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Lipid phase transition in planar bilayer membrane and its effect on carrier- and pore-mediated ion transport

(fluctuation phenomena/mixed-chain lipids/ionophores)

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ABSTRACT **Using mixed-chain lipids, we have recorded cooling and heating curves of planar bilayer membranes while they passed the lipid phase transition range. With unmodified planar bilayers, spontaneous current fluctuations are observed near the lipid phase transition temperature ($t_c \approx 29°C$). This effect coincides with the expected and measured decrease in membrane capacitance. Carrier (valinomycin)-modified planar bilayers exhibit near t_c an abrupt change from a high-conducting state above t_c to the state of bare membrane conductance below t_c. In contrast to this behavior, planar bilayers modified by pore-forming antibiotics (gramicidin A, alamethicin) do not show any peculiar effect at t_c. However, at 22–23°C a pronounced maximum in pore-induced conductance is seen. Whereas the gramicidin A pore abruptly stops stepwise fluctuations below ≈16°C, with alamethicin a few long-lasting pore and pore state fluctuations persist down to 10°C. It is suggested that the carrier may freeze out into the membrane/water interface. The effects observed with pore-forming substances, on the other hand, are interpreted in terms of lateral phase separation into pure lipid and lipid/antibiotic domains.**

Phase changes are well-known phenomena in artificial lipid/water systems (1) and biological systems (2). These phase transitions, which may play a role in triggering biological processes (3), can be induced by temperature changes or by interaction of ions with charged membrane lipids (4, 5). A great number of publications report on phase transition phenomena in pure and protein-loaded vesicles and liposomes. Due to the instability of planar bilayer membranes in the solid state, there are so far only two reports on electrochemical measurements in the freezing and melting range of planar lipid bilayer membranes. Experiments carried out on membranes from a 1:1 (wt/wt) mixture of dipalmitoylglycerol and distearoylglycerol in *n*-decane led to the interpretation that ion carriers became frozen and thus immobile within the membrane phase (6). On the other hand, the ionic conductance induced by the pore-former gramicidin was found to remain unchanged at the transition temperature t_c of 41°C. Recently, ion-conducting channels were reported to appear in unmodified planar bilayer membranes at the phase t_c of 59°C (7). Membranes were formed from a 1,2-distearoyl-glycero-3-phosphocholine/decane solution. There is also a paper, based on optical reflectivity measurements on membranes from monostearoylglycerol in *n*-hexadecane, which demonstrated an ≈70% increase in membrane thickness when the system was cooled below the t_c of 55°C (8).

Using saturated mixed-chain lipids with a t_c of ≈29°C, we succeeded in forming virtually solvent-free planar bilayer membranes below and above t_c. In this paper we report our investigations on pure and ionophore-modified planar bilayers of this type in the 10–40°C temperature range.

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MATERIALS AND METHODS

The following mixed-chain lipids were used: 1-stearoyl-3-myristoyl-glycero-2-phosphocholine (1,3-SMPC) and 1-hexadecyl-2-tetradecyl-*rac*-glycero-3-phosphocholine (1,2-HTPC). The synthesis of these lipids, which mainly followed procedures described by Eibl (9, 10), will be presented in a separate publication. Lipid purity was found to be ≥99%. The ratio of stearic acid to myristic acid was 1:1 as shown by quantitative gas chromatography (11). Notice the high purity of our lipids in comparison to the preparations of Keough and Davis (12). Their lipids (1-palmitoyl-2-myristoyl-glycero-3-phosphocholine and 1-myristoyl-2-palmitoyl-glycero-3-phosphocholine) were up to 20% impure due to acyl migration during synthesis.

Valinomycin was purchased from Calbiochem and the R_F 30 fraction of alamethicin (AL30) from Microbial Products Development and Production Laboratory, Porton-Salisbury, England. A sample of the R_F 50 fraction of alamethicin (AL50) was kindly provided by G. Jung, Tübingen. Gramicidin A purified by countercurrent distribution was a generous gift of E. Gross, National Institutes of Health, Bethesda, MD. Purified excitability-inducing material (EIM) was a generous gift of P. Mueller, Philadelphia.

