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CORRELATION BETWEEN MEMBRANE EXPANSION AND TEMPERATURE-INDUCED MEMBRANE FUSION

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Summary

For each phospholipid membrane, there is a characteristic phase transition temperature, and for each phospholipid spherical membrane, there is a specific 'fusion' temperature. In order to examine the possible correlation between temperature-induced membrane fusion and membrane expansion, the relationship between the physical states of phospholipid membranes at both temperatures have been investigated by the use of the monolayer system. Monolayer expansion studies have indicated that the increase in area per lipid molecule, caused by increasing the temperature from the phase transition to the fusion temperature, is approximately the same for five different phospholipids used. With the same temperature increase, phospholipid monolayers containing cholesterol did not expand appreciably. This correlates qualitatively with the greater inhibition of membrane fusion seen in the spherical phospholipid membrane systems when cholesterol was incorporated in the membrane.

The effect of pH on the expansion of phosphatidylserine monolayers was also studied in relation to membrane fusion phenomena. The shift in fusion temperature of the spherical phospholipid membranes due to the change of pH is explained by the shift in phase transition temperatures of lipid membranes. The expanded area per molecule in the monolayer caused by increasing the temperature from the phase transition to the fusion temperature was approximately the same irrespective of surface charge densities.

Introduction

A number of methods are currently being used to induce cell fusion [1,2]. These include: interaction with viruses [3—6], specific fusogens [7—11], spe-

cial macromolecules [12—16], divalent metal ions [1,17,18] and temperature changes [7]. It has been suggested that fusion is initiated by changes in membrane fluidity [19—22], surface tension or surface potential [23—26], membrane micellization [19,27,28], membrane instability [29] or molecular bridges [30,31]. In most instances, the actions of the fusing agents are not known, and the molecular mechanisms which occur during membrane fusion have not been elucidated. The main difficulty may be a consequence of the complex molecular structure of biological membranes. Recently, attempts to mimic the structure and functional properties of biomembranes have been made using model membrane systems [32—34]. The results of such studies suggest that artificially produced phospholipid membranes are very promising tools for studying particular molecular events relating to specific membrane functions.

It has been shown [21,22,29,35] that the fusion of phospholipid vesicles is related to membrane fluidity and instability associated with the gel-to-liquid crystalline phase transition of the lipid membranes. These fusion reactions are directly related to the strong binding of polyvalent ions with charged sites on the membrane. This polyvalent ion-induced membrane fusion has been studied with the use of other model membrane systems such as bilayer-vesicle [36—39], monolayer-vesicle [40] and the bilayer-bilayer [26,28,41,42] systems. On the other hand, spherical phospholipid bilayer membranes will fuse when the temperature is increased to a point characteristic for each phospholipid [26,28]. This temperature does not correspond to that of the phase transition of the phospholipid. The incorporation of lysophosphatidylcholine into phospholipid membranes lowered this fusion temperature by about 10°C, and incorporation of cholesterol inhibited the membrane fusion reaction drastically. It seems clear from the latter membrane fusion studies [26,28] that the temperature-induced membrane fusion corresponds to an increased state of fluidity and instability in the membrane, but not necessarily to the phase transition of the hydrocarbon region of the membrane. Breisblatt and Ohki [26] have proposed a mechanism of thermally induced membrane fusion which is based on an increased hydrophobic interaction at the interface between the two membrane surfaces in contact.

To test this proposal, surface tension experiments were performed with lipid monolayers at the air/water interface as a function of temperature. Firstly, keeping the surface pressure constant, the temperature was increased from the phase transition to their specific fusion temperatures, and then the increased areas per phospholipid molecule of the monolayers were measured. Another series of experiments was to measure the increase in surface pressure of monolayers with variation of temperature from its phase transition temperature to fusion temperature, keeping the area per molecule constant.

Effects of pH and cholesterol on membrane expansion as a function of temperature were also examined in relation to the temperature-induced membrane fusion phenomena.

