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Amino acid permeability of liposomal membranes measured by light scattering method: a simulation with a method of cumulants

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Summary

Four types of liposomes were formed from egg lecithin (PC), PC–cholesterol (2:1) (PC–Chol), PC–phosphatidic acid (9:1) (PC–PA) and PC-dicetyl phosphate (9:1) (PC–DCP). The purity of PC used was 88% and it included 9% lysolecithin. Observations of the light scatter 90° to incident indicates that the liposomes exposed to a hypertonic medium of amino acid undergo rapid shrinkage due to water loss and that a slower re-swell phase, dependent on amino acid entry into the intraliposomal spaces, follows. Taking into account the polydispersity of liposome size and its property, the volume dependent change was simulated with a method of cumulants. In PC liposomes, the order of rate constant for influx was Ala, Gly < Ser < His, Pro < Val < Leu < Phe and Overton's rule was held for amino acids. Their relative magnitudes were parallel with the hydrophobic scale of amino acids given by Nozaki and Tanford (1971). A 10-fold increase in the rate constant accompanied a −1430 cal/mol change in the additional free-energy of transfer from water to organic solvent. The PC–Chol, PC–PA and PC–DCP membranes had lower rate constants for amino acids in comparison with that of PC liposomes. Water permeability of PC–Chol membrane was lower than that of PC. Inclusion of cholesterol into lecithin showed its condensing and fluidizing effects. The addition of phosphatidic acid which has a higher transition temperature in comparison with that of lecithin increased water permeability. This was explained from a phase separation effect which occurs when two lipids with different transition temperatures are mixed.

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Introduction

By virtue of their versatile structure, composition and size, liposomes are recognized by many investigators as an attractive means of drug delivery in therapeutic and preventive medicine covering such aspects as cancer, antimicrobial and antiviral chemotherapy and immunopotentiation (Pagano and Weinstein, 1978; Gregoriadis and Allison, 1980).

Here, permeabilities of water and amino acids across several different liposomes have been measured with a light scattering method. Liposomes were made of egg lecithin (partially purified) (PC), lecithin–cholesterol (PC–Chd), lecithin–phosphatidic acid (PC–PA) and lecithin–dicetyl phosphate (PC–DCP). The light scattering method has been an effective means of determining solute permeabilities across biological membranes such as red cells (Rich et al., 1968), sarcoplasmic reticulum (Kometani and Kasai, 1978) and gastric vesicles (Rabon et al., 1980), and also water permeability across liposomal membranes (Bittman and Blau, 1972; Block et al., 1977). The method has an advantage against a popular isotope method because in the isotope method an additional dialysis permeability has to be taken into consideration, which makes the kinetic analysis of experimental results more difficult.

The aim of this paper is dual purpose. One is to obtain a reasonable mathematical formulation to systems whose physicochemical properties have polydispersity due to the presence of different sizes of liposomes and/or due to the presence of both multi- and single-lamellar liposomes. The other is to compare transport properties of amino acids with other thermodynamic properties such as hydrophobic scale of amino acids given by Nozaki and Tanford (1971), which will give grounds for the mathematical formulation. We wish to establish a practical way to describe transport properties of solute and solvent for liposomes with polydispersity.

Materials and Methods

Materials

A lecithin fraction was obtained from egg yolk of hens following a Faure (1950) method. The purity of lecithin fraction was 88% (mole ratio) on thin-layer chromatographies (Rouser et al., 1966). Other components were 9% lysolecithin, 1% phospatidyl ethanolamine and 2% a mixture of lysophosphatidyl ethanolamine and sphingomyelin. We used here this fraction as lecithin (PC) without further purification. Cholesterol (Chol), phosphatidic acid (D,L-α-dipalmitoyl) (PA) and dicetyl phosphate (DCP) were bought from Sigma. Until use, these materials were stored under nitrogen gas at −20°C.

Preparation of liposomes

Four different liposome suspensions were made of PC only, PC–Chol (2:1 in mole ratio), PC–PA (9:1) and PC-DCP (9:1). The lipid mixture in chloroform solution was deposited on the wall of round-bottom flask by removal of the organic solvent on a rotary evaporator. After the addition of 20 mM sucrose solution (1.9 μmol lipid/ml aqueous solution), the suspension was shaken by hand for a period of

1 h. The liposome solution was thereafter sonicated gently in a Branson B-42 bath at 25°C for 1 h. The effects of the shaking period on the size distribution of liposomes were discussed by Szoka and Papahadjopoulos (1980). Furthermore, the suspension was filtered through a 1.2 μm membrane made of cellulose acetate (Fuji film) to remove coagulated large liposomes and dusts. Fuji films include a small amount of glycerin which can be easily washed out and reported to have high filtration coefficients in comparison with made of cellulose nitrate (Shinkai and Nishimura, 1976). Negative stain electron micrographs with potassium phosphotungstate showed typical multi-lamellar liposomes accompanied by a small amount of single lamellar liposomes.

