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Fusion in Phospholipid Spherical Membranes

II. Effect of Cholesterol, Divalent Ions and pH

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Summary. Effect of cholesterol, divalent ions and pH on spherical bilayer membrane fusion was studied as a function of increasing temperature. Spherical bilayer membranes were composed of natural [phosphatidylcholine (PC) and phosphatidylserine (PS)] as well as synthetic (dipalmitoyl-PC, dimyristoyl-PC and dioleoyl-PC) phospholipids.

Incorporation of cholesterol into the membrane (33% by weight) suppressed the fusion temperature and also greatly reduced the percentage of membrane fusion. The presence of 1 mM divalent ions (Ca^{++}, Mg^{++} or Mn^{++}) on both sides or one side of the PC membrane did not affect appreciably its fusion characteristic with temperature, but the PS membrane fusion with temperature was greatly enhanced by the presence of divalent ions.

The variation of pH of the environmental solution in the range of 5.5~7.0 did not affect the membrane fusion characteristic. However, at pH 8.5, the fusion with respect to temperature was shifted toward the lower temperature by approximately 3 °C for PC and PS membranes, and at pH 3.0 the opposite situation was observed as the fusion temperature was increased by 6 °C for PS membranes and by 4 °C for PC membranes

The results seem to indicate that membrane fluidity and structural instability in the bilayer are important for membrane fusion to occur.

It has recently been reported that spherical bilayer membranes of the naturally occurring phospholipids [phosphatidylcholine (PC) and phosphatidylserine (PS)] undergo greater degrees of membrane fusion at temperatures specific for each phospholipid (PC–43 °C, PS–38 °C) (Breisblatt & Ohki, 1975). The incorporation of lysolecithin into these membranes lowers the characteristic fusion temperature by almost 10 °C. In this paper further results are reported using the spherical bilayer membrane system, which seem to indicate that both membrane fluidity as well as the creation of structural instability in the bilayer membrane are responsible for fusion in this model membrane system.

Membrane fluidity seems to be an important criteria for the fusion reaction. Poste and Allison (1971) have proposed a mechanism for fusion

which relies on the fluidity of the membranes as one of the prerequisites for the fusion reaction. Also the importance of fluidity for cell fusion has been suggested in the chemically and thermally induced fusion studies of Ahkong, Cramp, Fisher, Howell, Tampion, Verrinda and Lucy. Papahajopoulos, Poste and Schaeffer (1973) and Papahadjopoulos, Poste, Schaeffer, and Vail (1974) have also shown that phospholipid vesicle fusion is highly dependent on membrane fluidity.

Membrane fusion also appears to be closely related to structural changes which may occur in the membrane bilayer. Lucy (1964, 1970) has suggested that micelle formation in the membrane may be the key to the fusion reaction. Experiments that he and his group have conducted with lysolecithin and some short chain fatty acids seem to indicate that some perturbation of the bilayer structure may be important for the fusion reaction to occur (Poole, Howell & Lucy, 1970; Ahkong, Tampion & Lucy, 1974). This kind of perturbation could be produced by increased temperature, incorporation of some substances into the membrane, or by changing the ionic environment. The first two have been well documented both by Lucy's group and our laboratory (Breisblatt & Ohki, 1975) and further evidence is presented here. It has been suggested that changes in the ionic environment can result in conformational changes in the bilayer structure (Ohki & Aono, 1970; Ito & Ohnishi, 1974; Träuble & Eibl, 1974; Jacobson & Papahadjopoulos, 1975). Many authors have felt that divalent ions and in particular Ca^{++} ions may play an important role in the fusion process (Lucy, 1964; Woodin & Wieneke, 1964; Okada & Murayama, 1966; Poste & Allison, 1971; Poste & Reeve, 1972; Okada, Takeichi, Yasuda, & Masamichi, 1974). Recently, Papahadjopoulos, Vail, Jacobson and Poste (1975) have demonstrated that vesicle fusion is enhanced in the presence of calcium ions. Liberman and Nenashev (1972*a*; 1972*b*) and Neher (1974) have studied membrane adhesion in hemispherical membranes and have shown that calcium ions may play a significant role in the fusion process. Also, many of the mechanisms proposed to explain the fusion event have incorporated the idea that Ca^{++} ions are in some way associated with the fusion process (Poste & Allison, 1971). Other divalent ions have also been shown to play a similar role in the fusion process but to a lesser extent than Ca^{++} (Okada *et al.*, 1974).

The membrane structure could be changed by changing the other environmental parameters; changing the pH as well as the monovalent ion concentration can affect membrane fluidity as well as the bilayer structure. Träuble and Eibl (1974) have shown that changes in pH can

affect the phase transition temperatures (crystalline to liquid crystalline) of phospholipids which indicate changes in membrane fluidity.

Membrane fluidity can be increased by incorporating substances into the membrane such as short chain fatty acids and possibly lysolecithin (Haydon & Taylor, 1963; Poole *et al.*, 1970; Lucy, 1970) but membrane fluidity can be decreased by incorporating cholesterol into the membrane. Cholesterol creates a more rigid and constrained membrane structure when it is incorporated into the bilayer in the liquid crystalline state (Ladbrooke, Williams & Chapman, 1968; Papahadjopoulos, Nir & Ohki, 1971; Engelman & Rothman, 1972). Therefore, by the addition of cholesterol into the membrane, fusion can be analyzed from another perspective which may be important in the determination of a mechanism for the fusion process.

In this paper the effect of fluidity and instability of the membrane was studied on the fusion process in spherical bilayers. Cholesterol was added to the membrane forming solutions of PC and PS to test its effect on the fusion temperatures of these phospholipids. The role of divalent cations Ca^{++}, Mg^{++} and Mn^{++} on the fusion of PC and PS spherical bilayer membranes were also studied. The effect of these ions were examined for both symmetrical and asymmetrical distributions. Fusion was looked at in relation to pH variations. Some of the synthetic phospholipids of PC were also studied for their ability to fuse with increasing temperature. Since the synthetic phospholipids are more clearly defined than the natural phospholipids for their molecular composition, we can examine the effects of increasing chain length and unsaturation which are clearly related to membrane fluidity. In conclusion a mechanism for fusion is discussed in relation to both membrane fluidity and structural instability in the membrane.