Lipid phase transition temperatures of lipid/water emulsions were determined with a differential scanning calorimeter (Perkin–Elmer DSC 2) as described elsewhere. The values obtained from cooling curves are: 1,3-SMPC t_c = 30–27°C; 1,2-HTPC t_c = 31–27°C; from heating curves: 1,3-SMPC t_c = 30–33°C; 1,2-HTPC t_c = 30–34°C.

Planar bilayers were formed according to Montal and Mueller (13) by spreading lipid from a 5:1 (vol/vol) hexane/chloroform solution. The approach to an equilibrium state with respect to solvent content of the bilayer was monitored by capacitance measurements. Alternatively, membranes were formed from monolayers by using spread solvent-free vesicles (14). The Teflon septum was preconditioned with hexadecane dissolved in hexane. Otherwise membranes tended to become unstable.

The principle of the mechanical setup and the electronic equipment are described elsewhere (15). The electrical capacitance of the membrane was determined from the current relaxation trace after a voltage jump. In order to get a continuous signal of membrane capacitance, a 1-kHz alternating voltage signal of 1 mV amplitude was superimposed on the applied direct voltage. The resulting current signal was filtered in a narrow-band mode (Kemo VBF/8) and rectified.

Temperature was raised and lowered by using two thermo-

Abbreviations: 1,3-SMPC, 1-stearoyl-3-myristoyl-glycero-2-phosphocholine; 1,2-HTPC, 1-hexadecyl-2-tetradecyl-*rac*-glycero-3-phosphocholine; 1,2-DPPC, 1,2-dipalmitoyl-glycero-3-phosphocholine; 1,2-DOPC, 1,2-dioleoyl-glycero-3-phosphocholine; AL30 and AL50, alamethicin R_F 30 and 50 components; EIM, excitability-inducing material.

stats at different temperatures. Temperature changing rate was approximately 1.5°C per min within the 15–35°C range. Temperature was measured by a platinum resistor circuit with an accuracy of ±1°C.

RESULTS

Unmodified Planar Bilayer Membranes. The effects of the lipid phase transition on the electrical conductance and capacitance of solvent-free planar bilayer membranes have been investigated. Fig. 1 *A* and *B* shows cooling curves of a single-component lipid membrane. The current trace between 38 and 18°C is virtually constant with temperature except for spontaneous current fluctuations that occur in the small temperature range from 28 to 29°C. In heating curves the fluctuations are also observed, but between 30 and 32°C. Both temperature ranges correspond to the phase t_cs obtained by differential scanning calorimetry. Therefore, in cooling curves t_c indicates the temperature range from 28 to 29°C and in heating curves from 30 to 32°C, respectively.

In order to obtain information about the nature of these spontaneous fluctuations, we simultaneously measured the membrane capacitance using a superimposed 1-kHz alternating voltage signal of 1 mV amplitude. The narrow-band filtered and rectified current signal for the membrane of Fig. 1*A* is given in Fig. 1*B*. It is seen that a capacitance change occurs in the same temperature range as the current fluctuations. We suggest that the structural reorganization of the lipid molecules during phase transition may be the cause of the observed instabilities. Apparently, the transition of a bilayer membrane from the liquid-crystalline to the solid state and vice versa is

