopulation, it is not anticipated that the SUV would affect data interpretation, since they would be expected neither to entrap significant amounts of 6-CF nor to participate in the cooperative defect formation induced by DOL.

Vesicles containing 2:1 DOPE/DOPC (with and without DOL) were used in permeability studies and also sized using quasi-elastic light scattering (Model BI-90 Particle Sizer, Brookhaven, Holtsville, NY). Equivalent sphere diameters (ESD) approximated 500 nm. Although ESD varied considerably among preparations (as expected from the preparation method used), there was no correlation between DOL content and size. The pooled mean ESD of those vesicles containing DOL (565 $\pm$ 225 nm) was almost identical to that of DOL-free controls (545 $\pm$ 60 nm). Whether or not DOL was present, a small fraction of lipid (generally $< 5\%$ by weight) appeared as smaller particles (ESD < 100 nm), presumably because no attempt was made to size to a lower limit cutoff.

Vesicle morphology was examined by both confocal laser scanning fluorescence microscopy (CSFM) and scanning electron microscopy (SEM). For CSFM vesicles containing DOPE/DOPC (2:1 or 3:1, pH 7.4, 37°C, 40 μg/ml total lipid + 1 mol% Rhod-PE) in the presence and absence of DOL were immobilized in low-melt Agarose (23°C in 2.5% Agarose (T_{GEL} = 26°C) and examined by a CSFM (argon-ion laser excitation = 514 nm, OG 550 nm barrier filter, Model MRC-600, BioRad, Melville, NY). Using the microscope's ability to optically section along the z-axis, a series of optical section images ('z-series analysis') was utilized to either examine vesicles in various cross-sectional planes or to reconstruct a three-dimensional image as described by Morgan et al. (1992).

For examination with SEM, vesicles were placed on 1000 mesh carbon film-coated nickel grids, fixed with osmium tetroxide/thiocarbohydrazide (TCH) (Peters and Pohl, 1992), and critical point dried using liquid Freon TF and CO_2 as intermediate and transitional fluids, respectively. The specific technique utilizes an ultra-high resolution imaging mode for field emission scanning electron microscopy (FSEM) including a chromium coating by high-energy sputtering. The microscope (a modified Jeol JSM-890 cryo-microscope) provides an electron probe of ≈ 0.5 nm and operates at useful magnification (optimal scan density) of 300 000–500 000 $\times$, with excellent performance at maximum instrumental magnification of 950 000 $\times$.

2.2. Lipid phase behavior

Low angle X-ray diffraction was used to detect (L_α-H_{II}) lipid phase transitions. Fully hydrated vesicle dispersions (10 mg lipid/ml in pH 7.4, isotonic 10 mM Hepes) were concentrated 10-fold by centrifugation and transferred to 1–70 mm quartz capillary tubes. Samples were then exposed to a line-focused monochromatic X-ray beam (CuK_α, λ = 1.54 Å from a GX-18 rotating anode X-ray generator (Enraf Nonius, Bohemia, NY) using a single Franks mirror, nickel filter, and vertical and horizontal limiting slits.

Diffraction data were recorded on either a Braun one-dimensional position-sensitive electronic X-ray detector (Innovative Technology, Inc., South Hamilton, MA) interfaced to a Mi-

croVax II (Digital Equipment Corp., Maynard, MA) or a DEF-5 film stack (Kodak, Rochester, NY). A similar data reduction (background and geometrical corrections) has been described previously (Herbette et al., 1985). Briefly, an exponential function was fit to the background and subtracted in the integration routine. Since the entire lamellar reflection for each observed intensity was collected by the detector, the lamellar intensity functions from the samples collected with the electronic detector were simply Lorentz-corrected by a factor of $s = 2 \sin \Theta/\lambda$.

Data were recorded on Kodak DEF-5 film (Eastman Kodak Co.) and qualitatively examined to determine the high-angle acyl-chain packing of the samples and to verify the low-angle detector data.

2.3. Permeability studies

Vesicle permeability was evaluated by monitoring the increase in fluorescence intensity once the previously self-quenched concentrations of 6-CF had been diluted by release into the external milieu. MLV composed of 2:1 DOPE/DOPC, various concentrations of DOL, and 100 mM encapsulated 6-CF were prepared as described above. DOL-mediated H_{II}-phase formation was inhibited by preparation and maintenance of the vesicles at pH 9.5 to ensure unprotonated DOPE headgroups and limit the tendency toward H_{II}-phase formation prior to the initiation of each kinetic experiment. Following removal of unentrapped 6-CF by gel filtration chromatography (1 × 13 cm column of Sephadex G-50, Pharmacia, Piscataway, NJ), kinetic experiments were initiated by suspending vesicles in sufficient buffer (isotonic 10 mM Hepes, pH 7.4), to ensure sink conditions and reduce pH to 7.4. Efflux of 6-CF was followed fluorimetrically at 37°C (Model SF-330 Spectrophotofluorimeter: excitation, 470 nm; emission, 520 nm; Varian, Sunnyvale, CA). Once 50% of the entrapped probe had been released, self-quenching conditions were no longer maintained within the vesicles. At that point, the experiment was terminated and the vesicles disrupted by the addition of 0.1% Triton X-100 to determine total fluorescence intensity.

