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Fusion of dipalmitoylphosphatidylcholine vesicle membranes induced by concanavalin A

(membrane fusion rate/phase transition/spin label/freeze fracture)

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ABSTRACT **The temperature dependence of fatty acid spin label resonance spectra and freeze fracture micrographs of sonicated dipalmitoylphosphatidylcholine vesicles in the absence and presence of concanavalin A demonstrate a strong interaction of concanavalin A with these lipid membranes, which results in fusion of the vesicles. The rate of this reaction as followed with use of magnetic resonance exhibits a pronounced maximum at 36°, the midpoint of the phase transition range of dipalmitoylphosphatidylcholine vesicles. This maximum is discussed in terms of structural fluctuations, which are maximal in the phase transition range of the membranes.**

Fusion of membranes is a fundamental process involved in essential activities of cells, such as exo- and endocytosis, hormone secretions, and neurotransmitter release, as well as cell division, which formally corresponds to fission (the reverse process of fusion). The growth of cell membranes during the cell-cycle and differentiation seems to be accomplished in many instances by fusion of vesicles originating from the Golgi-apparatus (by fission) with the cell membrane. The formation of the nuclear membrane in the final step of mitosis is another example where membrane fusion plays a crucial role. For a review of the literature on membrane fusions in biological systems, see ref. 1.

In view of the high complexity of cellular systems, phospholipid vesicles have been employed as membrane model systems. Fusion of such vesicles with each other (2–7), with black bilayer membranes (8), and with cells (9–13) has been described, but a physical basis for the mechanism of fusion in terms of the molecular properties of the participating membrane components is lacking. In the present paper further experiments aimed at elucidating details of the fusion mechanism are described.

Because biological membrane fusions must be initiated by specific aggregation processes involving protein and lipid components (14–18), it appeared useful to us to work with a model system consisting of a defined phospholipid membrane and a defined aggregating agent. At the outset of the present work we thought that the following system might fulfill the requirements for such model investigations. (*1*) A few molecules of a glycolipid in the membranes of single-shelled phospholipid vesicles should serve as receptors for a lectin, which could bind simultaneously to several vesicles. A network of lectin-connected lipid vesicles would be the result of such binding. (*2*) Depending on the sort of phospholipid (charge, fatty acid length, etc.), it should be possible to drive fusion between the vesicles in the network by variation of appropriate physical parameters of the system, such as temperature or ionic concentrations, in the suspension medium. (*3*) In electron spin resonance (ESR) experiments, a small amount of fatty acid spin label (FASL) in the membranes of the small vesicles (250 Å diameter) should report the transition from the highly curved vesicle membrane to the resulting extended bilayer, inasmuch as the spectra of FASL in these two structures show pronounced shape differences.

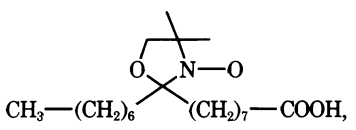
Concanavalin A (Con A) (for molecular properties of Con A, see refs. 19 and 20) was chosen as aggregating lectin; dipalmitoylphosphatidylcholine (DPPC) served as phospholipid; and bovine brain gangliosides as possible receptor structures for Con A.

To test the binding capacity of ganglioside-charged DPPC vesicles for Con A, we carried out a series of hemagglutination inhibition tests in which the ganglioside-DPPC vesicles were to compete with sheep erythrocytes for Con A. Unexpectedly, control experiments revealed that the presence of pure DPPC vesicles caused a strong inhibition of Con A-mediated hemagglutination. In addition, the precipitation of pure DPPC-vesicle suspensions by Con A indicated a direct interaction between Con A and the vesicles. In the following section, details of this interaction are described.