Materials and Methods

Phospholipids for the spherical membrane studies (PC-egg and PS-bovine) were purchased from Applied Science Laboratories (State College, Pa.) and Supelco Inc. (Bellefonte, Pa.). Applied Science Lab's samples of 10 mg were stored in chloroform solution, and Supelco Lab's samples of 25 mg were divided into three samples of 8.3 mg each which were stored in chloroform in the refrigerator at -15 °C. The synthetic phospholipids [dimyristoyl-PC (DMPC), dioleoyl-PC (DOPC), dipalmitoyl-PC (DPPC)] were purchased from Applied Science Laboratories and were stored in chloroform, except for DPPC which was obtained as powdered form and was prepared as needed. The purity of these samples was said to be more than 97% pure. All lipid samples were chromatographically pure

and showed only a single spot under TLC analysis. Preparation of the membrane forming solution was as previously reported (Breisblatt & Ohki, 1975). Cholesterol was purchased from Fischer Chemical (certified grade), and recrystallized in our laboratory by use of ethanol. 33% or 50% cholesterol by weight, was added to lipid samples of PC and PS. The cholesterol was dissolved in decane at 60 °C. The decane-cholesterol solution was then cooled down to room temperature before it was added to the rest of the membrane forming solution. In this case, also, the membrane forming solution had a constant lipid-solvent ratio; 10 mg of lipid and cholesterol in the solvent (0.4 ml chloroform, 0.3 ml methanol and 0.3 ml *n*-decane).

Spherical bilayers were formed in a layered concentration gradient containing a base solution of 4.5 M NaCl and an upper solution of 0.01 M NaCl. The inside solution for the membranes was 0.2 M NaCl. The procedure for preparing the membranes was similar to that of Pagano and Thompson (1967) and was modified by Breisblatt and Ohki (1975). Osmolarities of inside and outside of the spherical membrane was not measured but it was considered that they may have been approximately the same on both sides because the membranes apparently kept a constant size and position in the concentration gradient.

Membranes were observed through a low power microscope (Unitron MSF) and the bubble membranes were illuminated with a microscope illuminator (American Optics). Bilayer formation could be observed and was previously described (Breisblatt & Ohki, 1975). Temperature was varied during the experiment by using a water jacket and a thermoregulator (Neslab Instruments). pH measurements were taken at the beginning and end of each experiment to insure that the proper pH range was not exceeded, (pH 6.0–6.6 at 25 °C). Within this range of pH the same experimental results were obtained.

Membranes were formed in close proximity (within several mm) to facilitate membrane contact. We examined membrane contact and fusion with increasing temperature. The membrane contact was defined as the formation of a stable doublet and fusion was defined as the two cells (doublet) forming one. Fusion temperature (T_f) was defined as the temperature corresponding to the sharpest rise in the "percentage of fusion"-temperature curve. For each study approximately 10 membranes were made and the experiments for each phospholipid were repeated many times (more than 200 membranes for each phospholipid). All temperature dependent studies were made after bilayer formation was completed for all membranes. Temperature was increased from 25 to 65 °C successively and each study was completed in about 40 min (approximately 5 min was spent for a 5 °C increase in cell temperature) at 65 °C (70 °C for some of the studies). Experiments were also done in which membrane fusion was observed at fixed temperatures in the range of 25–60 °C. These experimental results were similar (with a 3 °C range) to the T_f's reported in this paper.

To study the effect of divalent ions, all salt solutions (4.5, 0.01, and 0.2 M NaCl) contained 1 or 10 mM of $CaCl_2$, $MgCl_2$ or $MnCl_2$. Equimolar concentrations of the divalent ions were placed on both sides of the membrane to test the symmetrical case, and to create an asymmetrical distribution; the salt solutions containing 1 mM of the divalent ions were placed on the outside of the membrane only. The pH was held constant at 7.0 with a 5 mM Tris buffer for all experiments with divalent cations.

The effect of pH on membrane fusion was studied and compared at different pH values: 3.0, 5.5, 7.0, and 8.5. The pH of all salt solutions was adjusted and checked with a pH meter (radiometer, London, Ohio) before and after each experiment. For the experiments at pH 8.5, a 5 mM Tris buffer (Sigma 121) was used to maintain the pH. The Tris buffer was titrated with HCl. Phosphate buffer 10 mM (potassium monobasic) was used to adjust solutions at pH's 5.5 and 7.0. The phosphate buffer monobasic was titrated with NaOH to obtain the desired buffering capacity. Experiments at pH 3.0, were adjusted using a 5 mM citrate buffer, which was titrated with NaOH to the proper pH. During the experiments the pH of the solutions remained fairly constant, varying

by only 0.1 pH unit. Except for the change in pH, membrane fusion experiments were conducted using the same procedure.

It should be mentioned that aging of lipid with time might attribute to instability of the membrane which may affect the degree of membrane fusion. However, this factor has not been taken into account in the results.

Results

The effect of cholesterol on the fusion of PC and PS spherical bilayers was studied with increasing temperature. It had previously been reported

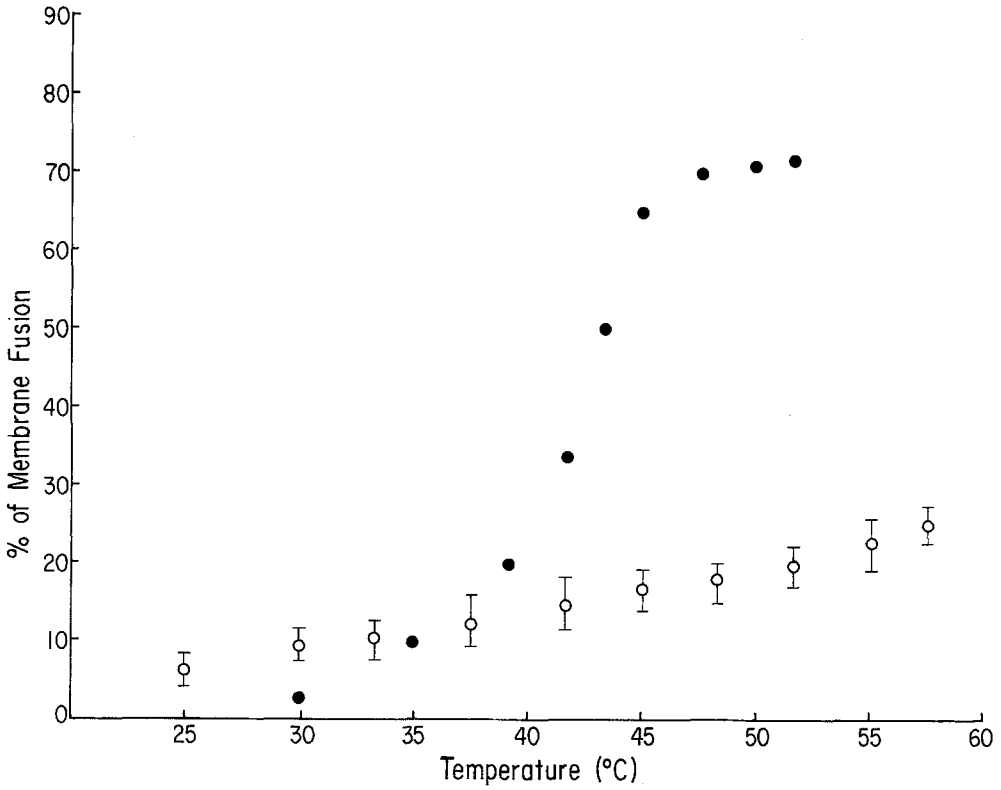

Fig. 1. Effect of cholesterol on PC membrane fusion. ● % of PC membrane fusion without cholesterol; ○ % of PC membrane fusion with cholesterol (33% by weight). Error bar was calculated by using the SE formula

$$\mathrm{SE}=\{\sum_{i}^{N}(x_i-\bar{X})^2/N-1\}^{1/2},$$

where x_i=the i-th experimental value, $\bar{X}$=the mean value of the experimental value, and N=the total number of measurements

