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Archaebacterial lipids: highly proton-impermeable membranes from 1,2-diphytanyl-*sn*-glycero-3-phosphocholine

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Unlike ordinary glycero-lipids such as egg-yolk lecithin, 1,2-diphytanyl-*sn*-glycero-3-phosphocholine, which was employed as a model lipid of archaebacterial acidophiles, gave liposomal membranes which were highly resistant to proton permeation in a wide temperature range (about 10–50°C); the permeability coefficient and activation energy of the flux were $0.7 \cdot 10^{-5}$ cm/s at 20°C and 24.5 kcal/mol, respectively. The high resistance to proton flow was attributed to a fluid and bulky isoprenoid chain-layer of the membrane.

Introduction

Many archaebacteria proliferate in such environments as volcanic hot springs, salt lakes, and acidic or alkaline spots [1–3]. For instance, *Thermoplasma acidophilum* grows optimally at 59°C and pH 1–2 [4]. It has been considered that the microorganisms appeared in the primeval biosphere, evolving or maintaining the unique lines to the present age in the extreme spots. At a molecular level, a most distinguished feature may be seen in the unusual lipid structure. While most membrane lipids of eubacterial and eukaryotes consist chiefly of fatty acids and glycerol with an ester bonding between them (Fig. 1), the archaebacterial lipids have isoprenoid chains of 20 or 40 carbon atoms which are connected to polyols (glycerol or nonitol) via ether bonds with an opposite stereochemistry at the *sn*-2 position to the conventional glycero-lipids [2]. It would be conceivable that the organisms have been adapted to the environment with the abnormal lipids to tolerate the extreme conditions. Previously we reported that 1,2-(3*RS*, 7*R*, 11*R*-phytanyl)-*sn*-glycero-3-phosphocholine (DPhyPC), a model lipids of the halophilic

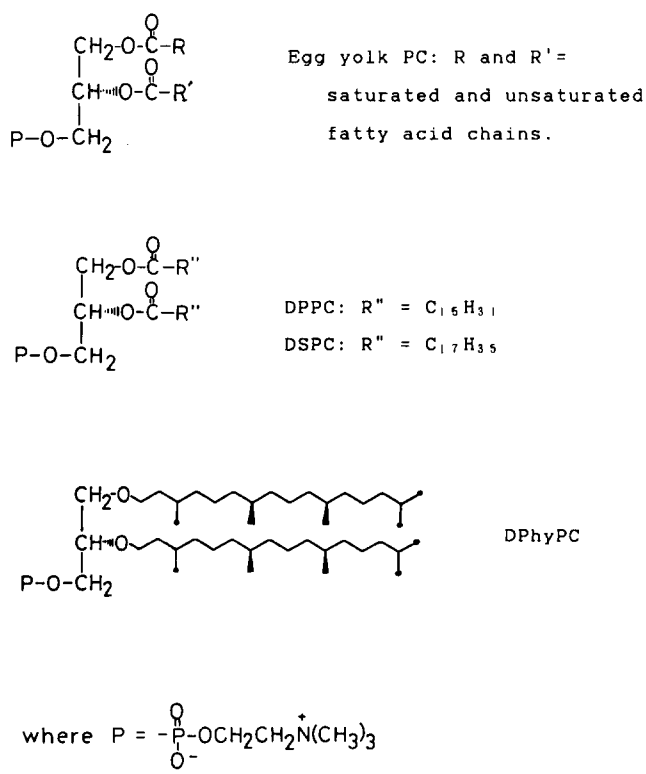

Fig. 1. Conventional lipids and archaebacterial model lipid, DPhyPC.

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Abbreviations: CF, 5(6)-carboxyfluorescein; DAPC, 1,2-diarachidonoyl-*sn*-glycero-3-phosphatidylcholine; DLS, light scattering measurement; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, myristoyl = n-$C_{13}H_{27}CO$-; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, palmitoyl = n-$C_{15}H_{31}CO$-; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine, stearoyl = n-$C_{17}H_{35}CO$-; DPhyPC, 1,2-diphytanyl-*sn*-glycero-3-phosphocholine (see Fig. 1); PA, phosphatidic acid; PC, phosphatidylcholine; P_T, a permeability coefficient of protons; SUV, small unilamellar vesicle; TEM, transmission electron micrograph; T_m, the temperature at midpoint in gel-to-liquid crystalline phase transition.

archaebacteria [5,6], gave the liposomal membrane which was highly impermeable to NaCl even at temperature as high as 70°C [7]. Since DPhyPC possess the core structure which is also common to acidophiles, we studied further on proton permeation for the membrane. The phenomenon, if confirmed, may provide a clue to preparation of lipid membranes for industrial uses.