Water permeability

All experiments of the light scattering were carried out at 25°C. Solutions were buffered to pH 7.4 by using 2.5 mM Tris-HCl. The principle of the light scattering method was to apply a sucrose osmotic gradient to liposomes suspended in 20 mM sucrose. For this purpose, the liposome suspension was mixed with a hypertonic 100 mM sucrose solution in a 1 : 1 volume ratio using a Durrum stop-flow spectrophotometer. 90° light scatter, observed as a change in transmittance at 550 nm, was monitored on a Tektronix storage oscilloscope as described elsewhere (Rabon et al., 1980). Final liposome size did not depend on the mixing speed when mixed rapidly by gas purge or slowly by hand pushing, suggesting that the large shear forces did not reduce the liposome size.

Time-dependent increases in the intensity of light scatter, Y, did not fit a single exponential equation. This is apparently due to the existence of the heterogeneity of the size and the permeability of liposome. Regarding this, Chan et al. (1973) have shown that the permeability coefficients for small and large liposomes ($\geq$ 100 nm) are different predominantly due to packing constraints.

In preliminary studies, it was found that a sum of two exponential terms can simulate the results. The simulation would be justified if the liposome suspension had been composed of two representative components; one component with a larger size and a higher permeability and the other with a smaller size and a low permeability. The actual distribution of liposome size, however, was very broad as seen in Fig. 1. Therefore, the simulation must be done with a sum of large number of exponential terms. Instead of using the sum of exponential terms, we used a cumulative expansion for the present system with polydispersity. The formulation:

$$Y = A\left\{1 - \left(1 + \mu_{2w} \cdot t^2/2\right)\exp(-k_w t)\right\} \quad (1)$$

is applicable to any experiments which yield a sum of or distribution of exponentials (Koppel, 1972), where k_w is the rate constant of water efflux and μ_{2w} the second expansion coefficient. The value of μ_{2w}/k_w^2 is the polydispersity index for the rate constant. The parameters in Eqn. 1, μ_{2w}, k_w and A were determined by the least-squares method.

Amino acid permeability

The liposome suspension was mixed with a 100 mM amino acid solution

(buffered to pH 7.4) in a 1:1 volume ratio. The initial rapid water efflux was monitored on the oscilloscope and the following slow phase was recorded on a servorecorder. It has been demonstrated that the re-swelling phase after the initial shrinkage is a function of solute entry under the condition that the efflux rate of sucrose is negligibly small in comparison with the solute entry. This condition was held mostly but not for some systems of amino acid–liposome. Under these conditions, the whole process after the mixing was able to be simulated with a following equation:

$$Y = A\{1 - (1 + \mu_{2w}t^2/2)\exp(-k_w t)\} - 1.2A\{1 - (1 + \mu_{2A}t^2/2)\exp(-k_A t)\} \qquad (2)$$

where k_w and k_A are the rate constants of water efflux and amino acid influx. The values of μ_{2w}/k_w^2 and μ_{2A}/k_A^2 are the polydispersity indexes for the k_w and k_A, respectively.

In Eqn. 2, the value of 1.2 was multiplied to A for the re-swelling process, because that the volume of maximally-swelled liposome had about 1.2 times the initial-unshrunken size. The volume of maximally-swelled liposome was determined as follows. The liposome suspension, pre-equilibrated with 20 mM sucrose, was mixed with water (buffered) in a 1:1 volume ratio and the resulting liposome volume was determined from a calibration curve as in Fig. 1 of a previous paper (Rabon et al., 1980).