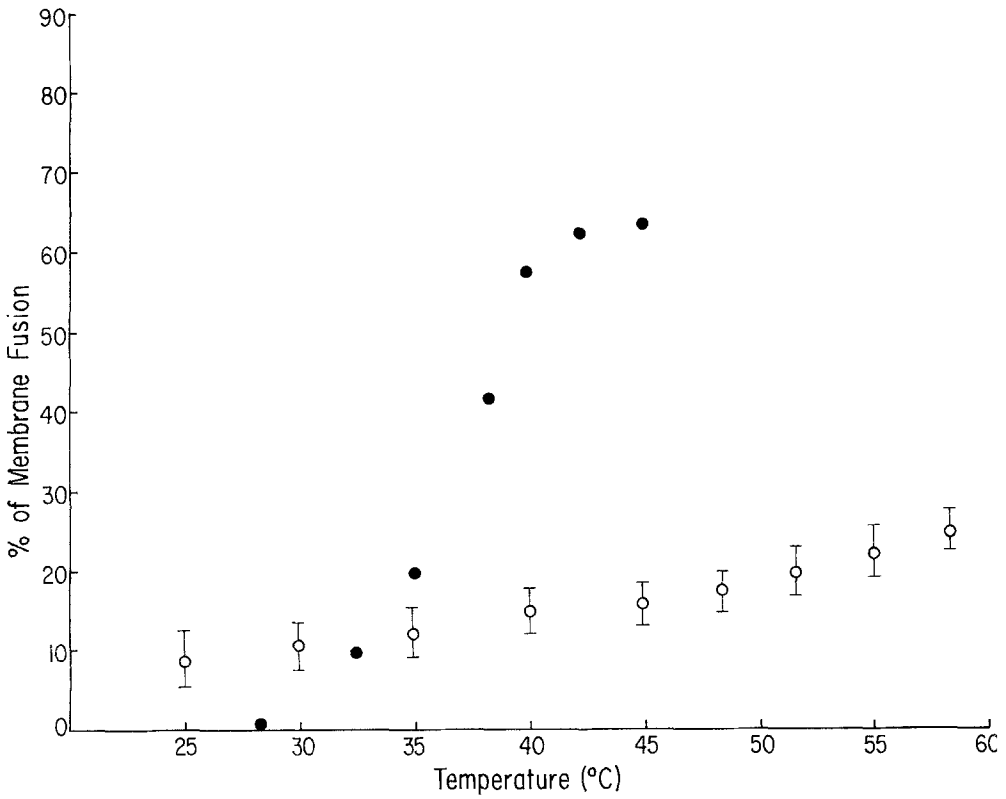

Fig. 2. Effect of cholesterol on PS membrane fusion. ● % of PS membrane fusion without cholesterol; ○ % of PS membrane fusion with cholesterol (33% by weight). Error bar was calculated from the SE formula

(Breisblatt & Ohki, 1975) that both PC and PS membranes demonstrated characteristic fusion temperatures (T_f-fusion temperature, PC−43 °C, PS−38 °C) for the case without cholesterol, where fusion temperature, T_f, was defined as that corresponding to the sharpest increase in *percentage of fusion* with respect to temperature. But in incorporation of cholesterol (both 33 and 50% by weight) into the membrane, these fusion temperatures were inhibited. As shown in Fig. 1, the percentage of membrane fusion is plotted for both PC membranes with and without 33% cholesterol. Fusion is greatly inhibited in the case with cholesterol (Fig. 1). Although membrane fusion increases over the temperature range of the experiment, the percentage of membrane fusion never rose above 30% for PC membranes. Fig. 2 indicates similar results for PS membrane fusion, in which a comparison is shown between PS membrane fusion with and without cholesterol (33%). Cholesterol concentrations of 50% by weight gave

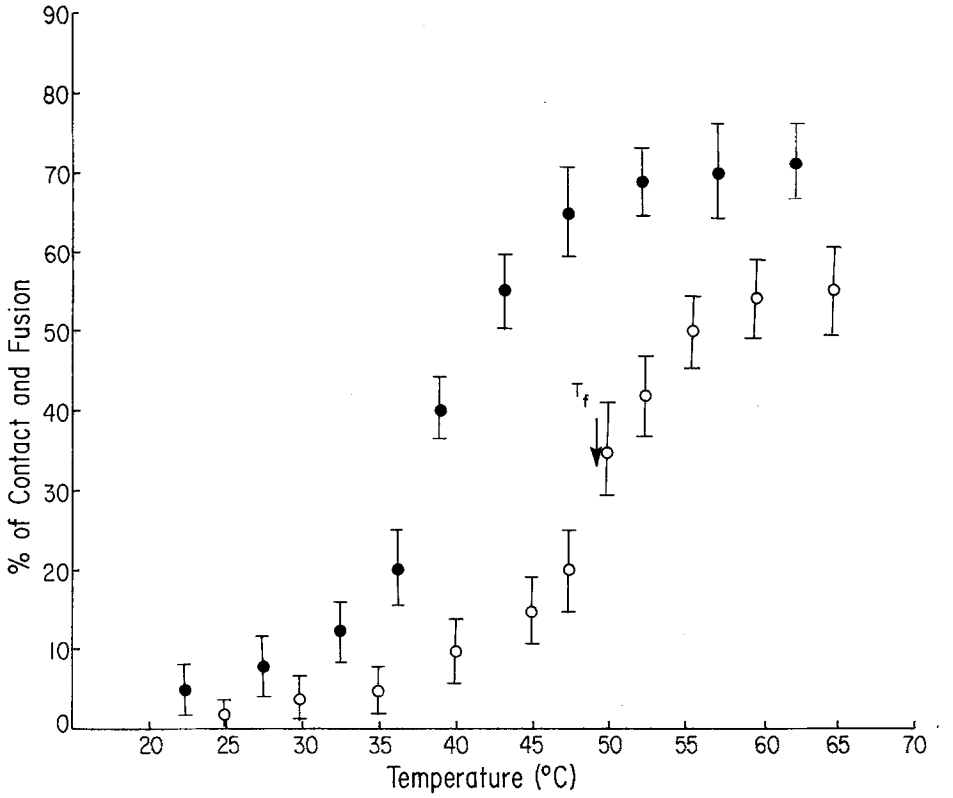

Fig. 3. Contact and fusion in DOPC membrane. ● % of membranes coming into contact; ○ % of membranes in contact that fuse; T_f fusion temperature (temperatures corresponding to the sharpest slope in the curve)