Materials and Methods

Materials and instruments

DPhyPC was prepared previously [7] *. DPPC and DSPC (purity at least 99%) were kindly donated by Nippon Fine Chemical, Takasago-shi, Japan. Egg-yolk lecithin of Avanti Polar Lipid, USA was purified prior to use by silica gel column chromatography. Aspartic acid (0.04 M) buffer (pH 4.0) and aspartic acid (0.04 M)/sodium chloride (0.22 M) buffer (pH 4.0) were prepared by diluting an aqueous mixture of aspartic acid (5.32 g), sodium chloride (0 or 12.85 g) and 1 M sodium hydroxide (22 ml) to 1 liter. Glycine (0.09 M)/NaOH buffer (pH 12) was obtained by diluting a mixture of glycine (6.76 g) and 1 M NaOH (110 ml) to 1 liter. Concentrations of the lipids in the vesicle suspensions were determined by a modified micro-phosphorus assay of Bartlett [8]. Instruments including an ultrasonicator, a pH meter, a transmission electron microscope and a light scattering spectrometer were the same as those used previously [7].

Liposomes

A lipid (5 mg) in a buffer such as 0.04 M aspartic acid buffer (pH 4.0) (2 ml) was sonicated at 50°C (DPPC), 60°C (DSPC), about 0°C (egg-yolk lecithin) or ambient temperature (DPhyPC) and at 30–40 W for 30 min. The resulting suspension was centrifuged at 2000 $\times g$ for 10 min to give the supernatant, which was applied subsequently to a Sephadex G-50 gel column (2 cm $\times$ 15 cm) equilibrated and developed with the same buffer. The initial fraction (about 3 ml) containing the lipid (checked by TLC) was subjected to proton-permeation study, transmission electron microscopic observation (TEM) and light scattering measurements (DLS). The TEM and DLS suggested that the liposomes were unilamellar vesicles having a number-average diameter of 910 $\pm$ 50 Å.

* DPhyPC used in the present study was the (*R*)-DPhyPC which was defined in Ref. 7. A further study showed that the (*RS*)-DPhyPC, which was a mixture of (*R*)- and (*S*)-DPhyPC's, gave actually a single homogeneous spot with a relative mobility (R_f) = 0.50 $\pm$ 0.8 in silica gel TLC (Merck, Art. 5735) using chloroform/methanol/water (65:25:4, v/v) as a developing solvent; thereby, the authors correct the previous description for the TLC result. All of the experimental results concerning (*RS*)-DPhyPC in the Ref. 7 were obtained by the use of the homogeneous product.

Proton permeability through liposome membranes

The procedure previously reported [9] was employed with minor modifications. The small unilamellar vesicles in the 0.04 M aspartic acid buffer (pH 4.0) (3.0 ml) were placed in a cell with two necks: one neck for a pH electrode and another as an inlet for aqueous sodium hydroxide (Fig. 2). The cell was flushed with nitrogen, and after sealing tightly the inlets with silicon rubber caps, immersed in a water bath thermostated at a suitable temperature ($\pm$0.5°C). Permeability measurement was started after quick addition of 3.5 M sodium hydroxide (about 15 μl) by means of a microsyringe to bring the pH close to approximately 8. Although the protons escaped from the vesicles were diluted by the external aqueous phase of the liposome suspension, the low buffer capacity of the pH 7 solution allowed the small proton leakage to be monitored readily by means of the pH meter. The amount of protons permeated from the liposomes was determined by means of an experimental relationship between pH of the buffer and added amounts of H^+ or OH^- ions at the temperature used (Fig. 3). After 1 h, 10 wt% of Triton X-100 (50 μl) was added to the liposome suspension in the cell in order to obtain the pH value at an infinite time