A mathematical model describing the release of permeant from homogeneous spheres was found adequate to describe 6-CF release from 2:1 DOPE/DOPC MLV. It was descriptive of the data and provided a permeability parameter useful for comparing various treatments in spite of the obvious fact that the MLV were neither spherical nor homogeneous. A homogeneous concentration distribution across the bilayers at time zero, unidirectional (outward) flux of 6-CF, non-steady-state conditions across the bilayers with increasing time and sink conditions at the vesicular surface constituted were all taken as initial boundary conditions. The following mathematical expression adapted from Crank (1975) was fitted to the reciprocal-variance weighted permeant release vs time data using the nonlinear regression program PCNONLIN (Statistical Consultants Inc., Lexington, KY).

$$M(t)/M(\infty) = 1 - 6/\pi^2 \Sigma 1/n^2 \exp(-\pi^2 n^2 P t) \quad (1)$$

where $M(t)$ and $M(\infty)$ represent the mass of permeant released at times t and ∞, respectively. The apparent permeability coefficient P, expressed in units of min^{-1}, is a relative measure of permeability which takes into account the radius of the vesicle from which solute is diffusing [P = (diffusivity)(partition coefficient)/(vesicle radius)^2].

2.4. Lipid mixing studies

Assays for vesicle-vesicle fusion or lipid mixing were based on the fluorescence resonance energy transfer (FRET) assay (Struck et al., 1981; Hoekstra, 1982). It relies upon resonance energy transfer between the two fluorophores when the emission band of NBD-PE (the energy donor) overlaps the excitation band of Rhod-PE (the energy acceptor). MLV composed of DOPE/DOPC (2:1) and varying concentrations of DOL (0–2%) were prepared as described above except that the two phospholipid fluorophores (NBD-PE and Rhod-PE, 1 mol% each) were added to the original lipid mixture. The vesicles were then mixed with 'unlabeled' MLV in a 1:3 ratio (maximum

dilution factor, 'N', = 4) in isotonic 10 mM Hepes at 37°C, pH 7.4. Fluorescence intensity (excitation = 470 nm) was followed over a period of 30 min at 37°C and the enhanced fluorescence of the donor NBD-PE at 530 nm monitored as dilutional effects secondary to fusion or lipid mixing with unlabeled MLV reduced FRET.

3. Results and discussion

3.1. Lipid bilayer destabilization

Fig. 2A and B shows the X-ray diffraction patterns for MLV containing 3:1 and 2:1 DOPE/DOPC, respectively, in the presence of various concentrations of DOL at pH 7.4. In the absence of DOL a 2-order pattern was evident (Fig. 2A). Peak spacings were integer, consistent with scattering from a well-defined lattice (such as a phospholipid bilayer) having a 62 Å unit cell repeat distance. Pure DOPC (a bilayer-forming phospholipid) produced a similar pattern either alone or in the presence of DOL (0–4%, not shown).

In the presence of DOL, a second phase became evident giving rise to peak spacings in the ratio of (1, $\sqrt{3}$, and 2), consistent with a mixture

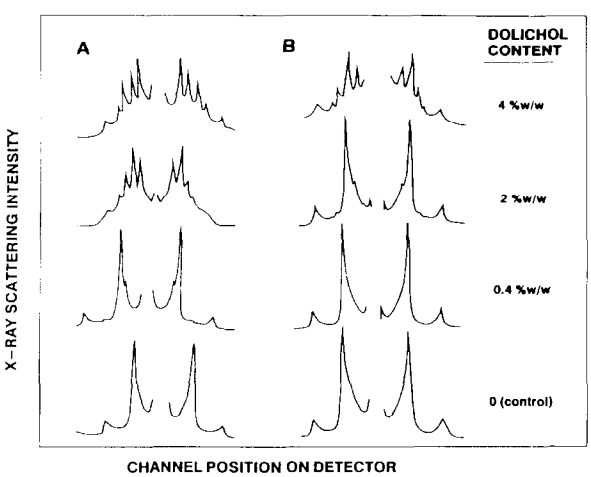


Fig. 2. Diffraction patterns for MLV containing 0–4% (w/w) DOL at 37°C for (a) 3:1 DOPE/DOPC; and (b) 2:1 DOPE/DOPC.

of lamellar and H_{II} phases. This was confirmed by observing identical 3-order spacings for pure DOPE, a nonbilayer-forming lipid known to exist in the H_{II} phase under physiological conditions (not shown). While the presence of mixed phases was clearly evident in the one-dimensional position-sensitive detector patterns at DOL concentrations as low as 0.4% in the 3:1 DOPE/DOPC system, 2% DOL was required before the H_{II} phase could be observed in the 2:1 DOPE/DOPC system (Fig. 2B). Thus, the propensity for H_{II}-phase formation increased with increases in either DOL or DOPE content.

Although Fig. 2B shows that the 2:1 DOPE/DOPC vesicles subsequently used in permeability studies showed little evidence of H_{II}-phase formation with the addition of < 2% DOL, this H_{II}-phase transition at 37°C was clearly evident on film exposures (two-dimensional images) for DOL concentrations as low as 0.4% (Fig. 3). A much higher detection sensitivity for phase transition was achieved using FSEM (see below).

The effects shown in Fig. 2 were also thermotropically inducible (Fig. 4). Increased temperature facilitated the formation of H_{II} phase in 2:1 DOPE/DOPC vesicles containing 4% dolichol, but had little or no measurable effect on similar vesicles prepared without the polyisoprenoid.