Finally, a possible molecular mechanism for the temperature-induced membrane fusion is discussed.

Materials and Methods

Chemicals

Bovine brain phosphatidylserine and egg phosphatidylcholine were purchased from Avanti Biochemicals Co., AL. Dipalmitoyl, dimyristoyl and dioleoyl phosphatidylcholines were purchased from Applied Science Laboratories, Inc., PA. All samples showed single spots on silica gel thin-layer chromatographic plates. Cholesterol was obtained from Fisher Chemical Co. in crystalline form and was recrystallized with ethanol to remove oxidized cholesterol. Other chemicals used were all reagent grade from Fisher Chemical Co.

Monolayer-spreading solutions consisted of these phospholipids dissolved in $CHCl_3$. The exact concentrations of phospholipid in the spreading solutions were determined by phosphate analysis [42]. The subphase solution was 0.2 M NaCl containing 1 mM Tris-HCl buffer for pH 7.0, and 1 mM sodium citrate for pH 5.5 and 3.0. The pH of the subphase solution was checked before and after the experiment, and it assured no appreciable change of pH of the solution. Water was distilled three times, including the process of alkaline permanganate treatment.

Methods

Monolayers were formed by spreading an aliquot (approx. 10 μl) of the lipid samples upon the surface of 0.2 M NaCl solutions in a Langmuir trough. The dimensions of the Teflon trough were 15×5 cm^2. A Teflon bar was used as the moving bar for compression or expansion of the monolayers. A Wilhelmy plate was made by fusing a platinum wire with a microscope cover glass ($18 \times 18 \times 0.2$ mm). The plate was thoroughly cleaned before and after the experiments.

The surface tensions of the aqueous phase and the spread monolayers were measured with a Cenco de Nouy surface tension meter connected to the Wilhelmy plate. The accuracy of the measurements was ±0.2 dyne/cm. The temperature of the solution in the trough was varied by circulating a mixture of antifreeze and water through a coil in the subphase solution. The temperature of the circulating mixture was controlled by a combination of a cooler (Haake model FK) and water circulator (Haake model FS). The experimental temperature was that of the subphase solution which was accurate to within ±0.5°C.

To study the expansion of lipid monolayers with respect to temperature, a monolayer was formed at the air/water interface to give a pressure of 25 dyne/cm. Keeping the film pressure constant (25 dyne/cm), the temperature of the cell was varied from 1 to 2 degrees above the phase transition temperature to its fusion temperature in order to avoid complication due to the conformation change of the lipid at the phase transition point. For dioleoyl phosphatidylcholine and egg phosphatidylcholine, of which the phase transition temperatures are below 0°C, the temperature was increased from 0°C to their fusion temperatures. These expansion curves observed were then extrapolated back to the respective phase transition temperatures.

Dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine monolayers were formed initially at low surface pressure (5 dyne/cm) on the maximum area of the trough at room temperature (approx. 23—24°C). After the lipid solvent was evaporated, the dimyristoyl phosphatidylcholine monolayers

were compressed to 25 dyne/cm at 25°C, and then the temperature was increased to the fusion temperature keeping the surface pressure constant. For dipalmitoyl phosphatidylcholine, the temperature of the subphase solution was increased from room temperature to 43°C and the monolayer was compressed to 25 dyne/cm at 43°C, and then the temperature was increased. For phosphatidylserine, the monolayers were formed initially at low pressure (5 dyne) at room temperature, then the temperature of the subphase solution was cooled to 8°C, the monolayer was compressed to 25 dyne/cm, and then the temperature was increased to fusion temperature. For dioleoyl phosphatidylcholine and egg phosphatidylcholine, the monolayers were first formed at low pressure at room temperature, cooled down to 0°C, then the film was compressed so that the surface pressure was slightly greater than 25 dyne/cm, and then the temperature was increased. Especially in the latter two cases, a number of π-T curves were obtained, and these curves were extrapolated back to their phase transition temperature. Those extrapolated π-T curves which give a surface pressure of 25 dyne/cm at −22°C for dioleoyl phosphatidylcholine and −7°C for phosphatidylcholine were accepted as representative experimental data.