METHODS AND MATERIALS

All experiments were carried out in water containing the following salt and buffer concentrations: 150 mM NaCl, 5 mM KCl, 20 mM Hepes [(*N*-2-hydroxy)ethylpiperazine-*N'*-2-ethanesulfonic acid]. The pH was adjusted with NaOH so that it was 7.6 at 22° and 7.4 at 43°. This pH range was chosen because above pH 7.4 no pH-dependent changes occur in the spectrum of FASLs in lecithin membranes (21).

DPPC-Vesicle Suspension. DPPC (Calbiochem) containing approximately 1 g of lysolecithin per 200 g as judged by thin-layer chromatography was used throughout the present work. DPPC suspensions in the above buffer containing 1 g of 6/7 FASL per 100 g DPPC,



were prepared as described in ref. 26. Sonication was carried out for 50 min in a waterbath sonicator at 50°. The resulting optically clear suspension was made 4 mM in EDTA. It consisted of single-shelled vesicles of 250–300 Å diameter as judged by electron microscopy and analysis of the autocorrelation functions obtained in laser-light scattering experiments (S. Aragon and J. van der Bosch, unpublished).

Con A Solutions. Solutions of Con A in the above buffer were always prepared immediately before use in an experi-

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; Con A, concanavalin A; FASL, fatty acid spin label; ESR, electron spin resonance.

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ment. After dissolving the protein, the solutions were centrifuged for 5 min in a clinical centrifuge and then filtered through a 0.22 μm Millipore filter. The resulting solutions were clear and remained so for at least 5 hr.

Reaction of DPPC Vesicles with Con A. At the bottom of a 3 ml test tube, 0.1 ml of a clear DPPC vesicle suspension (2 g/100 ml) was mixed with 0.1 ml of a Con A solution of appropriate concentration. For ESR measurements this mixture was transferred to a 50 μl capillary. For completion of the reaction the capillary was incubated for 5–10 hr at 25° and then heated in about 30 min from 25° to 39°. Then it was used for ESR measurements, which were performed at X band on a Varian E-12 spectrometer with temperature control equipment.

Measurement of Time Course of DPPC–Con A Interaction at Various Temperatures. For kinetic measurements in the ESR spectrometer two syringes (0.5 ml), one of which contained 0.25 ml of a DPPC-vesicle suspension (2 g/100 ml) and the other 0.25 ml of Con A solution (16 mg of Con A per ml), were placed in a thermoregulated aluminum block. In this block they were connected with a small mixing chamber which was connected directly to the capillary tube in the thermoregulated ESR cavity. The capillary terminated in a piece of plastic tubing. Under initial conditions the mixing chamber and capillary were filled with a DPPC-vesicle suspension (1 g/100 ml). After adjustment of the temperature in the mixing block and in the ESR cavity to the same value, the contents of the two syringes were mixed and transferred to the capillary in less than 1 sec by pushing the pistons of the syringes with a common slide. The time course of the reaction was followed at a fixed magnetic field.

Freeze Fracturing and Etching. A DPPC-vesicle suspension (0.5 ml of a 2 g/100 ml suspension) and a Con A solution (0.5 ml) containing 16 mg/ml of Con A were mixed and incubated at 36°. At various times after mixing, aliquots were transferred onto small gold plates at 36° and quenched immediately in Freon 22 at −150°. In a Balzers freeze-fracture chamber the samples were fractured at −115° and etched at the same temperature at 10^{-6} torr (1.33×10^{-4} pascals) for 3 min. Electron microscopic pictures of the carbon platinum replicas were taken with a Phillips E-200 microscope.

RESULTS

During experiments with Con A from different sources, it became clear that various concentrations of *free* Mn^{++} in the solutions caused changes in the interaction capability of Con A with DPPC vesicles. Therefore, all the following experiments were carried out with Con A from Pharmacia in the presence of 2 mM EDTA. Under these conditions the content of Mn^{++} tightly bound to Con A remained unchanged, as judged by ESR spectroscopy. A slow self-aggregation of Con A took place, resulting in visible precipitation of concentrated solutions in the course of days. As will be seen, this aggregating state of Con A is important for the interaction of this protein with DPPC vesicles.