The effects of DOL on phospholipid bilayer stability observed in this study are similar to those reported by other investigators using X-ray diffraction (Gruner, 1985; Valtersson et al., 1985), ^{31}P-NMR (Struck et al., 1981; Valtersson et al. 1985), and differential scanning calorimetry (Valtersson et al., 1985). For bilayers composed of mixtures of unsaturated PC's and PE's the intrinsic radius of curvature of the system which defines its tendency to form H_{II} cylinders as opposed to bilayers is critically dependent on overcoming the hydrocarbon-packing constraints associated with the H_{II} phase (Gruner, 1985). Such packing is facilitated by the small headgroup and 'wedge-shaped' structure of DOPE (especially at pH values sufficiently acidic to protonate the ethanolamine and neutralize the charge on the headgroup); by increases in temperature; and apparently by DOL, which, because of its hydropho-

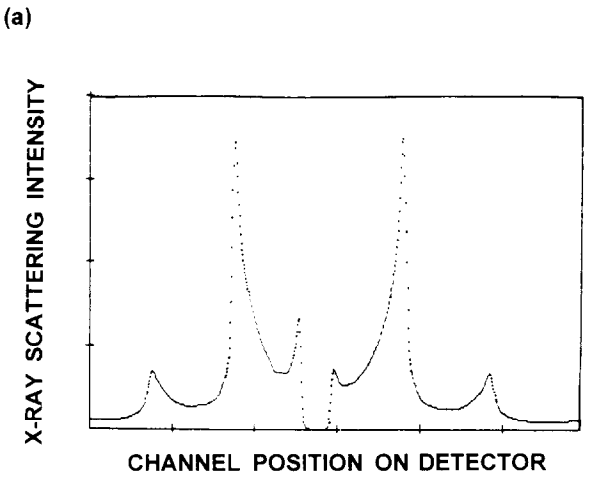


Fig. 3. Diffraction patterns for 2:1 DOPE/DOPC MLV containing 0.4% (w/w) DOL (37°C) using (a) electronic X-ray detector and (b) film stack (20 h exposure).

bicity, might be expected to intercalate within fatty acyl chains and relieve hydrocarbon-packing stresses that would otherwise work against formation of H_{II} phase (Gruner, 1985; Valtersson et al., 1985). These localized effects on lipid packing allow DOL to induce H_{II}-phase formation at temperatures well below those necessary for purely thermotropic transitions (Gruner, 1985; Valtersson et al., 1985; Van Duijn et al., 1986).

3.2. Permeability studies

Because of its potential importance in modulating drug flux either from vesicles and/or across

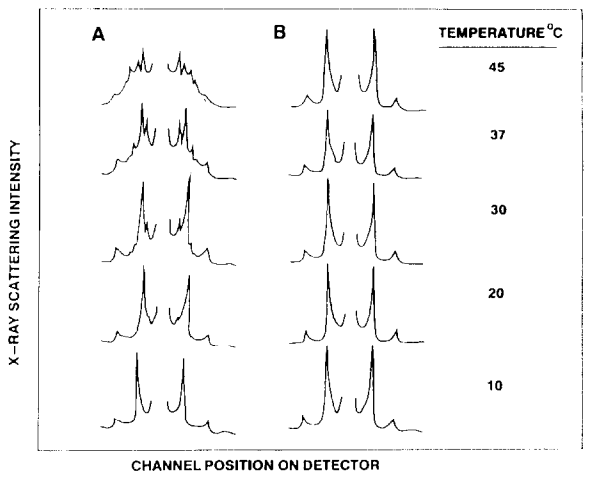


Fig. 4. Thermotropic induction of H_{II} phase (a) 2:1 DOPE/DOPC with DOL 4% (w/w); and lamellar diffraction patterns (b) for corresponding sample in the absence of DOL.

bilayer-containing membranes, the effect of the aforementioned phase transitions on vesicle permeability were determined. Profiles of percentage 6-CF released vs time for DOPE/DOPC vesicles (2:1, 37°C) containing various amounts of DOL are shown in Fig. 5. These data show the efflux of 6-CF from the vesicles to be correlated with, and strikingly sensitive to, the presence of small amounts of DOL incorporated within their

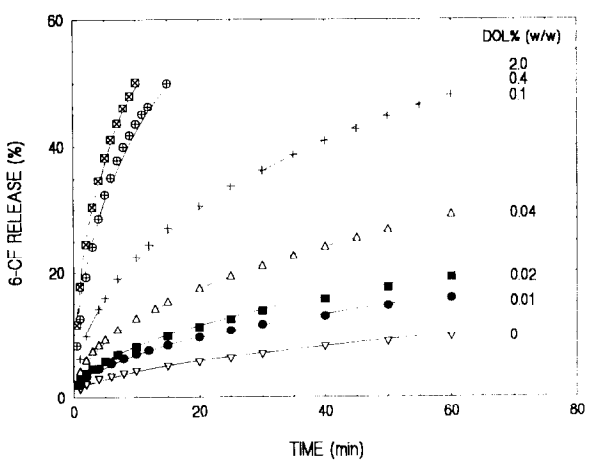


Fig. 5. Release of 6-CF from 2:1 DOPE/DOPC MLVs ± DOL at 37°C; (symbols and smooth curves represent observed data and PCNONLIN simulations, respectively).

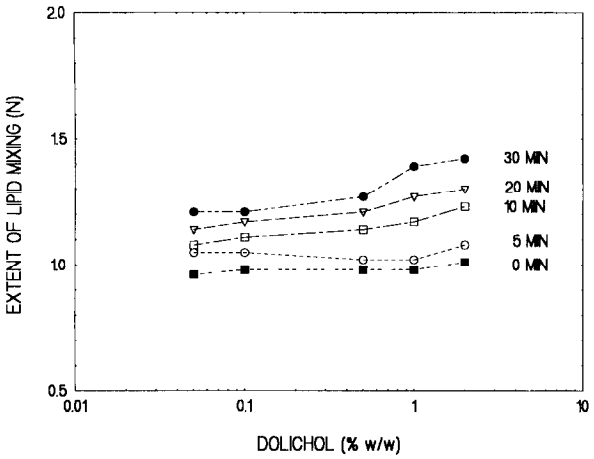


Fig. 6. Time-dependent lipid mixing of 2:1 DOPE/DOPC MLV as a function of DOL content.