Results and Discussion

From the monolayer expansion data, the ratio of area per lipid molecule (A_T) at temperature T to that (A_{T_c}) at the phase transition temperature was plotted against temperature. At the fusion temperature, the ratio of A_T to A_{T_c} was found to fall within the range of 1.09—1.14 for all phospholipids studied. The results are shown in Fig. 1 and Table I. From the expansion curves, the ratio of the area per molecule at the phase transition temperature to that at the fusion temperature was calculated. Table I contains the values of ΔA and $\Delta A \cdot \pi$ (constant, 25 dyne/cm) for different phospholipids. Here, $\Delta A \cdot \pi$ is the intrinsic surface energy increase per molecule. It is seen from Table I that both the increase in area per molecule ΔA and $\Delta A \cdot \pi$ are in the range of 7—8 Å^2 and 170—200 $\cdot 10^{-16}$ erg/molecule, respectively, for all phospholipids. It is interesting to note that the change of area per molecule is rather independent of the hydrocarbon chain lengths and the presence of double bonds in the lipid molecules. A similar situation may occur in lipid bilayer systems. As the temperature is increased, the bilayer membrane may expand.

From detailed ESR and NMR studies [44,45], a great deal of knowledge about the polar region of phospholipid bilayers has been obtained. McFarland and McConnell [44] have demonstrated that fatty acid chains near the polar head groups are rather rigid and oriented at a 30° angle relative to the normal to the surface of the bilayer above the phase transition temperature T_c. Other studies [46—48] have shown that the fluidity of the hydrocarbon chains near the polar group region increases as the temperature is increased. Ohnishi and Ito [49] have demonstrated, with ESR studies using spin-labeled phospholipids, that sudden changes in membrane configuration for phospholipid vesicles take place at 43° C for phosphatidylcholine, and at 38° C for phosphatidylserine. These temperatures are similar to the fusion temperatures observed in the spherical bilayer membrane system. Experimental evidence for the expansion of bilayer

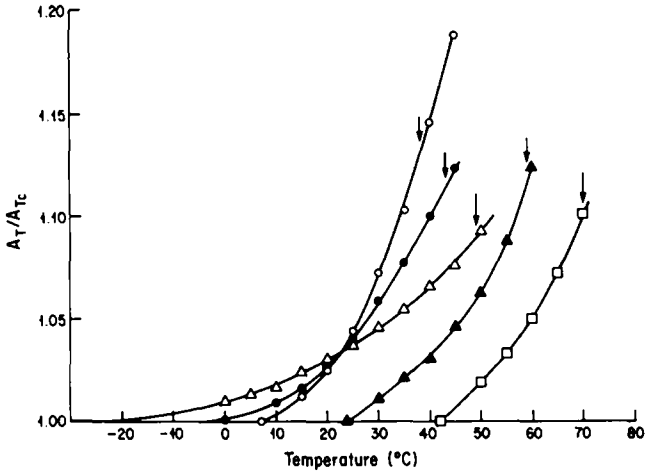

Fig. 1. Expansion curves for five different phospholipid monolayers at the air/water interface. The subphase was 0.2 M NaCl containing 1 mM Tris-HCl at pH 7.0. The ratio of the area per molecule at the fusion temperature to that at the phase transition temperature (solid-liquid crystalline) was plotted against temperature, keeping the surface pressure at 25 dyne/cm. ○, phosphatidylserine; ●, phosphatidylcholine; △, dioleoyl phosphatidylcholine; ▲, dimyristoyl phosphatidylcholine; □, dipalmitoyl phosphatidylcholine. The arrows indicate the 'fusion' temperature for respective spherical phospholipid membranes [26].

membranes with respect to increasing temperature has also been reported for the dipalmitoyl phosphatidylcholine membrane [48].