DPPC suspensions

Fig. 6a shows an example of the appearance of a freeze-fractured and etched sample of a suspension of sonicated vesicles without the addition of Con A. The fact that the vesicles form clumps of several thousand Ångström extension is most probably due to the freezing process, inasmuch as the vesicle suspension before freezing does not show any turbidity, which would be characteristic for particles of several thousand Ångström; nor are the autocorrelation functions obtained in laser-light scattering experiments (S. Aragon and J. van der Bosch, unpublished) indicative for the occurrence of such large vesicle aggregates.

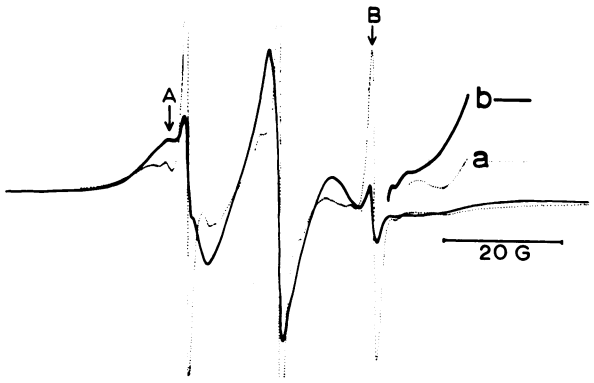

FIG. 1. 6/7 FASL-ESR spectra of DPPC suspensions (1 g/100 ml) at 30°. (a) unsonicated; (b) sonicated.

The 6/7-FASL-ESR spectrum of such a suspension is shown in Fig. 1 (spectrum b). It consists of two superimposed spectra, one of which arises from FASL in the phospholipid membranes, and the other from FASL in the water phase (22). For comparison the spectrum of the unsonicated state is also depicted in Fig. 1 (spectrum a). The following differences between the two states can be noted. (*1*) Judging from the B-signal intensities, the FASL concentration in water is higher by a factor of 5 in the unsonicated sample than in the sonicated one. (*2*) The apparent order parameter (23, 24), $S = (T_{\parallel}-T_{\perp})/(T_{zz}-T_{xx})$, decreases on going from the unsonicated to the sonicated state. Concomitantly, the intensity of peak A increases.

Interaction between DPPC vesicles and Con A

Upon mixing 1 volume of a clear DPPC-vesicle suspension (2 g/100 ml) with 1 volume of a clear solution of Con A (1–10 mg/ml) a thick white precipitate forms, which consists of a mixture of lipid and protein. To check the state of the lipid in this precipitate, we recorded ESR spectra between 20° and 45° and compared them with spectra of pure sonicated and unsonicated DPPC suspensions. The temperature dependence of peak A height (Fig. 2a) shows a phase transition near 40° for the unsonicated sample, in rough agreement with data from the literature (25–27). This sharp transition disappears upon sonication; instead, a broad transition range centered around 36° appears. Upon addition of Con A to the sonicated sample a transition at about 40° reappears, and the whole temperature profile becomes similar to the profile of the unsonicated sample. Similar considerations apply to the temperature profiles of peak B height (Fig. 2b). Going from high to low temperature a strong expulsion of FASL from the membrane into the water phase is observed during the transition in the unsonicated sample. No comparable behavior is observed in the sonicated state. Upon Con A addition to the sonicated sample the general behavior of the unsonicated state reappears.

A strong change upon addition of Con A to the sonicated sample is also observed in the temperature profile of the apparent order parameter (Fig. 2c). Below 40° the apparent order parameter in the DPPC–Con A mixture is even higher than in the pure unsonicated sample. This might be due in part to a reduced mobility of the fatty acid chains, and in part to spin exchange interactions caused by clustering of the FASL molecules in the presence of Con A.