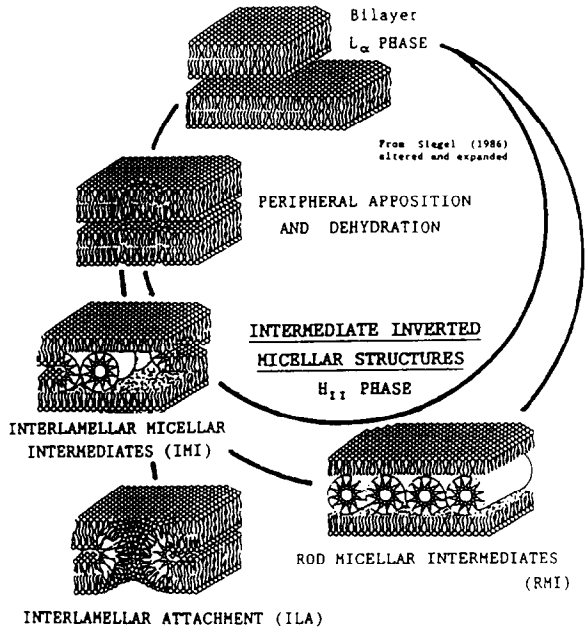


Fig. 7. Proposed interlamellar inverted micellar structures.

lamellae. For MLV containing 2% DOL, permeability was 200-fold higher than that of DOL-free vesicles for which only 8% of encapsulated 6-CF was released after 60 min. Efflux of 6-CF from vesicles composed of pure DOPC along with DOL at comparable concentrations (up to 2% w/w) was not significantly faster than control over a 4 h measurement period (data not shown).

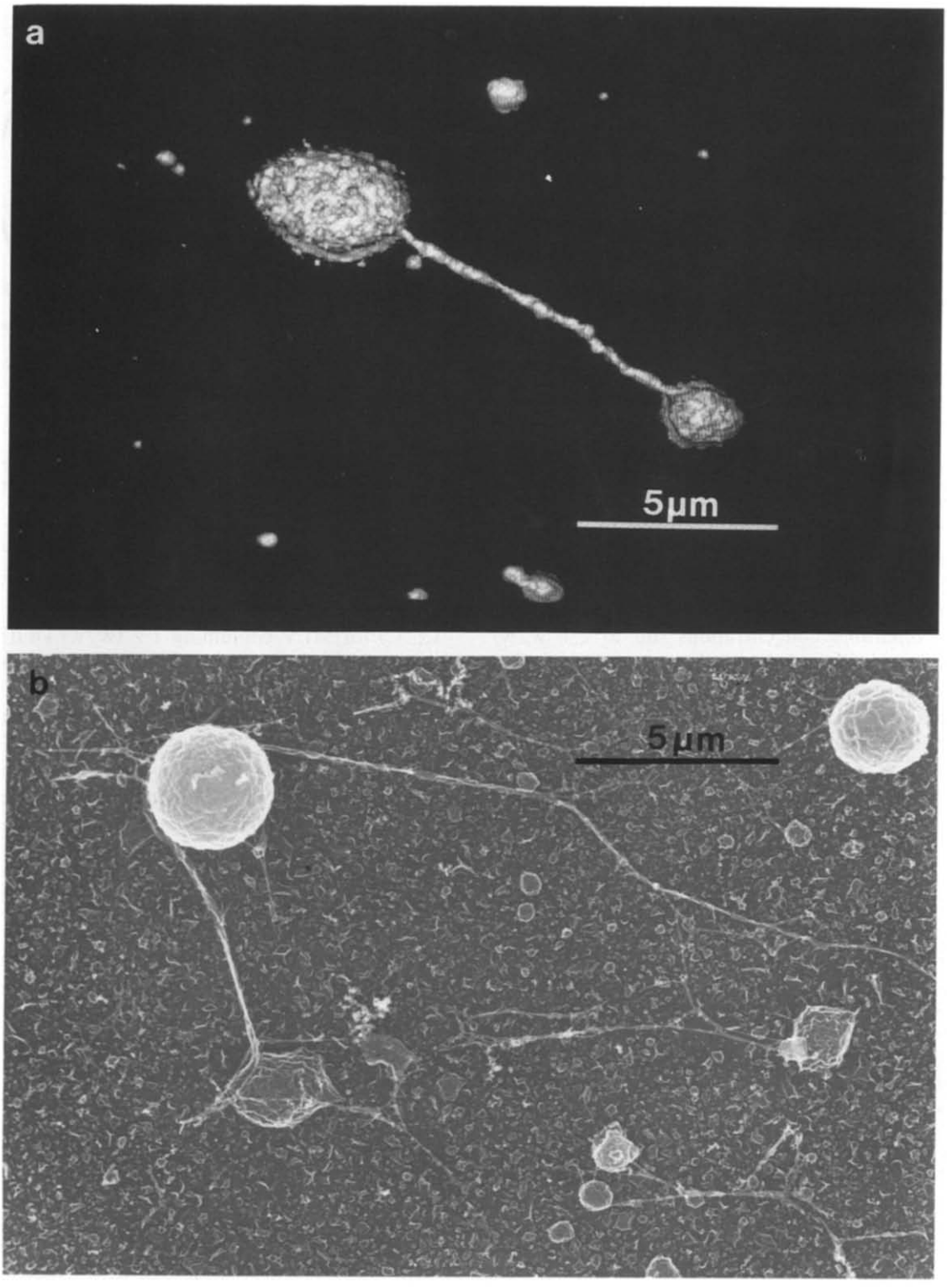
Since DOL induced substantial changes in membrane permeability at concentrations considerably lower than those at which H_{II} phase could be either detected or quantified, it was not possible to directly correlate changes in phase behavior with altered permeability. It was noted, however, that factors which promoted induction of H_{II} phase by DOL (i.e., DOPE content and temperature) also enhanced membrane permeability.