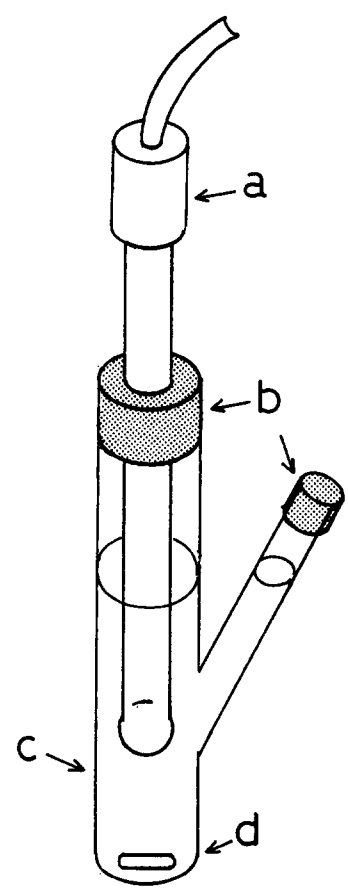

Fig. 2. A cell system for proton permeability measurements: *a*, a pH electrode; *b*, septum; *c*, an aqueous suspension of liposomes; *d*, a magnetic bar.

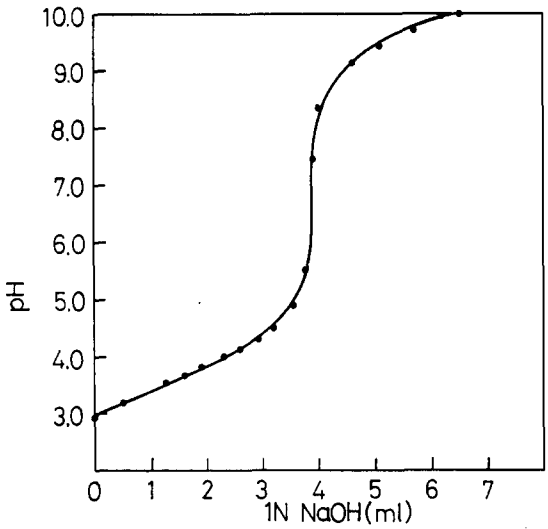

Fig. 3. Titration curves of 0.04 M aspartic acid with 0.1 M NaOH. Experimental titrations are shown by points.

or a total amount of H^+ which had been stored in the vesicles. As was described in the experiment by Nozaki and Tanford, we also observed the pH drift around pH 7 that rendered the estimation of proton leakage rate to be accurate only within ± 0.01 pH unit/min which was equivalent to $\pm 3 \cdot 10^{-7}$ cm/s in permeability coefficients at 20°C. Net flux, J_{H^+} (mol/cm^2 per s), and permeability coefficient, P_T (cm/s), were calculated by the equations:

$$J_{H^+} = 1.20 \cdot 10^{-6} \cdot 2(V_0 - V_t)/(M \cdot R \cdot A)$$

$$P_T = J_{H^+}/\Delta a_{H^+}$$

where V_0 and V_t are the volumes of 0.04 M NaOH at time = 0 and t, respectively, M is the apparent molarity of the lipid in the liposome suspension in the cell, R is an Avogadro's number, and A is the occupied area per lipid molecule in the membrane which was estimated by extrapolation of surface pressure–surface area curves on the area axis by the use of a Langmuir trough [10,11]; e.g., area/nm^2 (temperature in °C): DPhyPC, 1.03 (5), 1.05 (10) and 1.10 (20); egg-yolk PC, 0.71 (20) and 0.72 (25); DPPC, 0.48 (9)–0.51 (37); DSPC, 0.40 (5)–0.43 (35). $\Delta a_{H^+} = (1 \pm 0.05) \cdot 10^{-4}$ (mol/cm^3) (ΔpH was approx. 4 ± 0.02).