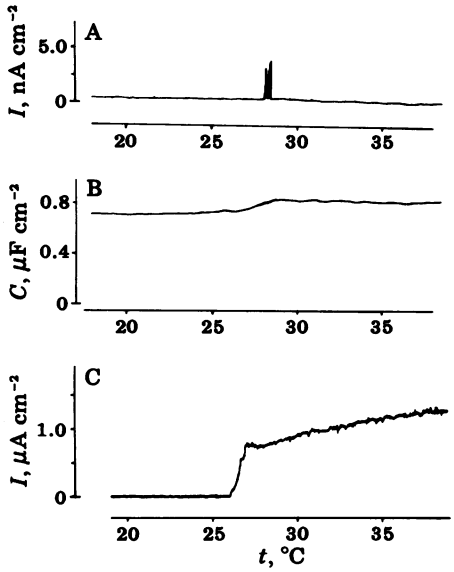

FIG. 1. Cooling curves of planar bilayer membranes showing transition effects near the phase-change temperature of the pure lipid/water dispersion. Bilayer forming method: Montal–Mueller technique (13); membrane area: 0.02 mm²; salt solution: 1 M KCl, pH 5.8; cooling rate: 1.5°C per min. (*A*) An unmodified 1,3-SMPC bilayer is cooled down from 38°C to 18°C. In the range of 28–29°C spontaneous current (*I*) fluctuations are observed at a constant applied voltage of 30 mV. (*B*) Same membrane as in *A*. Membrane capacitance *C* is measured by superposition of a 1-kHz alternating voltage signal of 1-mV amplitude to the applied direct voltage. The resulting current signal is filtered in narrow-band mode (Kemo VBF/8) and rectified. Notice that the capacitance change occurs at the same temperature at which spontaneous fluctuations are observed in *A*. (*C*) A 1,2-HTPC bilayer modified by the carrier antibiotic valinomycin is cooled down from 38°C to 19°C. Membrane current decreases until a minimum at 28–29°C is reached. Subsequently a dramatic change in current to bare membrane level occurs at 26–27°C. Valinomycin concentration: 1 μg cm⁻³; applied voltage: 50 mV.

associated with spontaneous fluctuations in membrane conductance.

Membrane capacitance was measured independently by applying voltage jumps and analyzing the resulting current relaxation traces. The obtained mean capacitance, C_M at temperatures $t > t_c$ is 0.83 ± 0.02 μF cm^{-2} and at $t < t_c$ C_M is 0.74 ± 0.04 μF cm^{-2}. This means that a change of 10–15% in membrane capacitance is associated with the structural changes involved in the phase transition of planar bilayer membranes composed of this type of mixed-chain lipids.

To check for a possible influence of solvent retained in the bilayer, the change of membrane capacitance with time was observed, utilizing membranes formed shortly after spreading of the lipid solution. At temperatures $t > t_c$, the membrane capacitance reached the constant value given above within 1–2 min. At $t < t_c$, on the other hand, mean membrane capacitance was initially observed to be about 0.6 μF cm^{-2}, and it increased in the course of 10–15 min to 0.74 μF cm^{-2}, which is the same value as is found after a membrane is cooled through the transition. To avoid hexane/chloroform as solvent, solvent-free lipid vesicles obtained by sonication of the lipid in aqueous solution were spread out at the air/water interface. The capacitances of the planar bilayers formed in this way were not different from the values described above. However, the membranes tended to break at $t < t_c$ after a short time. They were stable at $t > t_c$. We conclude that the capacitance values given are those of virtually solvent-free lipid bilayers.

Bilayer Modification by Valinomycin. Ion carriers such as valinomycin increase bilayer conductance up to several orders of magnitude (16). Fig. 1*C* shows the temperature dependence of the valinomycin-induced conductance of a 1,2-HTPC bilayer in 1 M KCl. The behavior is qualitatively the same with 1,3-SMPC membranes. A slight decrease in carrier-induced conductance with lowered temperature is observed down to *ca.* 28°C, which represents a small positive temperature coefficient in this case. After passing a weak minimum near 28°C the carrier-induced conductance suddenly disappears, and only the conductance of the unmodified lipid membrane is observed below 26–27°C. This behavior is virtually the same as described by Krasne *et al.* (6), using nonactin and valinomycin for their experiments. Careful inspection of the experimental data (Fig. 1 *A* versus *C*) indicates that the minimum in carrier-induced conductance appears in the same temperature interval as the spontaneous current fluctuations found in the case of unmodified bilayers. Notice that the considerable drop in conductance (Fig. 1*C*) is seen near the low-temperature end of the capacitance change (Fig. 1*B*). This conductance drop amounts to three orders of magnitude, which corresponds to a decrease in the observed current from 1 μA cm^{-2} to 1 nA cm^{-2}. Heating curves show qualitatively the same behavior, with a hysteresis shift of 2–3°C to higher temperatures.