similar results to those obtained with the lower cholesterol concentration (33%) for both PC and PS membranes. As previously reported, our measurement of membrane fusion is based on only those membranes that have come into contact (doublet) and then form a larger singlet. It should be noted that the real content of cholesterol in the membrane may not necessarily be those of the cholesterol percentages of the membrane forming solution. Also the incorporation of cholesterol into membranes of PC and PS increased their stability and raised the breaking temperature range[1] (45 ~ 50 °C for PS membrane, 52 ~ 66 °C for PC membranes without cholesterol) to 60 ~ 65 °C for both PS and PC spherical membranes.

[1] Here, the breaking temperature range means that most of the spherical membranes (more than 95%) break above this temperature range as described in the earlier paper (Breisblatt & Ohki, 1975).

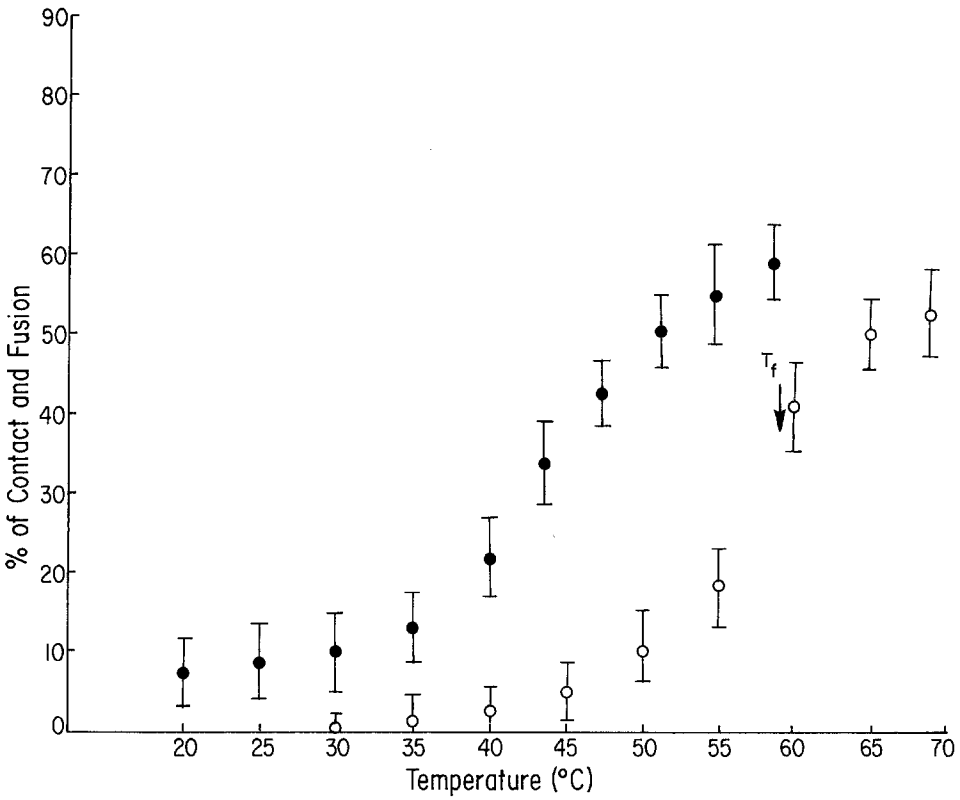

Fig. 4. Contact and fusion in DMPC membrane. ● % of membranes coming into contact; ○ % of membranes in contact that fuse

Fusion was also studied in some of the synthetic phospholipids of PC with increasing temperature. These phospholipids have a clearly defined molecular composition which allows us to evaluate the role of membrane fluidity in the fusion process (DMPC, 14 C per alkyl chain; DPPC, 16 C per alkyl chain; DOPC, 18 C (one double bond) per alkyl chain). Membrane contact and fusion of these phospholipids are shown in Figs. 3, 4 and 5. Each of these phospholipids demonstrates a characteristic fusion temperature (T_f) similar to those previously reported for PC and PS membranes. DOPC showed a sharp rise in membrane fusion at approximately 49 °C. For DMPC this temperature was 59 °C. The T_f for DPPC was not as clear cut as the others, because of rather gradual increase in fusion-temperature curve (up to 70 °C) and technical difficulty in observing fusion occurring above 70 °C. If one looks at the values obtained for the T_f for egg PC and some of its synthetics, the following pattern is seen: PC < DOPC < DMPC < DPPC. This order

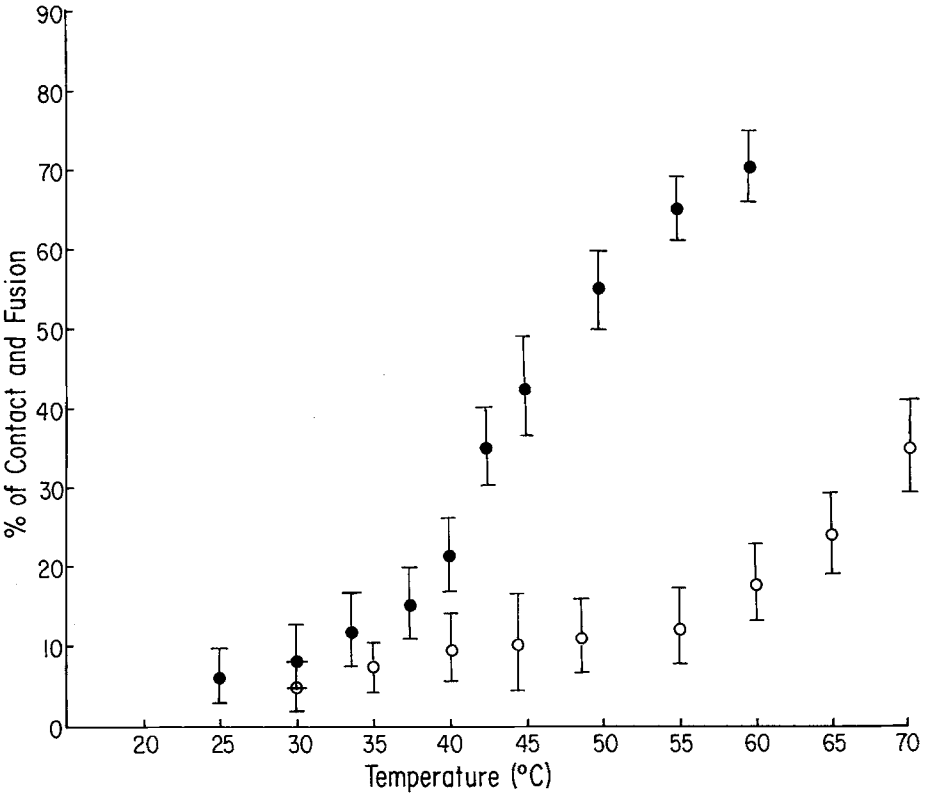

Fig. 5. Contact and fusion in DPPC membrane. ● % of membranes coming into contact; ○ % of membranes in contact that fuse