Liposomes in alkaline solutions

(A) Leakage of CF. A mixture of lipid (egg-yolk PC or DPhyPC; 5 mg) and 0.2 M CF (1 ml) was sonicated at 50°C for 30 min. During the process, the suspension was adjusted to pH 12 by 0.3 M NaOH. The resulting dispersion was applied to a gel column (2 cm × 25 cm; Sephadex G-50 (fine)) equilibrated with the glycine buffer (pH 12). SUVs bearing the fluorescent probe were eluted first (1.5–2 ml) and separated from free probe. The liposome suspension (30 ml) was then incubated at 30 or 50°C. An aliquot (30 μl), after suitable incubation time, was diluted with the glycine buffer to 3 ml which had been kept at the same temperature. After annealing the mixture for 15 min, the fluorescence intensity (I_t) at 520 nm with excitation at 470 nm (band width: excitation, 10 nm; emission, 5 nm) was measured. Finally, the sample solution was mixed with 10 vol% aqueous Triton X-100 solution (10 μl), and the maximum intensity (I^*) resulting from instantaneous rupture of the liposome was measured. The leakage extents (%) were calculated by $100 \times I/I^*$ as shown in Fig. 6.

(B) Hydrolysis study. The liposome suspensions (0.5 ml) of egg-yolk PC and DPhyPC, which were prepared in pure water in a manner similar above described, were mixed with the glycine buffer, then incubated at 30 or 50°C. The extent of the lipid hydrolysis was determined conveniently by the Fe^{3+}/hydroxylamine procedure [12]. The results are described in the caption of Fig. 6. Decomposition of DPhyPC was negligible.

Results

Proton permeability

A typical net H^+-flux in the DPhyPC liposome suspension, which was calculated from the pH change of the aqueous bulk phase, is shown in Fig. 4. The Arrhenius plot of the permeation rate of H^+ through DPhyPC liposome bilayer is shown in Fig. 5; the devia-

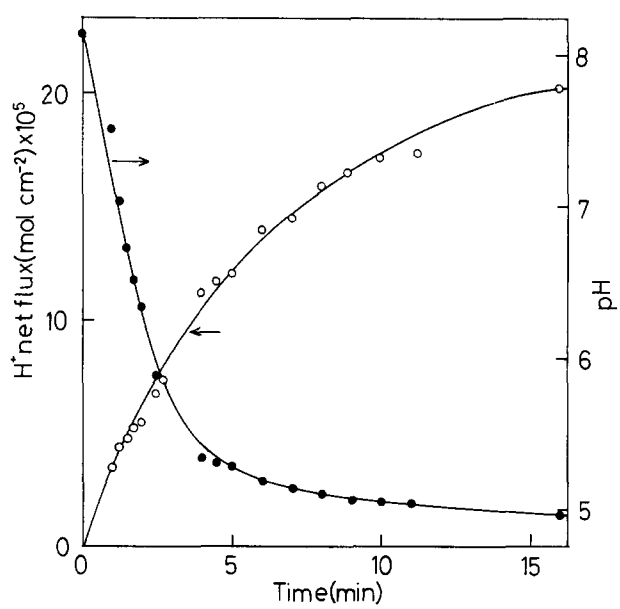

Fig. 4. A time course of net H^+-flux and pH in the DPhyPC-liposome suspension. The liposomes were prepared in 0.04 M aspartic acid (pH 4) and the bulk aqueous phase was adjusted to pH 8.16 at 20°C. The amounts of H^+ leaked from the vesicles to the aqueous bulk phase was estimated from the titration curves of Fig. 3. A pH 4.96 was observed upon addition of Triton X-100 to the suspension and was taken as a final pH which would have been attained at an infinite time.

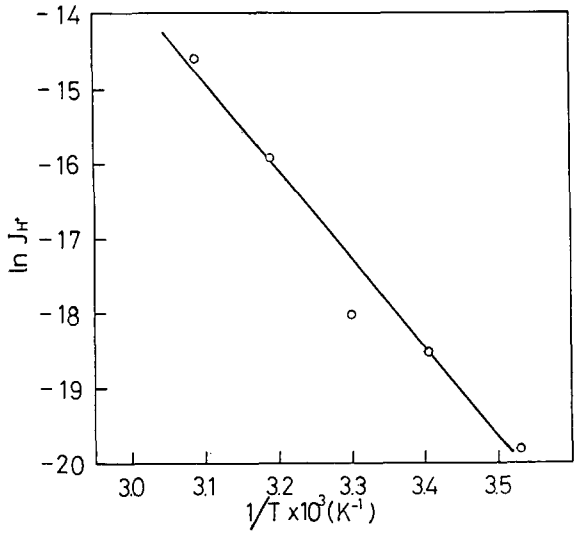

Fig. 5. An Arrhenius plot of net H^+-flux rates (mol cm^{-2} s^{-1}) in the DPhyPC liposome suspension vs. a reciprocal of the incubation temperature (K^{-1}).