The dependence of the extent of the reaction on the Con A concentration is shown in Fig. 3. First, it can be seen that

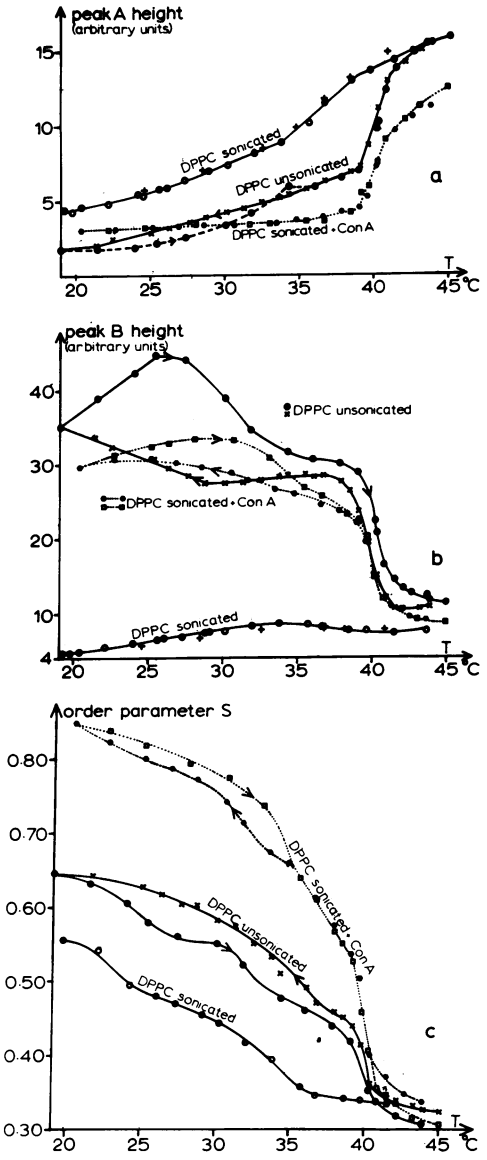

FIG. 2. Dependence on temperature of 6/7 FASL spectral parameters of sonicated, unsonicated, and Con A-treated sonicated DPPC suspensions. DPPC concentration: 1 g/100 ml; Con A concentration: 8 mg/ml. Rate of temperature change: 5 min/measured point. For explanation of peaks A and B, see Fig. 1; for order parameter S, see refs. 24 and 25.

in the absence of EDTA there is only a very small change of spectral parameters with rising Con A concentration. On the other hand, EDTA itself does not cause any reaction in the absence of Con A. Second, it is to be noted that α-methylmannoside (a sugar which binds to Con A with a binding constant of $K \approx 10^4$) reduces the extent of the reaction measured by spectral parameters. The absence of EDTA and presence of α-methylmannoside inhibit both self-aggregation and precipitation of pure Con A. As will be seen later on, electron microscopic pictures of freeze-fractured and etched samples of DPPC–Con A mixtures strongly suggest that the overall process, which gives rise to the observed spectral changes, is aggregation and fusion of the DPPC vesicles by means of Con A.

Kinetics of DPPC–Con A interaction

The kinetics have been recorded ESR spectroscopically as shown in Fig. 4. The steepest slope in the middle range of

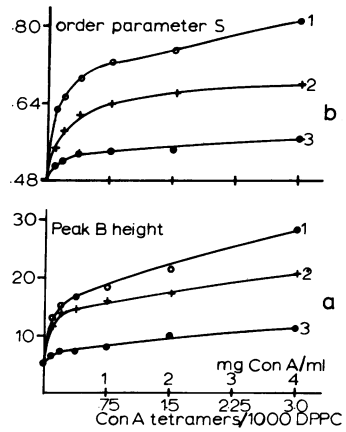

FIG. 3. Peak B height (a) and order parameter (b) of sonicated DPPC suspensions versus added Con A. DPPC concentration: 1 g/100 ml. (*1*) In presence of 2 mM EDTA; (*2*) in presence of 2 mM EDTA and 50 mM α-methylmannoside; (*3*) in absence of EDTA and α-methylmannoside. Temperature: 27°.

the curve is taken as a measure of the maximum reaction rate at a given temperature.