Bilayer Modification by Gramicidin. The polypeptide antibiotic gramicidin A induces an ionic conductance in planar bilayers by forming pores of helical structure (17). Fig. 2 demonstrates that the temperature-dependent behavior of carrier and pore-forming systems is completely different. The gramicidin-induced conductance (Fig. 2*A*) shows a small negative temperature coefficient down to *ca.* 23°C. Our observation of an approximately constant conductance value between 27° and 31°C agrees quite well with the experimental results of Krasne *et al.* (6), who did not see a conductance change near the transition temperature of their lipid membranes.

On further cooling remarkable effects are observed. About 6°C below t_c the gramicidin-induced conductance starts to decrease with lowered temperature, reaching a weak minimum at ≈21°C and a second maximum at ≈19°C. Finally, at 16–

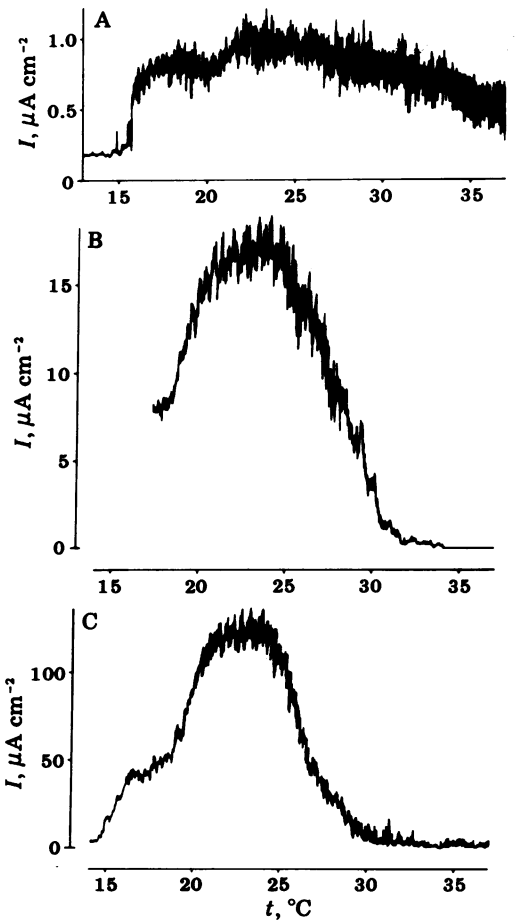

FIG. 2. Cooling and heating curves of modified planar bilayers, showing transition effects far below the phase change temperature of the pure lipid/water dispersion. General conditions are as for Fig. 1. (*A*) Cooling a 1,3-SMPC planar bilayer modified by the pore-forming antibiotic gramicidin A leads to slightly increasing current values down to 22–23°C, which is below the lipid phase t_c of ≈29°C. On further cooling a weak minimum and maximum are passed. At 16–17°C current drops to a nearly constant value which, however, is at least two orders of magnitude above bare membrane level. Gramicidin A concentration: 0.1 ng cm^{-3}; applied voltage: 20 mV. (*B*) A 1,3-SMPC bilayer modified by the pore-former AL30 and observed for some time at 37°C shows no pore activity under given experimental conditions. Decreasing temperature results in steep current increase, reaching a maximum at 23°C, which is below the lipid phase t_c. At 18°C the membrane broke. AL30 concentration: 0.2 μg cm^{-3}; applied voltage: 50 mV. (*C*) Heating curve of an AL30-modified 1,3-SMPC planar bilayer under same conditions as in *B* except for 1 μg cm^{-3} AL30 concentration. Membrane was formed at 14°C and then left for 15 min. By that time AL30 pore formation started. Increasing temperature with a heating rate of 1.5°C per min leads to curves qualitatively identical to those obtained by cooling. Maximum activity at ≈23°C is the most striking feature of those figures.