corresponds to that of phase transition temperature for PC. Phosphatidylcholine (egg) and DOPC are the most fluid of these phospholipids at 25 °C (solid-liquid crystalline phase transition temperature T_c is about −10 °C for egg−PC and DOPC (Ladbrooke & Chapman, 1969)), and characteristically they show the lowest T_f. DPPC (its T_c=41 °C) is the least fluid and indicates an extremely high fusion temperature, possibly more than 70 °C although it is not well determined experimentally, while DMPC (its T_c=23 °C) falls between these two groups.

The effect of divalent cations on the fusion of PC and PS spherical membranes was studied with respect to temperature. Experiments were done at two concentrations, 1 and 10 mM of divalent ions, and the chloride salts of these divalent cations were used for all solutions. In studying the symmetrical case, equimolar concentrations of the divalent ions were placed on both sides of the bilayer membrane. The effect of these cations on the fusion of PC and PS can be seen in Figs. 6 and 7. It is fairly

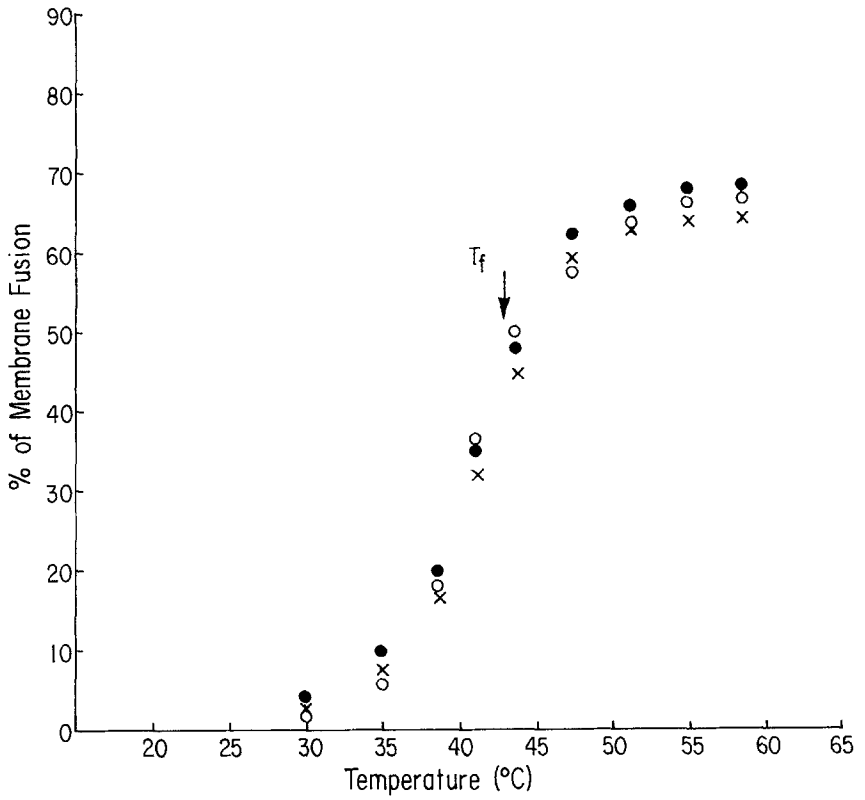

Fig. 6. Effect of divalent ions on PC membrane fusion; Symmetrical distribution of divalent ions (1 mM). ● Calcium; ○ Magnesium; and × Manganese. Maximum error is calculated as ±7% for an experimental point

clear from Fig. 6, that none of these divalent cations affects the fusion of PC membranes, as in all three cases the T_f for PC membrane fusion remains unchanged from those in the absence of divalent ions. But for PS membranes (Fig. 7), the divalent cations affect the fusion process by making it relatively independent of temperature. Therefore it appears as though the fusion temperature is abolished (a typical sharp increase with temperature is not seen in the curve). Ca^{++} (1 mM) exerted the strongest effect over PS membrane fusion among the divalent salts tested; about 40% membrane fusion occurred over all temperature ranges. Mg^{++} and Mn^{++} showed behavior similar to Ca^{++} except that they both show some temperature dependence. Mn^{++} exhibited more temperature dependence than the others. 10 mM concentrations of the divalent ions affected PS membrane fusion in a manner similar to the 1 mM concentrations. In all three cases the divalent cations affected PS membrane contact,

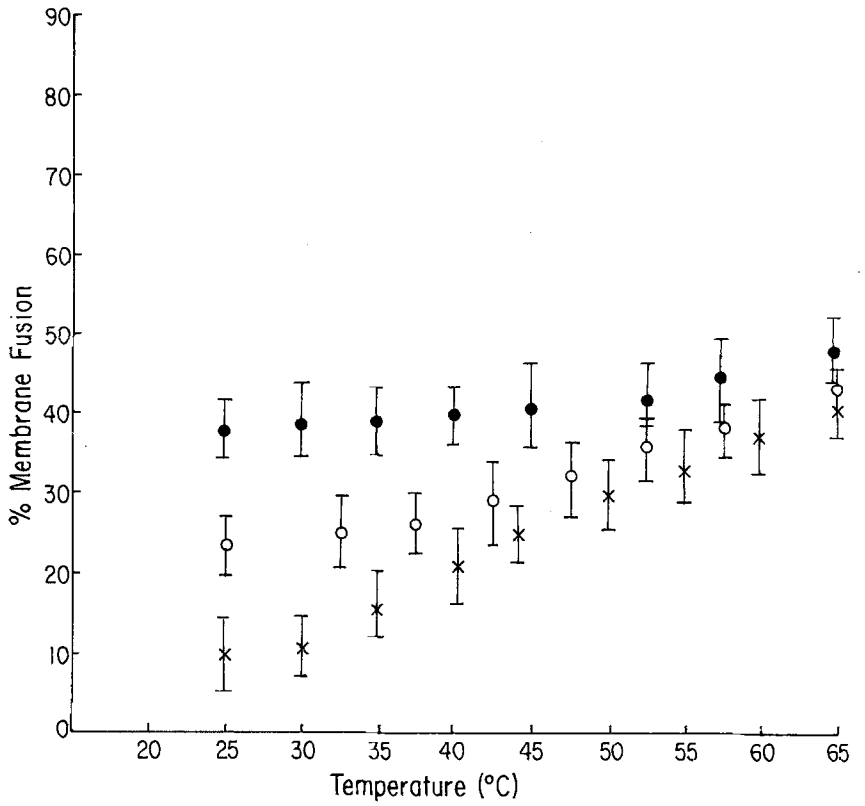

Fig. 7. Effect of divalent ions on PS membrane fusion; Symmetrical distribution of divalent ions (1 mM). ● Calcium; ○ Magnesium; and × Manganese

causing almost 90% of the membranes to form doublets and in many cases aggregate formation (more than two spherical membranes) did occur. While it is conceivable that the divalent cations may act as bridging agents between PS membranes there may be other explanations for the above observations which will be examined in the discussion section.