In Fig. 5, the normalized maximum rate of signal decrease is plotted versus temperature. A strong maximum is observed at 36°. At this temperature no significant spectral changes could be observed in the absence of Con A, even over a period of 20 hr. From these observations it can be said that the acceleration of DPPC-vesicle fusion by Con A is at least 2000-fold under the concentration conditions used in the present work.

A series of freeze-fracture samples has been taken at the temperature of maximum reaction rate (36°) before and 2–18 min after mixing Con A with DPPC vesicles. Electron-micrographs of the fractured and etched samples are shown in Fig. 6. It is obvious that during the course of the reaction the initial small vesicles disappear. Larger extended lipid structures become visible, which partly exhibit the striated appearance characteristic for well ordered lecithin membranes below their phase transition temperature (28). The etch faces of these structures are dotted with small particles, which probably represent Con A molecules or aggregates, inasmuch as they never have been observed in samples without Con A.

DISCUSSION

The present work demonstrates that Con A is able to interact with DPPC vesicles in concentration ranges of 1–20 Con A monomers [M (molecular weight) 25 to 30 × 10^3 (19, 20)]

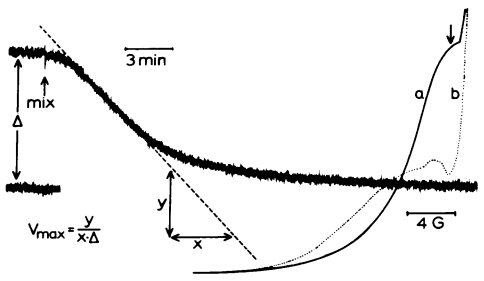

FIG. 4. Time course of peak A decrease upon mixing of equal volumes of a DPPC-vesicle suspension (2 g/100 ml) and a Con A solution (1.6 g/100 ml). At right, peak A is shown before mixing (a) and after completion of the reaction (b). The time course has been recorded at a constant field set as indicated by the arrow over peak A. Temperature: 35°.

per vesicle (Fig. 3). The final result of the interaction is fusion of the DPPC vesicles and formation of extended lipid structures (Fig. 6). The apparent order parameter (24, 25) of the membranes in this extended state derived from FASL spectra exceeds by far the order parameter of extended DPPC membranes in the absence of Con A (Fig. 2c). This fact and the electronmicroscopic evidence (Fig. 6) indicate that Con A is bound to the vesicles. Because this binding occurs under conditions which promote the self-aggregation of Con A, and because the Con A tetramer presumably has several binding sites for DPPC vesicles (as it has for certain monosaccharides), a network of Con A connected vesicles is very probably formed. In this network the vesicle surfaces are exposed to each other. This may be one means by which fusion between DPPC vesicles is enhanced in the presence of Con A. A second, perhaps still more important, factor is the influence of Con A on the molecular structure and dynamics of the vesicle membrane.

The time course of the reaction (Fig. 4) shows that there is an initiation period of 1–2 min during which the average mobility of the FASL is not much changed. In this period, binding of Con A and first fusion steps resulting in vesicles of still appreciable curvature might occur, as suggested by Fig. 6b. During the next 6–7 min the signal intensity decreases rapidly, indicating the emergence of extended membrane structures as shown in Fig. 6. Obviously a strong correlation exists between fusion and the immobilization of the spin label. Therefore, the rate of signal decrease was taken roughly for the rate of fusion. The temperature dependence of this rate (Fig. 5) shows a pronounced maximum at 36°, which is the midpoint of the rather broad transition range of DPPC vesicles (Fig. 2a).