Table 1 lists the permeability coefficients (37°C) for MLV containing 1% (w/w) DOL and varying amounts of DOPE. Permeability increased 50-fold as DOPE content increased from 50 to 66.6%. The effect was considerably less for vesicles containing less DOPE as evidenced by a far smaller 3-fold increase as DOPE content increased from 25 to 37.5%. Stepwise enhancement in apparent permeability was also observed in control MLVs (DOL-free) by increasing PE content in the bilayers from 25 to 66.7%, thereby reducing the effective size of the polar-headgroup region of the bilayers. Temperature, a second factor that induces H_{II} phase, also facilitated

Table 1
Permeability coefficients for DOPE/DOPC MLVs prepared DOL-free and with 1% (w/w) DOL [a]

DOPE (% w/w)	Permeability coefficients (min^{-1})		1% (w/w) DOL/ Control
	DOL-free control	1% (w/w) DOL	
25	6.59×10^{-7} (5.1)	9.19×10^{-7} (18.7) [b]	1.4
37.5	1.21×10^{-6} (16.7)	3.0×10^{-6} (32.9)	2.5
50	2.84×10^{-6} (13.0)	5.78×10^{-5} (9.9)	20
66.7	1.32×10^{-5} (34.0)	2.44×10^{-3} (14.5)	185

[a] Experiments run at 37°C in 150 mM NaCl/10 mM Hepes buffer adjusted to pH 7.4.
[b] Coefficient of variation.


a
5μm
b
5μm

DOL-induced increases in permeability. Permeability constants for 2:1 DOPE/DOPC vesicles induced by DOL (1% w/w) were 2 orders of magnitude higher at 37°C than at 4°C.

The present results are consistent with the findings of Lai and Schutzbach (1984) who used DOL to modulate the diffusional uptake of the cationic spin probe TEMPOcholine into MLV composed of soybean PE/PC 1:1. More recent studies have also shown enhanced leakage of calcein from phospholipid vesicles containing extremely high (5–10% w/w) concentrations of DOL and dolichyl phosphate (DOL-P) (Monti et al., 1987; Schutzbach and Jensen, 1989).

3.3. Lipid mixing

Fig. 6 shows the relationship among extent of lipid mixing between fluorophore-labeled and unlabeled MLV (2:1 DOPE/DOPC), DOL content (0.05–2%), and time. For DOL concentrations as low as 0.05% small amounts of lipid mixing, indicative of intervesicular interaction, were in evidence within 5 min. With increasing time, all preparations including control vesicles containing no DOL demonstrated diminished FRET and at 30 min showed 1.2 to 1.4-fold enhancements in degree of lipid mixing. Lipid mixing was considerably less for 2:1 DOPE/DOPC, fluorophore-labeled MLV when mixed in a similar 1:3 ratio with a population of unlabeled vesicles having smaller percentages of DOPE, and was negligible when the latter vesicles were composed totally of DOPC (results not shown), consistent with possible involvement of H_{II}-phase intermediates in the lipid mixing process.