tion from linearity was caused likely by experimental error, or ln $J_{H^+} \pm 0.1$–0.3. The representative permeability coefficients (P_T) at 20°C and the activation energies (E_a) for proton flow through the lipid bilayers are listed in the Table I *. DPPC- and DSPC-membranes, which were utilized as references for DPhyPC-membrane, allowed protons to permeate so fast around

* The deviation from linearity in Fig. 5 is within an experimental error of E_a, ±1.6 kcal/mol. Unlike E_a, ΔS^* (in practice, a pre-exponential factor of Arrhenius equation) was not acquirable as a reliable value because a small fluctuation of Δa_{H^+} from one liposome suspension to another affected profoundly the ΔS^*.

TABLE I

Proton permeability coefficient (P_T) at 20°C and activation energy (E_a) of proton flux in various lipid bilayers

Lipid	T_m (°C)	$10^5 \times P_T$ (cm/s)	E_a (kcal/mol)
DPPC [a]	41.5	4.7	15
DSPC [a]	55.0	1.5	26
Egg PC [b]	−15 ~ −7	2.0	14
DPhyPC [b]	< −20	0.7	25

[a] In a gel phase.
[b] In a liquid-crystalline phase.

and above the respective T_m values that their P_T values were only determinable from the fluxes measured for temperatures below the T_m values. It was found that the net flux in the buffer containing 0.22 M NaCl was exactly the same as the flux in the absence of NaCl in all kinds of the liposome systems, indicating that a diffusion potential across the membrane, if developed, had no significant effect on the proton flow, or protons might diffuse as neutral acid molecules (HCl) as suggested in Ref. 13.

Chemical stability of liposomal membranes

A phosphoester bond is much more stable under acidic and alkaline conditions than a carboester bond. In fact, the DPhyPC-liposomal membrane was hardly decomposed at pH 1–2 (by HCl) and pH 12 (by NaOH) at 30 and 50°C for at least a day. In contrast, the egg-yolk PC-membrane was degraded, especially significantly under pH 12, to increase turbidity of the aque-