Another series of experiments involved measuring the surface pressure increases of monolayers as a function of increasing temperature, keeping the

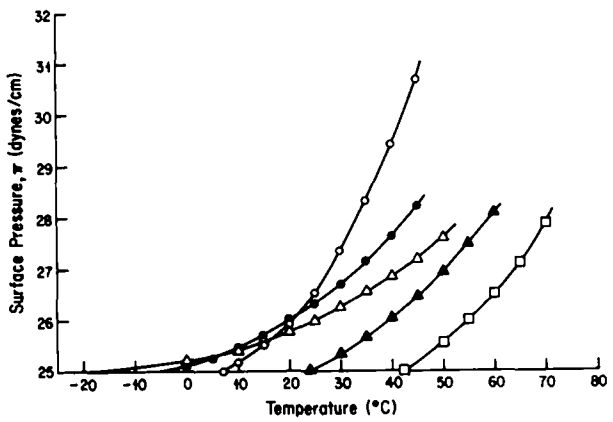

Fig. 2. Surface pressure-temperature relationship. The surface pressure was plotted against temperature when the area per lipid molecule was kept constant (59 $Å^2$ for phosphatidylserine, 70 $Å^2$ for phosphatidylcholine, 80 $Å^2$ for dioleoyl phosphatidylcholine, 60 $Å^2$ for dimyristoyl phosphatidylcholine and 64 $Å^2$ for dipalmitoyl phosphatidylcholine). At these areas per molecule, each lipid monolayer gave a surface pressure of 25 dyne/cm at the phase transition temperature (shown in Table I), ○, phosphatidylserine; ●, phosphatidylcholine; △, dioleoyl phosphatidylcholine; ▲, dipalmitoyl phosphatidylcholine; □, dimyristoyl phosphatidylcholine.

TABLE I

DOPC, dioleoyl phosphatidylcholine; PS, phosphatidylserine; DMPC, dimyristoyl phosphatidylcholine; PC, egg phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine.

Phospholipid	Phase transition temperature (T_c) (°C) *	Fusion temperature (T_f) (°C) **	Area per molecule at phase transition temperature (A_{T_c}) (Å^2)	Area per molecule at fusion temperature (A_{T_f}) (Å^2)	A_{T_f}/A_{T_c}	$\Delta A = A_{T_f} - A_{T_c}$ (Å^2)	$\pi_{T_c} \cdot \Delta A$ ($\times 10^{-16}$) (erg/molecule)
DOPC (pH 7)	−22	49	80 ± 0.6	87.2 ± 0.6	1.09	7.2	180
PS (pH 7)	7	38	59 ± 0.4	67 ± 0.5	1.135	8.0	200
DMPC (pH 7)	23	59	60 ± 0.4	67.1 ± 0.5	1.12	7.1	176
PC (pH 7)	−7	43	70 ± 0.5	77.7 ± 0.6	1.11	7.7	192
DPPC (pH 7)	41	70	64 ± 0.4	71.3 ± 0.5	1.11	7.3	181
PS (pH 3)	23	43			1.14		
PS (pH 5.5)	10	39			1.126		

* Ref. 53.

** Ref. 26.

TABLE II

DOPC, dioleoyl phosphatidylcholine; PS, phosphatidylserine; DMPC, dimyristoyl phosphatidylcholine; PC, egg phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine.