17°C the gramicidin-induced current drops down to a nearly constant value that lies, however, at least two orders of magnitude above bare membrane level. When we examined the stepwise fluctuations of single pore events (not shown) we found a monotonic increase in the mean pore lifetime towards lower temperatures and the appearance of a maximum in the pore formation rate. Below the temperature of conductance drop, current fluctuations do not take the form of clean steps. In a further publication we will report single-pore data in detail.

The heating curve of the gramicidin A pore system shows a shift of the current trace in Fig. 2*A* to higher temperatures, similar to the situation with valinomycin. The appearance of a shoulder in the rising part of this current–temperature characteristic demonstrates its equivalence to the cooling curve.

Bilayer Modification by Alamethicin (AL30, AL50). The polypeptide antibiotic AL30 forms voltage-dependent pores in planar bilayer membranes that can adopt several different conductance states (15, 18–20). Fig. 2 *B* and *C* shows AL30-induced current traces with a pronounced temperature dependence. Both the heating and the cooling curves exhibit maxima near 23°C, which implies a negative temperature coefficient above and a positive temperature coefficient below 23°C for the AL30-induced membrane conductance. Whereas under the given conditions of Fig. 2*B* no pore formation is observed at the initial temperature of 37°C, a steep conductance increase appears at lower temperature. Although stable membranes could be formed below as well as above 23°C, experimentally it was difficult to cool or heat through the range of maximum conductance. At 19–20°C the AL30-induced conductance seems to reach a constant level (Fig. 2*B*), but we did not succeed in cooling down to the 10–14°C range without breaking the membrane. Fig. 2*C* represents a heating curve of an AL30-modified 1,3-SMPC membrane. At 14°C, a membrane voltage of 50 mV, and the given AL30 concentration, pore fluctuations started a short time after membrane formation. With rising temperature the pore-induced conductance increases, showing a shoulder between 17 and 19°C and reaching a maximum at 23–24°C. Towards higher temperatures the conductance decreases again, showing single-pore behavior at the end of the trace. The experimental results were virtually identical with the natural analogue AL50.

At higher temperatures (>30°C) single-pore measurements (not shown; to be presented elsewhere) revealed short-lived pores with short mean pore state lifetimes. Lowering temperature lengthens mean pore state lifetimes, but the increase in the mean lifetime of the fluctuating pore aggregate appears to be much more distinctive. Below 23°C the frequency of pore formation is strongly reduced. In the temperature range 9–12°C pore state fluctuations lose their stepwise character after some time as in the case of gramicidin A. A comparable effect is observed with the alamethicin pore in frog muscle membrane at ≈8°C (B. Sakmann and G. Boheim, unpublished results).

An additional cooling experiment with valinomycin and AL30 simultaneously present at low concentrations demonstrated the independence of these two ion-translocating systems. Whereas the carrier-induced conductance abruptly vanished near 27°C, AL30 pore fluctuations did not start until 25°C.

A few preliminary measurements were carried out with EIM, a protein of high molecular weight that forms ion-conducting pores in bilayer membranes (21). The temperature dependence of the EIM-induced conductance was qualitatively similar to that observed with gramicidin A and AL30 with respect to a maximum value near 23°C. Additionally, stable stepwise single-pore fluctuations could be observed down to 10°C.