Results

Size distribution

Size distribution of the PC liposome was determined from negative stain electron micrographs (Fig. 1). The average diameter of the liposomes was 0.14 ± 0.09 μm (average ± S.D.) (129 observations). The maximum size was under 0.5 μm probably due to the long gentle sonication and filtration with 1.2 μm filter. We did not

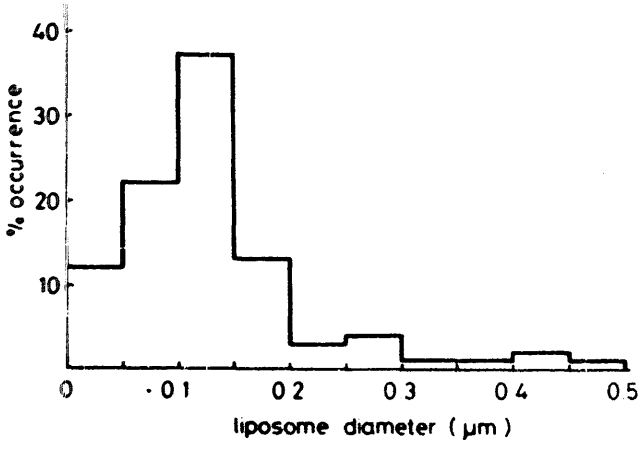

Fig. 1. Size distribution determined from a negative stain electron micrograph with potassium phosphotungstate.

elaborate the size distribution in small unilamellar liposomes (20–50 nm) which are osmotically insensitive (Johnson and Butress, 1973).

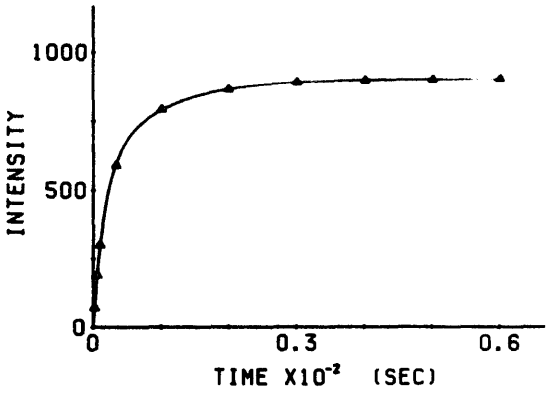

Fig. 2. A time-dependent change in the intensity of 90° light scatter (arbitrary scale) due to the liposome shrinkage. PC–Chol liposome suspension equilibrated with 20 mM sucrose was mixed with an equal volume of 100 mM sucrose solution. The marks of ▲ show the experimental points read from a continuous curve on the oscilloscope and the solid curve shows the simulation result.

Water permeability

Marks of ▲ in Fig. 2 show experimental points read from a continuous curve of the shrinking process on the oscilloscope when the PC–Chol liposome suspension with 20 mM sucrose was mixed with 100 mM sucrose solution. The solid line in the figure shows the simulation result with Eqn. 1. Rate constants, k_w, and the polydispersity indexes for 4 different types of liposome are listed in Table 1.

The addition of cholesterol into the lecithin significantly decreased the water permeability (74% decrease in k_w), whereas the addition of phosphatidic acid and dicetyl phosphate increased the water permeability (63% and 20% increases in k_w, respectively).

The water permeability was also obtained from experiments of amino acid permeability using Eqn. 2. Table 2 lists the average values of k_w and μ_{2w}/k_w^2 from the experiments. There are good correlations between corresponding values in Tables 1 and 2.

Table 1

Rate constants of water efflux k_w and their polydispersity indexes μ_{2w}/k_w^2 from Eqn. 1. Values are averages ± S.E. (no. of observations)

Types of liposomes	$k_w(sec^{-1})$	μ_{2w}/k_w^2
PC	2.00 ± 0.05 (2)	0.41 ± 0.01 (2)
PC–Chol	0.51 ± 0.05 (2)	0.56 ± 0.07 (2)
PC–PA	2.70 (1)	0.50(1)
PC–DCP	2.40 ± 0.03 (2)	0.88 ± 0.07 (2)

Table 2

Rate constants of water efflux k_w and their polydispersity indexes, μ_2/k_w^2. Values are averages ± S.E. (no. of different amino acids)

Types of liposomes	$k_w(sec^{-1})$	μ_{2w}/k_w^2
PC	2.09 ± 0.06 (8)	0.39 ± 0.03 (8)
PC-Chol	0.46 ± 0.02 (4)	0.44 ± 0.05 (4)
PC-PA	2.57 ± 0.15 (4)	0.55 ± 0.05 (4)
PC-DCP	1.79 ± 0.24 (4)	0.79 ± 0.10 (4)