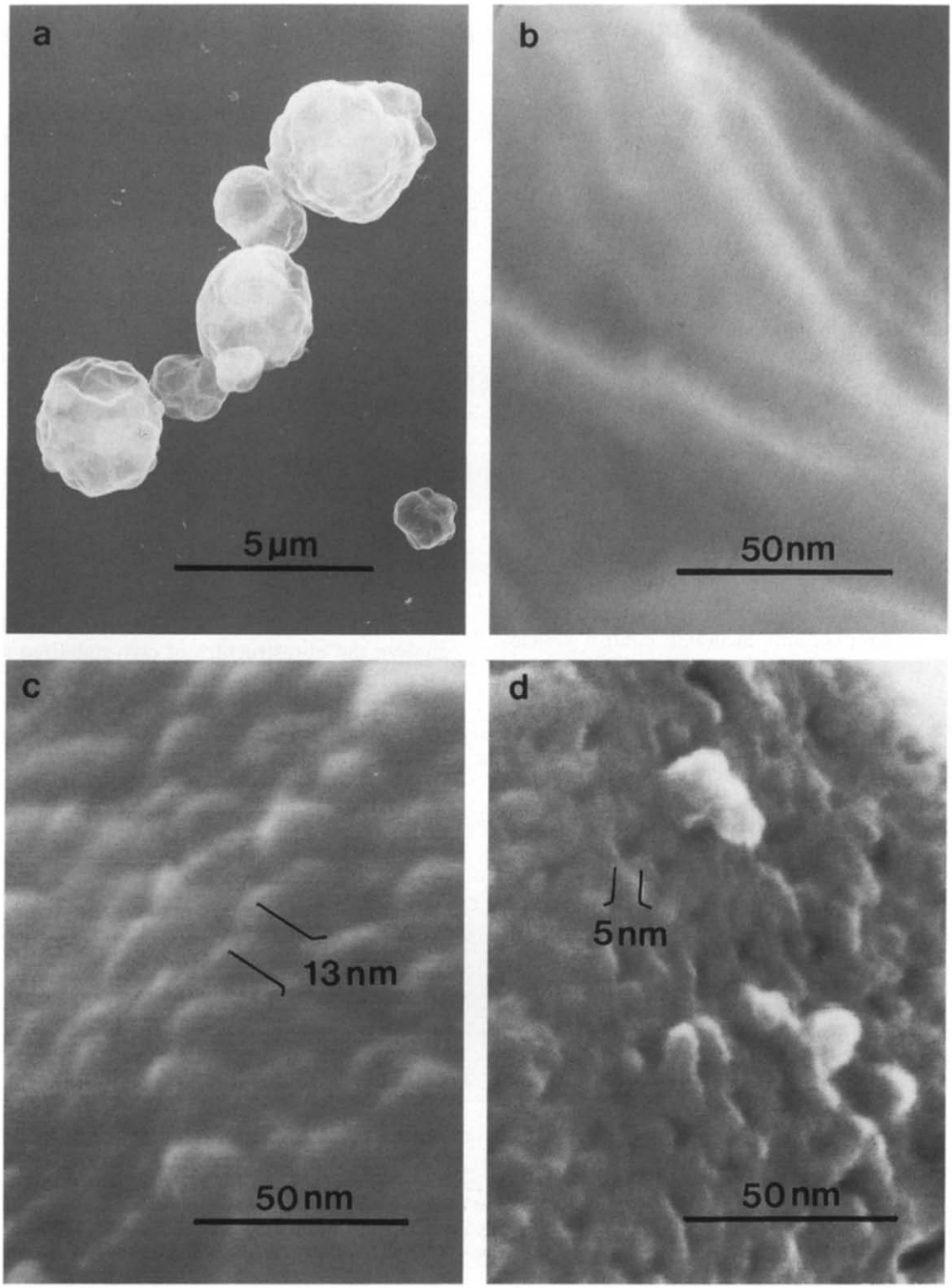
3.4. Morphologic observations

It is recognized that the L_α-H_{II} phase transition, which may be an important intermediate step in the bilayer fusion process (Cullis and De Krujff, 1980; Cullis et al., 1980; Siegel, 1984, 1986a; Seddon, 1990) is an interlamellar event. It occurs between closely apposed bilayers and involves lipid contact (local dehydration in the immediate vicinity of bilayer apposition) and formation of an 'interlamellar micellar intermediate (IMI)' (Siegel, 1984, 1986b; Seddon, 1990). Siegel (1984) has pointed out that once formed, IMI can revert back into the original two bilayers or, more rarely, fuse with outer monolayers to form 'hourglass shaped' conduits between bilayers called interlamellar attachments (ILA) (Fig. 7). Seddon (1990) and Siegel (1984) also suggest that during L_α-H_{II} phase transitions, IMI can coalesce into cylinders of H_{II}-phase phospholipid called rod micellar intermediates (RMI).

Consistent with these postulations, scanning microscopies were used to confirm the presence of hexagonal phase-like ultrastructures and fine structural membrane modifications which might have been a result of such phase transitional events. CSFM was applied to study the fine structure of fully hydrated samples and FSEM to analyze the ultrastructure of cryo-stabilized samples. Together these technologies permitted interpretation of fine structural changes at the level of phase transition ultrastructure while providing for control of preparation artifacts.

Vesicles composed of 3:1 DOPE/DOPC underwent profound changes following prolonged incubation (15 min) at physiological temperatures (37°C) and pH (7.4). Long filamentous connections were formed (Fig. 8a), often connecting vesicles in a specific manner (Fig. 8b). Unilamellar vesicles of similar or lesser size appeared to be stripped from the MLV, often remaining in contact with the parent vesicles through filaments having tubular characteristics. Both secondary structures seemed to disintegrate into much

Fig. 8. Microscopy of 3:1 DOPE/DOPC vesicles prepared at 10°C, pH 9.5 and incubated for 15 min at 37°C, pH 7.4. (a) Three-dimensional confocal microscopy of vesicles immobilized in low-melt Agarose™. Serial optical sections were collected at 0.5μm intervals. Data were reconstructed and displayed in three dimensions using isosurface rendering (section 2). (b) Scanning electron microscopy of vesicles after cryo-stabilization and drying. Elongated filaments of various diameters similar to those seen in (a) were found connecting MLV and unilamellar vesicles.


a
5 µm
b
50nm
c
13nm
50nm
d
5nm
50nm

smaller features adhering to the vesicle support used for microscopy (Fig. 8b).

In order to investigate the cause of these changes, vesicles maintained at pH 7.4 were analyzed before and after shorter incubations (1 min) at somewhat lower temperature (30°C) (Fig. 9). The control, maintained at a higher pH (9.5), showed no apparent morphological changes (Fig. 9a,b). After only 1 min at pH 7.4, however, ultrastructural changes of some of the vesicles were evident (Fig. 9c,d). Large areas of the membranes were studded with small elevated bumps approx. 10–15 nm in diameter. There was no apparent regularity and the minimum center-to-center distance between them was approx. 13 nm. Other areas of the surface displayed small holes approx. 5 nm in diameter, often seen within an elevated collar. These structures resemble in size and shape unilamellarly covered interlamellar micellar intermediates (IMI) and interlamellar attachments (ILA) (see Fig. 7). Such phase transitional ultrastructures were previously seen only with TEM (Verkleij, 1984; Siegel et al., 1989), but could be revealed here for ultrahigh magnification surface imaging. The ability of the DOPE/DOPC system to form some of the postulated phase transition intermediates may also extend to rod micellar intermediates (RMI) which are good candidates for explaining the assembly of filaments seen in the same preparations.