DISCUSSION

Experimental data presented in *Results* demonstrate that it is possible to obtain stable planar, virtually solvent-free bilayer membranes 20°C below the lipid phase t_c by using mixed-chain lipids (with two different fatty acids per lipid molecule) of the 1,3-SMPC or 1,2-HTPC type. The results of Krasne *et al.* (6) obtained in a small range around t_c were confirmed, even though these authors used solvent-containing membranes made with a mixture of diacylglycerols. Experiments with 1,2-dipalmitoyl-glycero-3-phosphocholine (1,2-DPPC) failed to give stable, frozen planar membranes. A reason for this difference in lipid behavior might be the relative length of the acyl chains. If there is a difference of four CH_2 groups in the two acyl chains for a symmetrical 1,3-lecithin, opposing acyl chains in the bilayer may interlock and thus stabilize each other.

Unmodified Planar Bilayer Membranes. The current traces in Fig. 1 *A* and *B*, obtained from the same membrane, show that the spontaneous conductance fluctuations and the membrane capacitance change occur within the same temperature range. This range coincides with that of the lipid phase transition measured by differential scanning calorimetry. The data indicate a capacitance change of 10–15%, which would correspond to a thickness change of the membrane hydrocarbon core from $d = 2.25$ nm above t_c to $d = 2.5$ nm below t_c (assuming a dielectric constant of $\epsilon = 2.1$). Experiments with vesicles composed of 1,2-DPPC were reported to reveal a local maximum of the $^{22}Na^+$ self-diffusion rate in the temperature range of the phase transition (22). There exist two explanations for this effect. First, the increase in permeability may arise at the boundaries between solid and fluid domains because of differences in the packing of lipid chains (23). Second, the permeability increase may be correlated with an increase in lateral compressibility of the bilayer that arises from the difference in the cross-sectional area of lipid chains above and below t_c (24). Our observations of fluctuations involve large conductance changes, which seem to indicate the presence of a few defects of quite extended size rather than many disturbances. In order to get more insight into the underlying molecular mechanisms it may be useful to analyze the power spectral density of these fluctuations. The report recently published by Antonov *et al.* (7) is consistent with our results. These authors observed single channel fluctuations in unmodified 1,2-distearoyl-glycero-3-phosphocholine/decane membranes at the phase t_c of 59°C.

Bilayer Modification by Valinomycin. The most pronounced feature of Fig. 1*C* is the abrupt drop in valinomycin-induced conductance to the conductance level of the pure lipid bilayer about 2°C below t_c. This is consistent with the data of Hsu and Chan (25), who studied the interaction of valinomycin with unsonicated 1,2-DPPC bilayers by delayed Fourier transform proton magnetic resonance and pulsed nuclear magnetic resonance spectroscopy. The effects of 2 mol % valinomycin (1 antibiotic per 50 lipid molecules) on the bilayer phase transition of 1,2-DPPC are to broaden slightly the transition range and to lower the temperature of the phase transition by 1°C. Hsu and Chan concluded that the position of the carrier molecule had to be near the polar head group region at the bilayer/water interface, except for the case in which a cation–carrier complex is translocated across the membrane. In our situation the carrier-mediated ion transport is blocked completely below 26–27°C, which matches closely the phase t_c of the pure lipid. Most probably the valinomycin molecules have been frozen out. Substantial amounts of valinomycin remaining in the hydrophobic lipid core would be expected to depress the temperature of conductance drop strongly as observed with gramicidin A and AL30.

The number of carrier molecules that interact with bilayers painted from a diphytanoyl-glycero-phosphocholine/decane solution was measured by fluorometric and electrical methods, using the analogue dansyllysine-valinomycin (26). At a carrier-induced conductance of $\lambda_0 = 20\ \mu S\ cm^{-2}$ (see Fig. 1*C*) a number of $n_0 = 0.3\text{–}2.0 \times 10^{12}$ carrier molecules per cm^2 of membrane area is obtained. The corresponding number of lipid molecules amounts to approximately 2×10^{14} per cm^2. Thus the resulting ratio of 1 carrier per 100–600 lipid molecules is not too different from that used by Hsu and Chan (25), assuming that valinomycin and its analogue behave similarly.