Phospholipid	Surface pressure at T_c (π_{T_c}) (dyne/cm)	Surface pressure at T_f (π_{T_f}) (dyne/cm)	$\Delta\pi = \pi_{T_f} - \pi_{T_c}$ (dyne/cm)	$A_{T_c} \cdot \Delta\pi$ ($\times 10^{-16}$) (erg/molecule)
DOPC	25 ± 0.2	27.5 ± 0.2	2.5	200
PS	25 ± 0.2	29 ± 0.2	4	236
DMPC	25 ± 0.2	28 ± 0.2	3	180
PC	25 ± 0.2	28 ± 0.2	3	210
DPPC	25 ± 0.2	27.9 ± 0.2	2.9	186

area per molecule constant. The results are given in Fig. 2 and Table II. The magnitudes of $A\Delta\pi$ are almost the same for all phospholipids used, where $\Delta\pi$ is the difference in surface pressures at phase transition temperature and fusion temperature. It is seen from Tables I and II that the quantity $A\Delta\pi$ is slightly greater than $\pi\Delta A$. This difference may come from the non-ideal situation of the two-dimensional molecules in the monolayer.

The charge of the membrane seems to have an effect on membrane fusion. The charged lipid membrane was more expanded than the uncharged one, and it was experimentally observed [50,51] that the charged lipid also had a lower phase transition temperature than the uncharged one. For example, the charged phosphatidylserine membrane at pH 7.4 has a phase transition temperature of approx. 7°C, whereas the same phospholipid membrane at pH 3.5 had a phase transition temperature of about 20°C [50]. The shift in the phase transition temperature due to the change of surface charge densities for phosphatidic acid membranes has been explained by the change in surface electrostatic energy [52]. It has also been found that the fusion temperature (38°C) of the phosphatidylserine spherical membranes increased to 39 and 43°C, when the pH of the solution was decreased from 7.0 to 5.5 and to 3.0, respectively [26].

Expansion of the phosphatidylserine monolayers at pH 3.0 and 5.5 from their phase transition temperatures (23°C at pH 3.0 and 10°C at pH 5.5) to the fusion temperature (43°C at pH 3.0 and 39°C at pH 5.5) was also investigated. The phase transition temperatures of 23°C for pH 3.0 and 10°C for pH 5.5 were estimated using the phase transition temperature data presented by MacDonald et al. [51] for dipalmitoyl phosphatidylserine membranes. The former value corresponds to that obtained by interpolating the data of phase transition temperatures for phosphatidylserine membranes observed by others [50]. The results are shown in Fig. 3. As in the previous case (pH 7.0), the values of $\pi\Delta A$ are approximately the same irrespective of the pH of the subphase solution and the same as those obtained for the other lipids used.

The monolayer expansion was drastically inhibited due to the presence of cholesterol in the membrane. Expansions of phosphatidylcholine and phosphatidylserine monolayers in the presence of equimolar cholesterol are shown in Fig. 4. The great suppression of monolayer expansion suggests that the fusion of cholesterol-incorporated bilayers will be inhibited greatly, which agrees with