Fusion was also studied from the standpoint of an asymmetrical divalent cation distribution. This was accomplished by placing salt solutions containing 1 mM divalent cations on the outside solution of the membrane and the results obtained are seen in Figs. 8 and 9. By imposing the asymmetrical distribution the fusion of both PC and PS membranes was affected. Although ionic strength due to the asymmetric distribution of divalent ions was not compensated, PS membrane fusions were similar to those observed in the symmetrical case, except that the percentages of membrane fusion observed were 10–25% higher than in the symmetrical case depending on the divalent cation used.

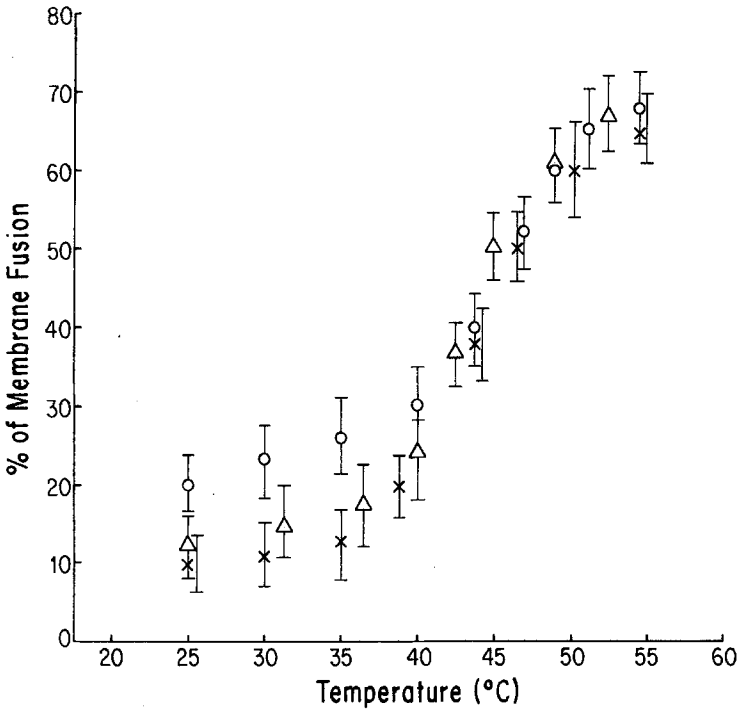

Fig. 8. Effect of divalent ions on PC membrane fusion; Asymmetrical distribution of divalent ions (1 mM). ○ Ca^{++}; △ Mg^{++}; × Mn^{++}

The order of effect of the divalent cations on PS membrane fusion is as follows: $Ca^{++} > Mg^{++} > Mn^{++}$ (Fig. 9). For PC membranes fusion curves similar to those without divalent ions are obtained (Fig. 8) and fusion temperature remains constant at 43 °C. Ca^{++} produces the greatest change in the PC and PS fusion curves (Fig. 8 and 9) as a greater percentage of membrane fusion is observed at the lower regions of the curve. Similar effects are seen for Mg^{++} and Mn^{++} (Fig. 8 and 9) as a greater percentage of membrane fusion is observed at the lower regions of the curve. Similar effects are seen for Mg^{++} and Mn^{++} (Figs. 8 and 9) but to a lesser extent than those seen for Ca^{++}.

By creating an asymmetrical distribution some instability may have been introduced which, especially in the case of PS membranes, may have contributed to the increased percentage of fusion while in the case of PC it is able to affect the percentage of membrane fusions without affecting the T_f. It has previously been demonstrated that an asymmetrical distribution of divalent cations (Ca^{++}) can lead to instability in

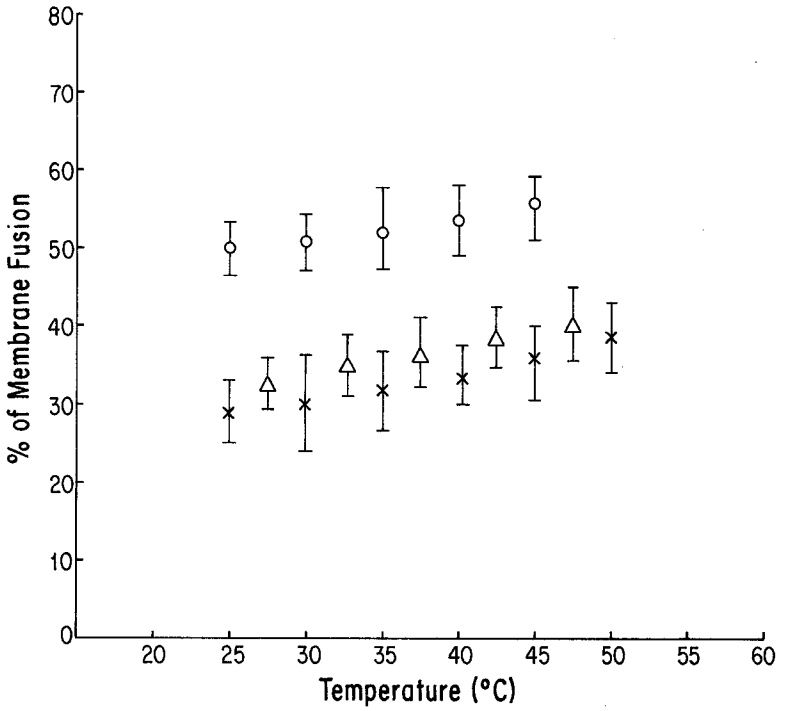

Fig. 9. Effect of divalent ions on PS membrane fusion; Asymmetrical distribution of divalent ions (1 mM). ○ Ca^{++}; △ Mg^{++}; and × Mn^{++}

the bilayer structure of the membrane (Ohki & Papahadjopoulos, 1970). It is also significant that membrane breakage was higher than in the case without Ca^{++}, which was what one might expect due to the nature of the asymmetrical distribution of Ca^{++}. In addition it was observed that there was no clear breakage range for the membranes, as membrane breakage was almost independent of temperature.