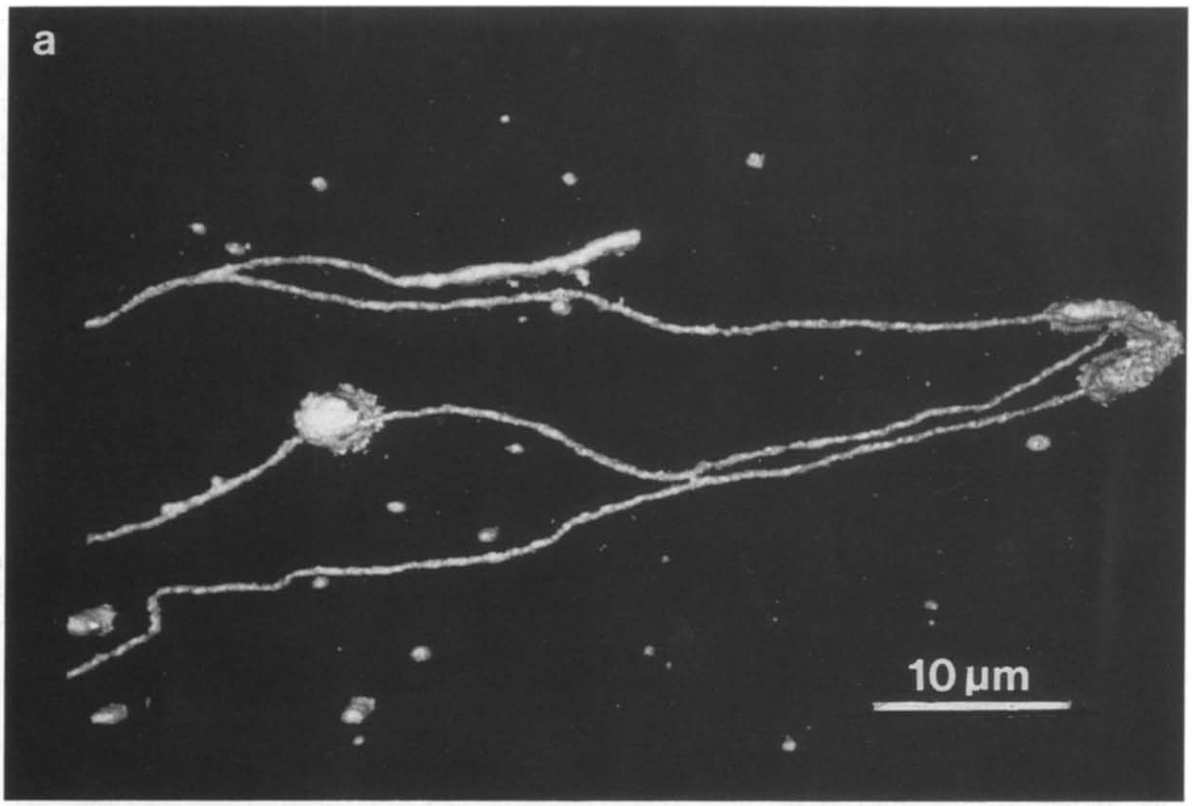
ILA formation could account for the leakage rate observed in 6-CF release experiments (Fig. 5) and would be the initiation process for an 'onion skin'-like stripping of the outer bilayer from MLV. The connecting filaments observed here are indicative of interbilayer phase transitions within the MLV. However, the prevalence of small particles seen after prolonged incubation at the higher temperature of 37°C might be indicative of continuous stripping and disintegrating of outer doublets of bilayers connected through ILA.