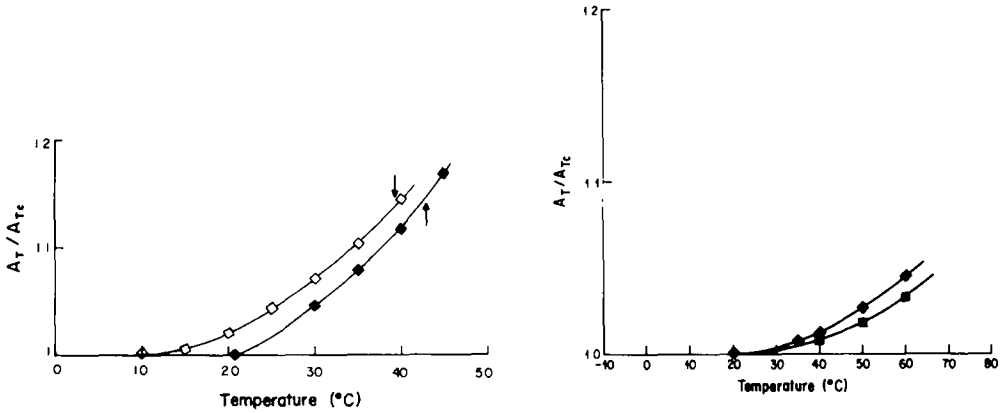

Fig. 3. Expansion curves of phosphatidylserine monolayer at pH 3 and 5.5. The ratio of the area per molecule at an experimental temperature to that at the phase transition temperature, keeping the monolayer surface pressure constant at 25 dyne/cm, is plotted with respect to temperature. ■, pH 5.5; □, pH 3.0. The arrows indicate the fusion temperature of respective spherical phospholipid membranes [26].

Fig. 4. Expansion curves of phosphatidylserine and phosphatidylcholine monolayers in the presence of equimolar cholesterol in the monolayers. The ratio of the area per molecule at any temperature to that at the phase transition temperature (corresponding to pure phosphatidylcholine and phosphatidylserine) was plotted against temperature. ◆, phosphatidylserine/cholesterol; ■, phosphatidylcholine/cholesterol.

the experimental observation in the phospholipid spherical membrane systems. This is further evidence that the membrane expansion is related to temperature-induced membrane fusion events.

From the above studies, we have seen that the increase in area per molecule may be the major factor for the temperature-induced membrane fusion. As a consequence of an increase in the area per molecule, there are two accompanying factors: (1) an increase in the area of the hydrocarbon phase exposed to the aqueous phase, and (2) disorder of structural water at the membrane surface. These factors may render the membrane surface more hydrophobic. At the critical temperature, this new configuration occurring in the membrane may become sufficient to induce membrane fusion of two closely apposed membranes.

It should be noted, however, that the degree of the increase in area per molecule in the lipid monolayers may not be the same as that in the lipid bilayers because the molecular environments are different between the bilayer and the monolayer formed at the air/water interface. In the case of a bilayer system, when the temperature is increased, both surface pressure and the surface area per molecule of the bilayer membranes would be altered.

It is found here that the intrinsic energy increases of the monolayer during the change of temperature from the phase transition to the fusion temperature, $A\Delta\pi$ or $\pi\Delta A$, which does not include the water surface energy, were about the same for all phospholipids examined in this work. The main change in the physical state of a bilayer due to the change of temperature seems to be the change in area per molecule, rather than the change in film pressure due to lipid hydrocarbon chains. However, further investigation is necessary to conclude explicitly the above statement.

The effect of lipid dissolution on the surface properties has been neglected

while studying π-A isotherms and monolayer expansions. However, this is justified because the dissolution rates of phospholipids are very small and negligible for the temperature and time limits of the experimental conditions used. The organic solvents possibly retained in the bilayer and monolayer membranes may play some role in membrane fusion and thermal membrane expansion. However, Breisblatt and Ohki [26] clearly obtained distinct differences in fusion temperature for different phospholipid spherical membranes which seem to indicate that the role of the organic solvent may not be a predominant factor for this membrane fusion. Also, the rate of thermal expansion of phospholipid monolayers is different for each phospholipid, and correlates well with the fusion data; this indicates that the organic solvent probably does not play an predominant role in these fusion and expansion processes. Nevertheless, it should be borne in mind that the different behaviors of fusion events observed in phospholipid membranes with and without organic solvents may be attributed to the solvent effect. The choice of a constant pressure of 25 dyne/cm for the monolayer expansion studies was rather arbitrary. If we chose a different value for constant pressure, the magnitude of the expanded area per molecule of the lipid monolayer would be different when the temperature is varied from the phase transition to the fusion temperature. However, as far as the relationship between the relative magnitude of the membrane expansion and the fusion temperature is concerned, the choice of the pressure does not affect the final argument.

Although there are several assumptions which ought to be clarified in the future to understand the molecular mechanism of fusion, the result obtained from our monolayer studies may be relevant for a qualitative analysis of membrane fusion.

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