Other workers (3, 7) have found a similar maximum for the rate of fusion of pure dimyristoylphosphatidylcholine

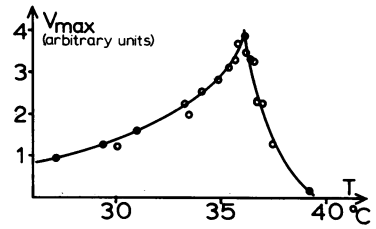

FIG. 5. Maximum rate of peak A decrease (V_{max}) versus temperature. V_{max} has been obtained, as shown in Fig. 4.

(DMPC) vesicles in the temperature range of the DMPC-phase transition.

In studies of sugar transport across membranes of fatty acid auxotrophic *Escherichia coli* mutants, optima of the transport rate have been observed at the onset temperatures of lateral phospholipid phase separations (30, 31). Similarly, enhanced cation permeabilities have been observed at the transition temperature in pure DPPC (32, 33) and in DPPC systems that contain valinomycin (33). An explanation for these optima has been proposed (30) in terms of the enhanced lateral compressibility and associated fluctuations in packing and density of the membrane lipids at the phase transition. A maximum of lateral compressibility has been demonstrated in DPPC monolayers at the phase transition (34). We wish to apply this idea to the maximum fusion rate observed in the present work and in the DMPC system (3, 7).

In order for two bilayer membranes to fuse with one another, it is necessary that the hydrophobic interiors (the fatty acid chains) contact one another. In a pure phospholipid bilayer in the fluid state this is an unlikely event because the bilayer membranes normally assume a conformation that minimizes the exposure of the hydrophobic groups to the