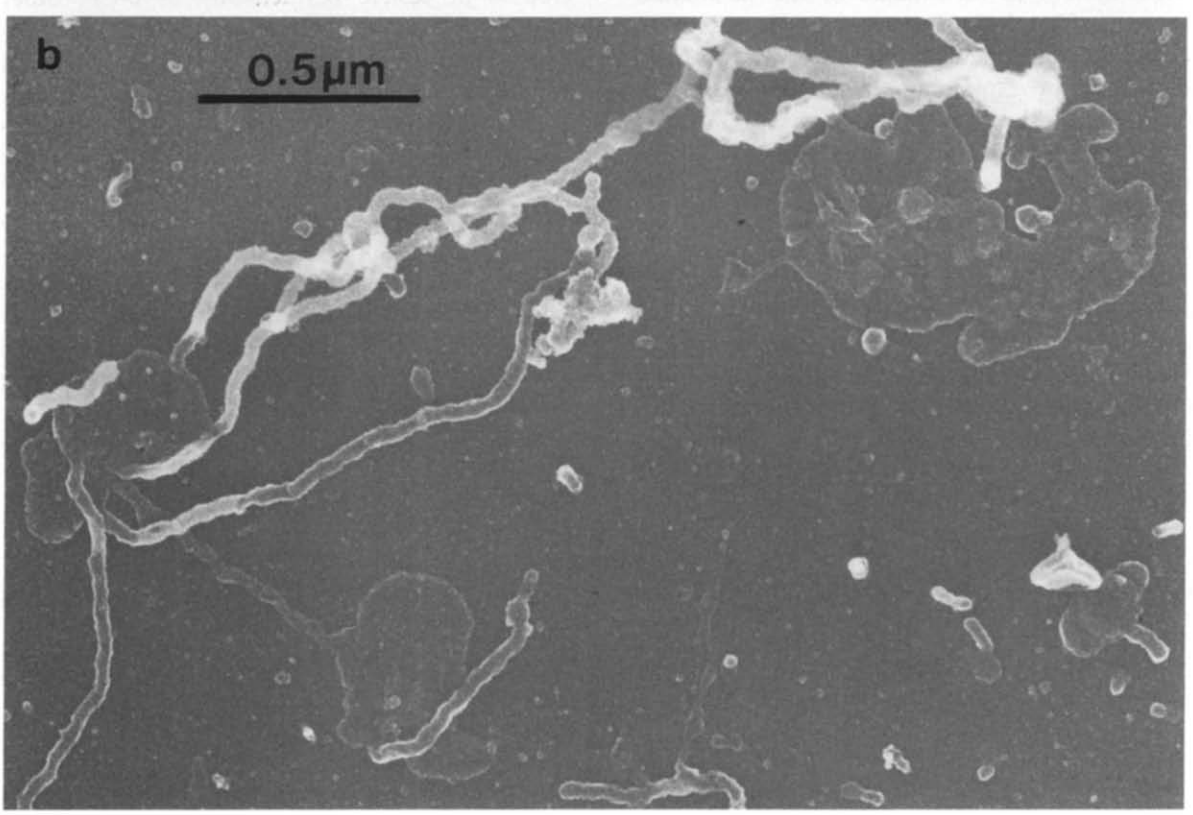
DOL (2% w/w, Fig. 10a; 1% w/w, Fig. 10b) was found to accelerate 'remodeling' of MLV and formation of tubules. At high magnifications, the preparations revealed unilamellar vesicles connected with and studded by thin tubules approx. 30–40 nm in diameter. Smaller particles, but not tubules, were seen on vesicles as well as on the support. The relatively large diameter of the tubules might be interpreted as a coaxial assemblage composed of multiple RMI. Larger tubules seen in samples with and without DOL measured up to several hundred nanometers in diameter and were hollow as observed in FSEM.

To summarize, DOL incorporated into DOPE-containing bilayers even at very low concentrations can induce or promote the formation of H_{II} phase which increases the permeability of the bilayers. At higher concentrations of both species H_{II} phase growth patterns in at least two dimensions are evident with a resultant gross alteration in vesicle morphology.

While the present data are consistent with DOL-induced phases changes effecting the increases in vesicle permeability to 6-CF, other factors such as headgroup size were almost certainly involved as well. While phospholipids of varying headgroup sizes were not systematically examined in the present study, when the percentage of the phospholipid constituent having the smaller headgroup (DOPE) in the DOPE/DOPC vesicles was increased (from 25 to 66.7%) in the absence of DOL, permeability increases were observed. Although the increases were only a fraction of those observed when the same compositional change was effected in vesicles containing 1% DOL, they occurred in spite of the fact that no H_{II} phase was detected in these DOL-free vesicles at any of the compositions studied. One

Fig. 9. Ultrahigh resolution field emission scanning electron microscopy of 3:1 DOPE/DOPC vesicles mixed at 10°C, pH 9.5. After incubation for 30 min at 30°C, pH 9.5, the vesicles remained intact (a) and, under high magnification, exhibited a smooth bilayer surface (b). After an additional 1 min incubation at 30°C at pH 7.4, the ultrastructure of the outer bilayer from some vesicles was altered. Both 10–15 nm diameter blebs (c), and 5 nm diameter holes (d) were observed, consistent with phase transitions occurring within the outermost vesicle bilayer.


a
10 µm


b
0.5µm

might anticipate, therefore, that systems employing protonated phosphospholipids incapable of forming H_{II} phase, might produce results similar to those observed here in DOL-free systems containing DOPE.

The phenomena observed in this study have implications for liposomal drug delivery of therapeutic peptides and proteins. Since the primary structure of vesicles appears to grow into thread-like filaments from IMI and ILA forms generated through H_{II} phase cylinders, it may become possible to trigger a convective release of macromolecules such as therapeutic peptides and proteins from liposomes via controlled formation of a series of ILA between adjacent lamellae of MLV. Such a process would extrude the entrapped material through aqueous conduits in series, obviating the requirement that large and generally hydrophilic materials diffuse across lipid bilayers to reach the external liposomal milieu. Experiments are currently in progress to develop a triggering mechanism for using polyisoprenoids, such as DOL, to release entrapped peptides and proteins.

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Fig. 10. Microscopy of 2:1 DOPE/DOPC vesicles prepared with DOL (1 or 2% w/w) at 10°C, pH 9.5 and incubated for 30 min at 37°C, pH 7.4. (a) Three-dimensional confocal microscopy of DOL vesicles (2% w/w) immobilized in low-melt Agarose™. Serial optical sections were collected at 0.5μm intervals. The data were reconstructed and displayed in three dimensions using isosurface rendering as described in section 2. Fully hydrated preparations revealed long fibrillar elements with a diameter of 100–400 nm extruding from vesicles. (b) Scanning electron microscopy of DOL vesicles (1% w/w) after cryo-stabilization and drying. 30–40 nm diameter filaments originating from MLV and often connecting to other vesicles as well as large numbers of small unilamellar vesicles were evident.

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