Amino acid permeability

Rate constants of solute influx across the liposomal membrane were determined on 8 amino acids; i.e. Gly, Ala, Val, Leu, Phe, Ser, Pro and His. In Fig. 3, the time-dependent change in the light scatter is shown when the PC suspension with 20

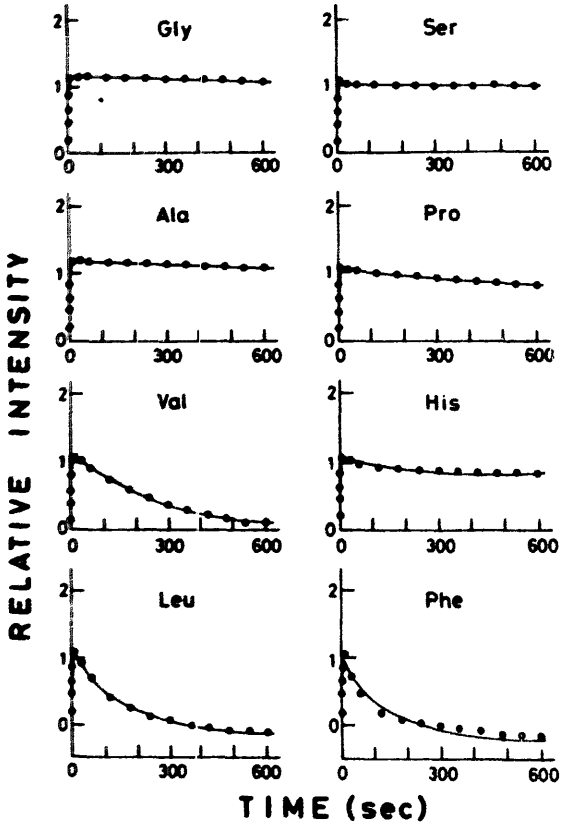

Fig. 3. Time-dependent changes in the intensity of 90° light scatter (arbitrary unit) when PC liposome suspension equilibrated with 20 mM sucrose was mixed with 100 mM amino acid solution in a 1 : 1 volume ratio. Initial rising phases are due to water efflux and the latter slow decreasing phases reflect the influx of amino acid. Zero intensity is taken at the unshrunken state of liposome. The marks of ● show the experimental points read from continuous records at 0.1, 0.3, 0.5, 1.0, 10, 30, 60, 120, 180, 240, 300, 360, 420, 480, 540 and 600 s. Although points at 0.2, 0.4, 0.8, 20, 40 and 50 were read, they are not drawn. Solid curves show the simulation results in which all points were equally weighted.

Table 3

Rate constants of amino acid influx k_A and their polydispersity indexes μ_{2A}/k_A^2

Amino acids	Types of liposome, k_A and μ_{2A}/k_A^2							
	PC		PC–Chol		PC–PA		PC–DCP	
	$k_A(sec^{-1})$	μ_{2A}/k_A^2	$k_A(sec^{-1})$	μ_{2A}/k_A^2	$k_A(sec^{-1})$	μ_{2A}/k_A^2	$k_A(sec^{-1})$	μ_{2A}/k_A^2
Phe	1.1E-2 [a]	0.39	2.2E-3	0.38	5.4E-3	0.65	4.4E-3	0.69
Leu	7.0E-3	0.27	9.4E-4	0.32	4.1E-3	0.64	2.7E-3	0.54
Val	2.8E-3	0.13	2.7E-4	0.014	1.6E-3	0.47	9.8E-4	0.17
Pro	4.9E-4	1.73	– [b]		7.8E-5	0.17	1.0E-4	0.10
Ser	3.2E-4	7.75	–	–	–	–	–	–
Ala	1.4E-4	0.25	–	–	–	–	–	–
Gly	1.3E-4	0.063	–	–	–	–	–	–
His	9.5E-4	3.02	–	–	–	–	–	–

[a] For example, 1.1E-2 means 1.1×10^{-2}.

[b] Values were not obtained by the light scattering method because that the permeability of these amino acids were not significantly larger than that of sucrose.