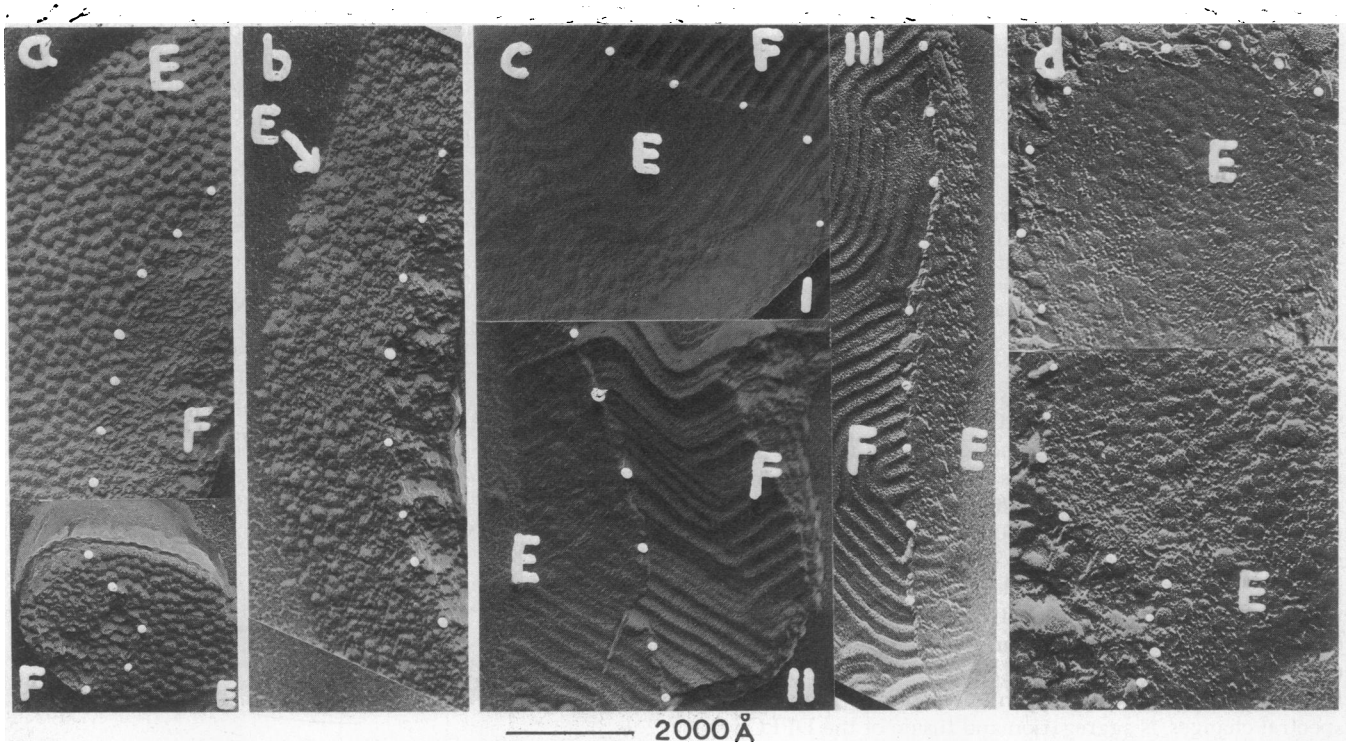

FIG. 6. Electronmicrographs of freeze-etch samples of various states of Con A-induced DPPC-vesicle fusion at 36°. (a) Sonicated DPPC suspension without Con A; (b) same suspension 2 min after Con A addition; (c) same suspension 7 (1), 10 (11), and 18 (111) min after Con A addition, showing larger ordered lipid domains on the fracture and etch faces; (d) same suspension 18 min after Con A addition, showing large smooth lipid domains on the etch face, dotted with Con A particles. White dotted lines show border lines between fracture (F) and etch (E) faces.

aqueous environment. Similar statements apply to bilayer membranes in the solid state. In extended phospholipid bilayer membranes, solid and fluid domains can co-exist in equilibrium within the plane of the same membrane. These domain boundaries are typically relatively few, indicating that they have a high free energy; partial exposure of hydrophobic groups may thus be involved, and domain boundaries might act as sites for the initiation of membrane fusion. We imagine that the phospholipid molecules in the domain boundaries have conformations (hydrocarbon chain kinks, headgroup conformation) that are distinct and perhaps intermediate between those in the fluid and solid domains. Intermediate conformations of lipid regions can also be produced by time-dependent membrane fluctuations involving transitions between the fluid and solid lipid states. Judging from the temperature jump studies of Träuble (35), the relaxation time of such fluctuations has a maximum at the phase transition temperature. Adam (29) has developed a kinetic theory for lipid phase transitions which describes this maximum. This theory is based on the generation of gauche conformations ("kinks") in the fatty acid chains and takes into account single and pair distributions of straight and kinked chains. It is then reasonable to assume that at the phase transition temperature both the number and lifetime of lipid molecules with intermediate conformations is maximal. The result of such fluctuations would be an irregular vesicle profile and surface pattern. In other words, this state of the membrane allows for a variety of surface patterns which may be induced by interaction with Con A and/or other vesicle surfaces during aggregation and fusion.

Experimental and theoretical evidence has been presented that proteins bound to membranes can produce intermediate lipid conformations (36–38). Such perturbed lipid "halos" surrounding membrane proteins reach maximal size as one approaches the lipid transition temperature from above (37). Thus, a protein-catalyzed fusion event can also show a sharp maximum at the phase transition temperature. Therefore, in addition to the already described aggregating effect of Con A, this protein may stabilize intermediate lipid conformations that facilitate fusion.

We think that the present investigation has some relevance to membrane fusions that occur in biological systems. Membrane proteins and/or divalent cations possibly act to destabilize and to interconnect membranes; the membrane lipids may exist in a state of intermediate fluidity and thereby enhance the structural fluctuations discussed above. This view is supported by the fact that the rate of fusion of cells in culture can be influenced by variation of temperature as well as of lipid and divalent cation concentrations (39), indicating that these factors also play an important role in biological membrane fusions.

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