The effect of pH on the fusion of both PC and PS membranes was studied, as shown in Figs. 10 and 11. Fusion was studied at four different pH values; 3.0, 5.5, 7.0 and 8.5. For both PC and PS membranes, the T_f (43 °C, PC; 38 °C, PS) for membrane fusion (at pH 6.0 ~ 6.6) remained constant at pH's 5.5 and 7.0, and the fusion curves were very similar to those reported for PC and PS with temperature. However, at pH 8.5 a shift of about 3 °C toward a lower temperature occurred in both the PC and PS fusion curves and this naturally decreased the T_f by approximately 3 °C. At pH 3.0 another shift in the fusion curves of PC and PS is seen in Figs. 10 and 11, but in this case the T_f for membrane

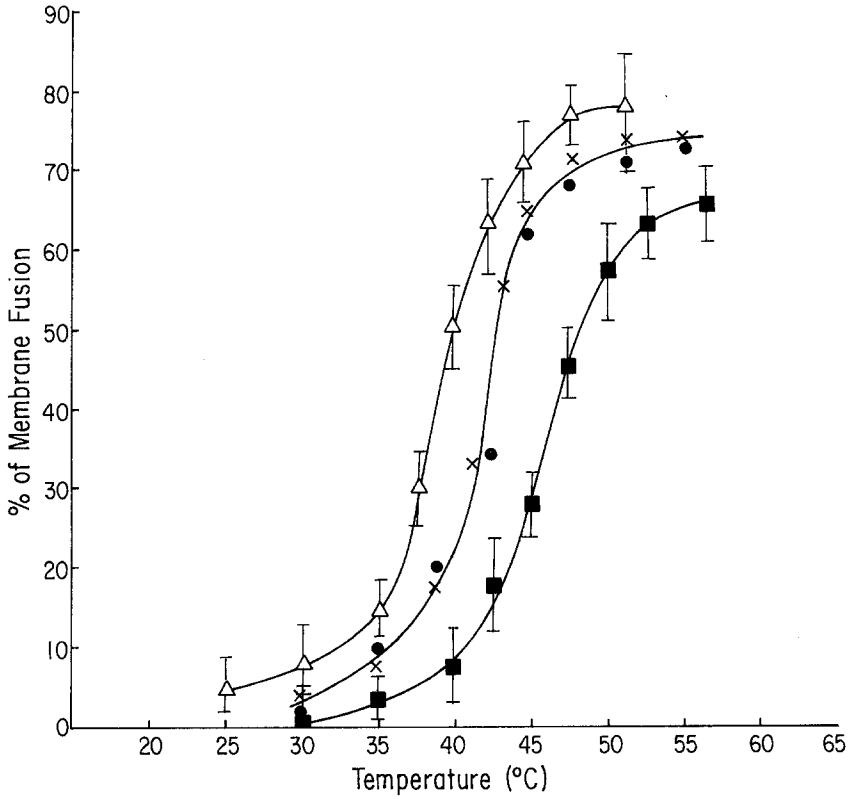

Fig. 10. Effect of pH on the fusion of PC membranes. △=pH 8.5; ●=pH 7.0; ×=pH 5.5; and ■=pH 3.0

fusion has been increased by almost 6 °C for PS and 4 °C for PC membranes, respectively. The reproducibility of these results was extremely good.

Discussion

The present studies suggest that membrane fluidity as well as structural instability in the bilayer may be important for the fusion process to occur. A previous report showed that the addition of lysolecithin to spherical bilayer membranes, which caused instability and possible increased fluidity of the membranes lowered the T_f for PC and PS membrane fusion by almost 10 °C (Breisblatt & Ohki, 1975). Our present study indicates that the addition of cholesterol into the bilayer membranes of PC and PS abolishes the T_f and strongly inhibits the fusion process.

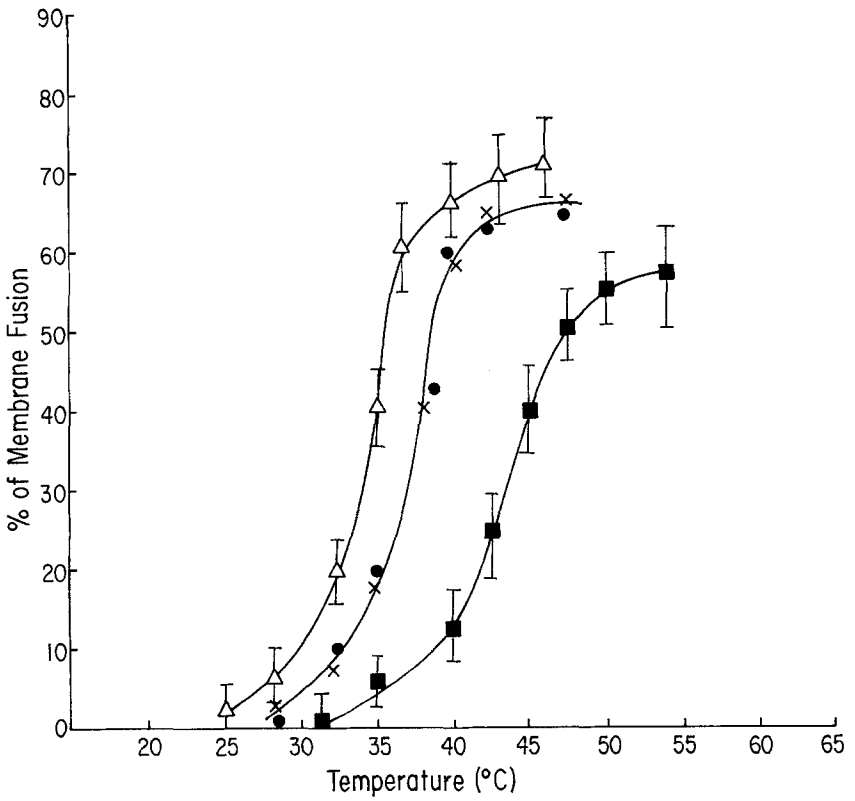

Fig. 11. Effect of pH on the fusion of PS membranes. △=pH 8.5; ●=pH 7.0; ×=pH 5.5; and ■=pH 3.0

It is known that the addition of cholesterol into the membranes decreases membrane fluidity, namely making the membrane (hydrocarbon chain packing) more rigid and constrained (Ladbrooke *et al.*, 1968; Papahadjopoulos *et al.*, 1971; Engelman & Rothman, 1972). By doing so the freedom of movement of the hydrocarbon chains of the lipid molecules is decreased and the possibility of structural change occurring in the membrane is suppressed. Therefore, cholesterol incorporation into the membrane should be expected to suppress the membrane fusion reaction.