Bilayer Modification by Gramicidin. In going beyond the temperature range accessible experimentally to Krasne *et al.* (6), we also observed freezing effects with the gramicidin-induced membrane conductance (Fig. 2*A*). The most striking effects are the conductance maximum near 23°C and the conductance drop at 16–17°C. In contrast to the situation with valinomycin, a conductance level about two orders of magnitude above bare membrane conductance remains at temperatures below 16°C. Single-pore behavior in this temperature range gives evidence that the remaining conductance does not show stepwise fluctuations. A possible explanation of this fact might be given by a complete freezing of the membrane and the generation of structural defects between domains of different composition. Additional investigations on ion selectivity should reveal whether the ion pathways are the familiar gramicidin pore structures or unspecific boundary leaks.

A detailed study of the influence of gramicidin A on aqueous 1,2-DPPC dispersions with differential scanning calorimetry was carried out by Chapman *et al.* (27). Introducing small amounts of the polypeptide into the lipid caused a broadening of the phase transition range from 1.5°C to 7–8°C (half-width of the main calorimetric endotherm). The width of the phase transition became less sensitive to a further addition of the polypeptide if the ratio of lipid to gramicidin was smaller than 20. The maximum of the gramicidin-induced conductance observed by us was ≈6°C below t_c (Fig. 2*A*).

Chapman *et al.* (27) claimed that the calorimetric traces indicated the presence of a broad component superimposed on a narrower component on both the heating and cooling curves. This was interpreted as a heterogeneity of lipid populations. Starting at t_c, a separation into two phases seems to occur. One phase reveals a sharp transition near t_c and may consist of virtually pure lipid. The other is characterized by a wider transition and is composed of polypeptide molecules and some associated boundary lipid. With our method of conductance measurements, changes in the membrane system can be observed only if they modify gramicidin A pore properties. Fig. 2*A* allows identification of the phase of wide transition in Chapman *et al.* (27) with that containing gramicidin A pores. The occurrence of the sharp transition near t_c and the concomitant freezing of pure lipid domains should involve a lateral phase separation with gramicidin A becoming more concentrated in the still fluid antibiotic/lipid phase. Because gramicidin A pore formation depends on the second power of polypeptide concentration (28), one would expect a characteristic increase in the gramicidin-induced conductance below t_c. However, Fig. 2*A* does not show an additional conductance increase starting at t_c. This may be due to the low diffusion rate of molecules in a frozen lipid matrix, which would prevent gramicidin from becoming concentrated within the time scale of our experiments if, for example, the liquid phase is finely dispersed in the frozen matrix. Because our system does not seem to be in a thermodynamic equilibrium below t_c, it is not possible to decide at the moment if the isobaric melting diagram of gramicidin A and 1,3-SMPC indicates complete miscibility in both the fluid and frozen phases with a liquidus and solidus curve enveloping the two-phase zone. Alternatively, complete miscibility only in the fluid state and phase separation in the solid state can be imagined. Finding answers to such questions definitely requires more experimental efforts. The small negative temperature coefficient of gramicidin-induced conductance between 25 and 35°C seems to be a superposition of three effects: positive temperature effects on single-pore conductance and on the equilibrium constant of dimerization (29) and a negative temperature effect from the change of gramicidin concentration during the development of the new phase.

Simultaneous fluorescence and conductance studies of planar bilayer membranes containing the analogue dansyl-gramicidin C were reported by Veatch *et al.* (30). On the basis of their results with 1,2-dioleoyl-glycero-phosphocholine (1,2-DOPC)/decane membranes (cf. figure 7) and a pore density of 10^7 per cm^2 derived from our Fig. 2*A*, a concentration of 10^9 gramicidin molecules per cm^2 of membrane area is calculated. With

approximately 2×10^{14} lipid molecules per cm^2 this yields a ratio of 2×10^5 lipid molecules per gramicidin. Because Chapman *et al.* (27) used at most 100 lipid molecules per gramicidin, the gramicidin concentration in our experiments seems to be three orders of magnitude below this value.