Table 4

Relative magnitudes of several amino acids in each type of liposomes

Amino acids	k_A/k_A(Phe) [a]			
	PC	PC–Chol	PC–PA	PC–DCP
Phe	1.00 [b]	1.00 [b]	1.00 [b]	1.00 [b]
Leu	0.64	0.42	0.75	0.61
Val	0.25	0.12	0.30	0.22
Pro	0.045	–	0.014	0.023

[a] k_A(Phe) is the rate constant of Phe influx.
[b] Defined to be unity.

mM sucrose was mixed with 100 mM amino acid solution in a 1 : 1 volume ratio. Marks of ● show the experimental points read from the original continuous curves. Simulations were done during a period of 9–10 min starting from the mixings. Solid lines show the simulation curves obtained with Eqn. 2 (parameters in Table 3). Similar experiments with PC–Chol, PC–PA and PC–DCP liposomes were done and results are listed in Table 3 (figures are not shown).

Amino acid permeabilities depended on their side-chain structure. In the PC–liposome, the order of magnitudes of k_A was: Phe > Leu > Val > His > Pro > Ser > Gly, Ala.

In the PC–Chol, PC–PA and PC–DCP liposomes, k_A for Ser, Ala, Gly and His were so small that these could not be determined under the condition that the sucrose permeability is negligibly small in comparison with these of amino acids. In these liposomes, the order of k_A for Phe, Leu, Val and Pro was the same with that of the PC liposome.

Relative magnitudes of k_A for Phe, Leu, Val and Pro in the same liposome showed a systematic change. They are approximately independent of the types of liposome as shown in Table 4 in which the rate constants of each amino acid were divided by the k_A for Phe. This suggests that the extent of hydrophobicity of amino acid is a significant factor in determining the permeability.

The amino acid permeability also changed according to the types of liposome. The order of k_A for the same amino acid was PC > PC–PA > PC–DCP > PC–Chol, which were independent of kinds of amino acid (Table 3). It is interesting to note that the corresponding order for water, k_w, was PC–PA > PC–DCP, PC > PC–Chol.

Discussion

It has been demonstrated that the cumulative expansion used in Eqn. 1 and 2 is applicable to present shrink–reswell processes of liposome which has polydispersity in regard to its size and permeability.

Klein et al. (1971) have shown that, using liposomes prepared from a mixture of PC–Chol–phosphatidyl serine (3 : 4 : 1), there is a correlation between the water–*n*-octane partition coefficient and the rate of efflux for amino acids except histidine

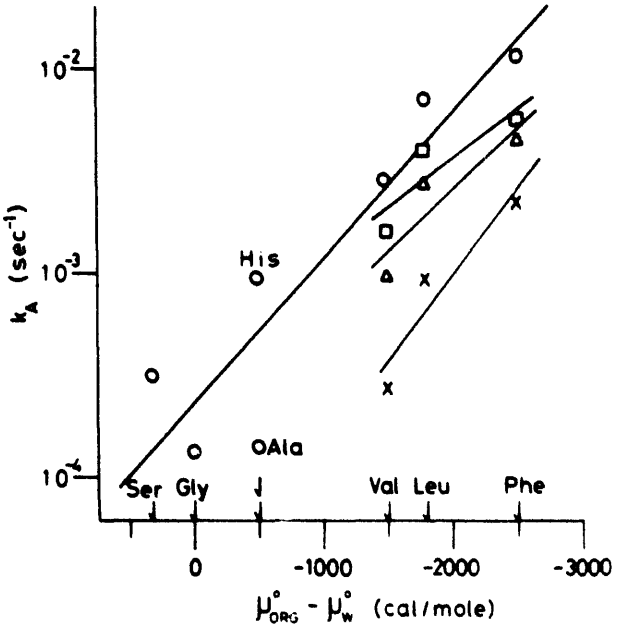

Fig. 4. Relationships between the rate constant of k_A and the $(\mu^\circ_{org}-\mu^\circ_w)$. The values of $(\mu^\circ_{org}-\mu^\circ_w)$ (see the text for the meaning) were taken from the paper by Nozaki and Tanford (1971). Linear lines were drawn by the last-squares method. ○, PC liposomes; ×, PC–Chol liposomes; □, PC–PA liposomes; and △, PC–DCP liposomes.

from isotope efflux measurements (tested amino acids; Phe, Met, Leu, Ile, Ala, Gly, His and norleucine).