Further support for the importance of membrane fluidity is demonstrated by our spherical bilayer fusion studies with some of the synthetic derivatives of phosphatidylcholine. As evidenced by the T_f's found for these phospholipids (DOPC, 49 °C; DMPC, 59 °C; DPPC, above 70 °C) it is clear that the fluidity must play a role in the differences observed for membrane fusion. DPPC, which has the longest saturated hydrocarbon chains of those phospholipids studied, has the highest T_f [it also

has the highest phase transition temperature (T_c; 41 °C) and least fluid with increasing temperature], while DOPC which contains one double bond on each alkyl chain has a fusion temperature very much comparable to egg PC (T_f: 43 °C). DMPC, as expected, falls in between these two with its T_f. The only question which might arise concerns why DOPC, which has 2 double bonds, has a higher fusion temperature than egg PC (1 double bond). This could possibly be explained by the fact that a great deal of heterogeneity is found in PC (egg) concerning chain length and degree of unsaturation. This could lead to the lower fusion temperature observed for PC. Therefore, the results agree well with what one might expect if fluidity is one of the key factors involved in fusion process.

The effect of divalent cations on spherical bilayer fusion with increasing temperature, relates both membrane fluidity and structural instability to the fusion event. In examining the symmetrical case, it is clear that none of the divalent cations (Fig. 6) has any effect on the fusion of PC membranes. This is predictable because PC behaves as a neutral species and divalent ions are not likely to be adsorbed on the membrane strongly (Rojas & Tobias, 1965; Hauser & Dawson, 1967; Seimiya & Ohki, 1973). On the other hand, PS which has a negatively charged polar group will adsorb the divalent cations (more than the neutral species) which could affect the fusion process through both a binding and screening phenomena. As indicated in Fig. 7, fusion of PS membranes is affected, as the T_f for PS membrane fusion is abolished and, at lower temperatures, fusion is enhanced. For PS membrane, the relatively temperature independent process for fusion may be due to the stabilization of the acidic phospholipid membrane by Ca^{++}, which is well documented in the literature (Ohki, 1969; Jacobson & Papahadjopoulos, 1975). However, the fairly high percentage of fusion for the overall temperature range may be due to another reason. Hauser, Phillips and Barratt (1975) have recently shown by the NMR technique that the interaction of divalent cations with phospholipid polar groups causes a water exclusion effect, which makes the phospholipid-divalent ion complex appear to be more hydrophobic in nature. It appears therefore that there are two main factors contributing to our membrane fusion: instability of the membrane and hydrophobicity of the membrane surface which is related to membrane fluidity, as well as increase in surface tension. Although Ca^{++} stabilizes the membrane which in turn decreases membrane fluidity, it is considered that the acidis phospholipid membrane surfaces become more hydrophobic with Ca^{++} adsorption at the surface than those with-

out Ca^{++}, that is, the surface may become a higher energy state. The high aggregation rate among PS spherical membranes corresponds probably to the latter factor (high free energy surface) which also contributes to the high percentage of fusion. On the other hand, low temperature dependency with respect to fusion percentage may correspond to low fluidity of the membrane due to possible calcium ion binding with the phospholipids. This interpretation also goes along with the results obtained with phospholipid vesicles due to Ca^{++} concentration (Papahadjopoulos *et al.*, 1974).

By creating an asymmetrical distribution of the divalent cation with respect to the membrane, another factor, membrane instability, has been added. For PS membranes, membrane breakage was extremely high. This could be due to membrane instability which has been well documented with planar bilayer membranes (Ohki & Papahadjopoulos, 1970). PS membrane fusion results were similar to those obtained in the symmetrical case, except that the percentages of membrane fusion were increased by 10–20%. This increase could be interpreted by the additional instability factor which was created by the asymmetrical distribution of Ca^{2+}. In the case of PC membranes the asymmetrical distribution of the divalent cations caused some effect on membrane fusion. Membrane breakage also was higher than without the divalent cations, and at lower temperatures membrane fusion was increased, although the T_f was not affected. From the study on PC planar bilayer membranes, the asymmetrical distribution with this concentration of Ca^{++} did not show an appreciable change in electrical conductance as well as membrane instability (Ohki & Papahadjopoulos, 1970). It is possible that divalent ions change the nature of the PC membrane surface which influence the membrane fusion reaction. However, this change may not be large enough to be detected as a change in electrical conductance or instability of the planar bilayer. As a whole the experiments with divalent cations may suggest that the creation of some instability as well as possible rearrangement in membrane structure are important factors for the fusion process, to a large extent, for PS membranes and to a lesser extent, for PC membranes.

The change of the pH also seems to affect membrane fluidity as well as stability. As shown in Figs. 10 and 11, the shifts of the fusion curves as well as the fusion temperature were observed at lower and higher pH's. At pH 8.5 the T_f was decreased by approximately 3 °C for both PC and PS membranes. But at pH 3.0, we observed the opposite situation as the T_f for PS membranes was increased by 6 °C, and for

PC membranes the increase was 4 °C. Träuble and Eibl (1974) have also pointed out that changes in pH would affect the phase transition temperature of phospholipids through changes in membrane fluidity. The opposite situation is observed at low pH as there is a decrease in membrane fluidity and the resulting effect is to increase the phase transition temperature. Our results seem to parallel these observations. Membrane stability is also affected: at pH 8.5 the phospholipid membranes become very unstable and there is a high degree of membrane breakage (Ohki, 1969). This problem was not observed at pH 3.0 as membranes formed were very stable. At pH's 5.5 and 7.0 there was no effect on the T_f and the fusion curves were similar to those previously reported. This would clearly indicate that neither membrane fluidity nor stability was affected by pH changes in this range.

It seems fairly obvious from the present experimental results that membrane fluidity and structural instability are closely related to fusion in this model membrane system. And at the same time, it has been shown that as membrane fluidity is increased, more instability may arise in the membrane bilayer. This was previously demonstrated with the effects of temperature and lysolecithin, and the results presented here with divalent ions, cholesterol and pH seem to offer further support. By increasing membrane fluidity, the freedom of motion of the hydrocarbon chains of lipid molecules could be increased so as to allow the hydrocarbon chains of the lipid bilayer to reveal the membrane surface. This increase in the hydrophobic surface area would cause instability in the bilayer structure and possibly form what we have termed a semi-micelle configuration (Breisblatt & Ohki, 1975). If this occurs, hydrophobic interactions between apposed membrane surfaces could lead to membrane fusion as we have recently postulated (Ohki & Breisblatt, 1975). This study may provide some support for the above proposal fusion reaction in this model membrane system being the result of increased hydrophobic interactions between the two apposed membrane surfaces.

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