Bilayer Modification by Alamethicin. The pronounced effect of the lipid phase transition on AL30-induced ion transport can be seen in Fig. 2 *B* and *C*. The cooling curve (Fig. 2*B*) exhibits a temperature-dependent density increase from virtually no pores at 35°C to a maximum of *ca.* 10^5 pores per cm^2 at 23°C under the given experimental conditions. A similar strong density change is seen in the heating curve (Fig. 2*C*) from *ca.* 10^6 pores per cm^2 at 24°C to approximately 1 pore per cm^2 at 34°C. This would correspond to a formal activation energy of the AL30-induced conductance of $\approx$1000 kJ mol^{-1} between 24 and 34°C. This behavior contrasts sharply with the relatively weak temperature dependence of an AL30-induced conductance in a 1,2-DOPC/decane membrane as observed between 4 and 25°C (15). Single-pore data indicate an increase in the mean pore lifetime with lowered temperature; however, this effect does not seem to account for the entire conductance increase observed in Fig. 2 *B* and *C*. Rather, an increase in the pore formation rate seems to occur, contrary to the situation with 1,2-DOPC/decane membranes (15). Because an increase in the pore formation rate with raised AL30 concentration has been reported (15), we think that the structural modifications associated with the lipid phase transition lead to domains that are more concentrated in AL30 than the homogeneous lipid membrane above t_c. AL30-induced conductance depends on the 9th to 10th power of AL30 concentration (15, 19, 20), which would easily explain the effect described above.

The addition of 1% alamethicin to unsonicated vesicles made from 1,2-DPPC does not produce significant changes in the phase transition temperature, according to Lau and Chan (31), other than to broaden somewhat the temperature range of the transition. This seems to indicate that most of the lipid freezes near t_c and that a second fluid phase of AL30 and some 1,3-SMPC is formed in the same way as discussed above for gramicidin A. Although a true thermodynamic equilibrium may not be established in this case either, we believe that we have observed lateral phase separation in the planar bilayer.

Differential scanning calorimetric measurements have not been carried out using AL30/lipid/water mixtures. Furthermore, the actual concentration of AL30 at the membrane interface and the ratio of inactive to pore-forming molecules are not known. Therefore, we cannot estimate at which AL30 concentration lateral phase separation starts. We suppose that a single AL30 aggregate suffices to disturb the ordering of surrounding lipid and thus lower its freezing temperature. The occurrence of stepwise AL30 pore state fluctuations down to 12°C and also the observation of fluctuating EIM pores at this temperature range seem to confirm this concept.

Recently Fringeli and Fringeli (32) reported experiments with multilayers from 1,2-DPPC/AL30 at a molecular ratio of 80:1 that were carried out by means of infrared attenuated total reflection spectroscopy. In order to reach an equilibrium distribution of AL30 between the membrane and the water phase, the system was kept at 25°C up to a few days. Subsequently it was measured at this temperature, which is 16–17°C *below* the phase t_c of 1,2-DPPC. A comparable temperature for our lipids is about 12°C, a region in which AL30 pore fluctuations start to lose their stepwise character. It is known for 1,2-DOPC/decane membranes that a weakly voltage-dependent conductance appears in the presence of AL30 (15). Whether the remaining conductance in our system at 12°C is of the nature of this weakly voltage-dependent conductance or due to unspecific boundary leaks is not known to us at present. Fringeli and Fringeli (32) claimed to have found a ratio of 80 lipid molecules per AL30 within the membrane phase and that the AL30 molecules were in a conformation spanning the membrane. On the basis of our experimental data we think that such high AL30 concentrations may lead to the formation of quite large frozen patches of AL30 associated with some lipid and that these patches are dispersed in a frozen matrix of virtually pure lipid. The conformation of the AL30 molecule in such frozen patches may well be different from that in a fluctuating pore. This indicates that the experiments of Fringeli and Fringeli (32) were not carried out under conditions comparable to those used so far in black lipid membrane experiments.

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