Katz and Diamond (1974) have shown that the non-electrolyte partition coefficients between lecithin and water are qualitatively similar to those summarized by Overton's rules for permeation and partition in other non-polar solvent–water systems. Nozaki and Tanford (1971) reported the value of the additional free-energy of transfer from water to organic solvent, $(\mu^\circ_{org} - \mu^\circ_w)$, that is generated when the side-chain is substituted for the hydrogen atom of a glycyl residue. A relationship between log k_A and $(\mu^\circ_{org} - \mu^\circ_w)$ for PC liposomes is shown in Fig. 4. The straight lines were drawn by the least-squares method. Proline data was deleted because the value of $(\mu^\circ_{org} - \mu^\circ_w)$ for proline was not available. The straight line indicated that the partition coefficients are the major determinant of the relative magnitude of k_A. That is, a simple Overton's rule is held (Orbach and Finkelstein, 1980). A 10-fold increase in the k_A accompanied a -1430 cal/mol change in the $(\mu^\circ_{org}-\mu^\circ_w)$ for the PC liposome which was calculated from the value of slope in Fig. 4. From the theory of absolute rate processes (Glasstone et al., 1941), the ratio of rate constants between two species, p and q, is expressed as follows:

$$k_p/k_q = \exp(-(\Delta\mu^\circ_p - \Delta\mu^\circ_q)/RT) \quad (3)$$

where $\Delta\mu^\circ = \mu^\circ_{org} - \mu^\circ_w$. Eqn. 3 predicts that the 10-fold increase in the rate constant accompanies the change of -1380 cal/mol in the $(\mu^\circ_{org}-\mu^\circ_w)$, which is in a good agreement with the present value of -1430 cal/mol. This indicates that the values

of $(\mu^{\circ}_{org}-\mu^{\circ}_{w})$ given by Nozaki and Tanford are useful and reliable.

Proline also is suggested to follow Overton's rule from Table 4. Introducing the experimental value of k_A for proline into the linear relationship, the value of $(\mu^{\circ}_{org}-\mu^{\circ}_{w})$ was calculated to be -500 cal/mol.

In the PC liposome, k_A for alanine was almost equal to that of glycine. A similar, but more significant finding was reported that $k_{Gly} > k_{Ala}$ in the lysosomal membrane (Lloyd, 1971).

A linear relationship between log k_A and $(\mu^{\circ}_{org}-\mu^{\circ}_{w})$ had almost the same slope with that of PC liposome.

For PC–PA and PC–DCP liposomes, the relationships between log k_A and $(\mu^{\circ}_{org}-\mu^{\circ}_{w})$ for Phe, Leu and Val were curving and the slopes were different from those of PC and PC–Chol liposomes.

As expected, the addition of cholesterol into lecithin caused decreases in both permeabilities of water and all amino acids tested. Träuble (1971) developed a molecular model for the movement of water across lipid bilayers. In his model, structural defects, kinks, arising from gauche-trans-gauche conformations in the paraffin chains, diffuse along the chain, and water molecules can reside in the free volume created by the kinks and diffuse across the membrane. Hence, the fluidity of the hydrocarbon chains governs water permeability. Because the addition of cholesterol decreases the fluidity (Oldfield and Chapman, 1972; Shimshick and McConnell, 1973), the addition causes a decrease in the water permeability. Our present findings on water permeability are in accord with previous findings (Bittman and Blue, 1972; Block et al., 1977).

It has been suggested that cholesterol molecules, when introduced into the lecithin bilayer, enter into specific interactions with lecithin molecules (2 : 1 complex) (Engelman and Rothman, 1972) and that packing of the molecules becomes more condensed (Demel et al., 1967). Apparently, the present decrease in the permeabilities of amino acid is due to the condensing effect.

The addition of phosphatidic acid and dicetyl phosphate decreased the amino acid permeabilities, whereas the addition of phosphatidic acid increased water permeability significantly. Since the charged lipid decreases the hydrophobicity of bilayers, even if it makes a separate phase from lecithin as discussed later, the partition barrier from the aqueous phase into the lipid phase becomes harder than that of lecithin itself. This explains the decreases in permeabilities of amino acid.

There is a question as to why the addition of phosphatidic acid increased water permeability. Dipalmitoyl phosphatidic acid (the cheapest material commercially available) has a lipid phase transition temperature, T_c, of 67°C (Jain and Wagner, 1980). The T_c of egg yolk lecithin is -7 to -14°C (Ladbrooke et al., 1968). When these two lipids in which one has a gel state and the other a liquid-crystalline state at room temperature are mixed, a phase separation occurs (Kruyff et al., 1973). The gel phase has almost no permeability against the water molecule (Block et al., 1976). Present higher water permeability in PC–PA liposomes can be best explained by a postulate that water permeability through the regions of mismatch at the boundaries between discrete gel and liquid-crystalline phases is much greater than that in the liquid-crystalline state.

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