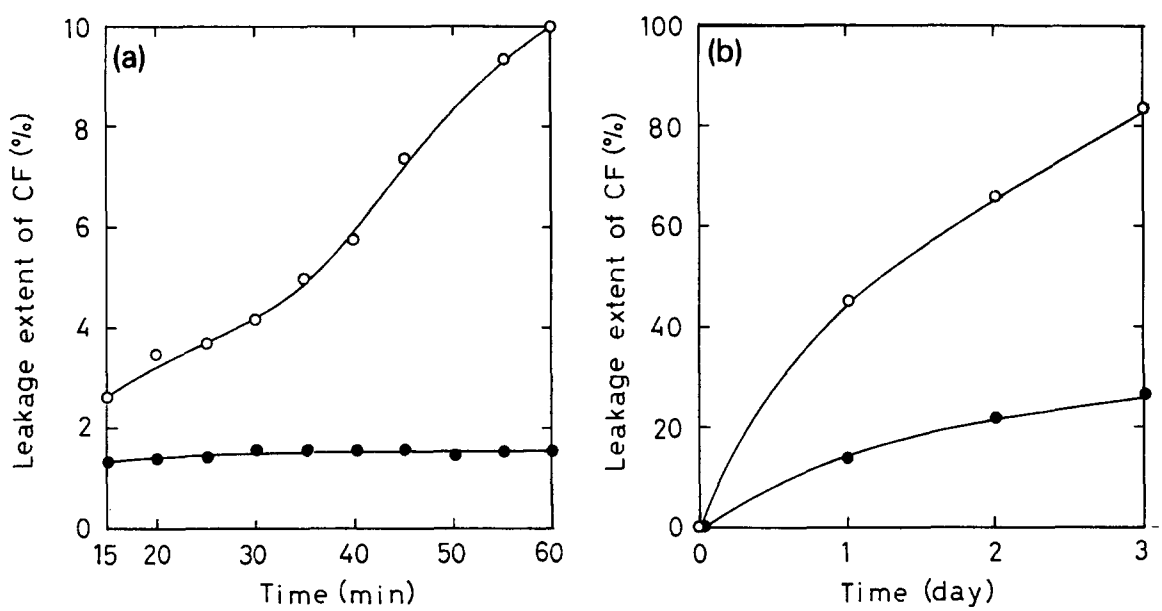

Fig. 6. Leakage of CF from liposomes at pH 12 at 30°C (panel b) and 50°C (panel a) as a function of time (= incubation time minus annealation time of 15 min); ●, DPhyPC liposomes; ○, egg-yolk PC liposomes. Extent of hydrolysis of egg yolk PC (%/incubation time): 41% (1 day), 50% (2 days) and 57% (3 days) at 30°C; 7% (10 min), 13% (20%) and 15% (60 min) at 50°C. Hydrolysis of DPhyPC was less than 5% at 50°C after three days.

TABLE II
Proton or proton-hydroxide permeability of various lipid membranes

Lipid (membrane system)	Temp. (°C)	P_T (cm/s)	Ref.
Egg PC (LUV)	rt	$1.7 \cdot 10^{-4}$	18
Egg PC (LUV)	rt	$3 \cdot 10^{-9}$ [a]	9
Soy azolectin (SUV)	25	10^{-3}–10^{-4}	19
Mitochondoria	rt	10^{-3}	20
PC/PA (9:1) (LUV)	rt	$3.2 \cdot 10^{-4}$	21
DMPC (SUV)	35	10^{-3}–10^{-5}	22
DAPC (SUV)	–	$1.8 \cdot 10^{-5}$	23

[a] Proton permeability.

ous suspension. Such decomposition resulted in releasing substances trapped in the vesicle interior. Leakage extents of CF-probes from egg-yolk PC- and DPhyPC-liposomes at pH 12 is typically shown in the Fig. 6; the hydrolysis extents of the lipids are described in the captions.

Discussion

Though many studies have concerned with proton-flow across the lipid membranes (Table II), an effect of temperature on proton-permeability has not been investigated well. As shown in Table I, we measured the permeability of liposomal membranes of DPPC, DSPC and egg PC to obtain P_T values in the order of $(1.5\text{–}5) \cdot 10^{-5}$ cm/s at 20°C, which agreed with the literature values. It was found generally that protons permeated more readily with increasing temperature, especially at around T_m values. It appeared also that gel phase (G phase)-membranes were not always better as a barrier than liquid crystalline phase (LC phase)-membranes. For instance, (a) the DPPC-membrane of 35°C (G phase) was as leaky as the membrane of 45°C (LC phase); (b) at 30°C, the DPPC-membrane (G phase) was more leaky than the egg lecithin-membrane (LC phase). The passage of protons or neutral HCl molecules might be through defect structures in the gel membranes or hydrogen bonded chains of water in its rigid hydrocarbon-layers (*vide infra*).

DPhyPC, which was used in this paper as the model lipids of acidophiles, furnished the very stable liposomes (SUV) in a quantitative yield. In addition, the membrane was much tighter to the passage of protons than the membranes of conventional lipids such as DPPC, DSPC and egg-yolk lecithin; e.g., P_T was as small as $(0.7 \pm 0.2) \cdot 10^{-5}$ cm/s at 20°C and E_a was as large as 24.5 ± 1.6 kcal/mol. The same DPhyPC liposomal membrane was highly impermeable to NaCl and tolerant in an aqueous satulated solution of NaCl [7]. Notably, the DPhyPC membrane did not exhibit any detectable gel-to-liquid crystalline phase transition at least from −20°C to 70°C. Thus, the membrane existed in a fluid state over the wide temperature range, perhaps owning to steric interference of the methyl groups in the ordered packing of the isoprenoid residues or a small energy difference between the *trans* and *gauche* rotamers [14,15]. Although the small permeability in the lipid membranes having isoprenoid chains has not been explained, it might be ascribed to exclusion of such proton-conductive pathways as microscopic icebergs of water or some form of a hydrogen bonding chain of water from the interstices in the hydrocarbon moieties [16,17].

From a viewpoint of chemical stability, the DPhyPC-membrane was stable in wide pH range at ambient temperature. By contrast, the liposomal membranes from the conventional diester-type lipids were degraded readily under acidic and alkaline conditions because of hydrolytic fission of the carboester linkages (Fig. 6). Industrial application of the acid-tolerant membrane of DPhyPC is under examination.

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