

rescent varicosities observed in this study. It seems possible that these ventrolaterally placed perikarya may be the source of the constant pattern of catecholamine varicosities observed in the cat.

These results indicate the existence of marked interspecies differences in the distribution of catecholamine varicosities within the brainstem reticular formation of the rat and cat. In addition, recent evidence indicates that marked differences also exist in other areas of the cat brain (5). Prior to these findings it frequently has been assumed that the distribution of varicosities within the rat brain was representative of other mammals. However, based on the present study, it seems necessary to thoroughly examine other mammalian species for the purpose of comparison of the distribution of biogenic amines (10).

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10. Personal communication with David Felten, Department of Anatomy, University of Pennsylvania School of Medicine, has indicated that marked dissimilarities also occur in the distribution of both catecholamine perikarya and varicosities within the brains of the rat and squirrel monkey.
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below its transition temperature, whereas no detectable change was observed at this temperature for gramicidin A.

Planar bilayer membranes (19) were formed in a thermostated chamber from mixtures of 20 mg of glyceryl dipalmitate and 20 mg of glyceryl distearate in 1 ml of decane (hereafter referred to as GD membranes) (20). When in use, the mixture was maintained at the temperature of 58°C. The lipids were 80 percent pure, the remaining 20 percent being chiefly mono- and triglycerides of the same chain length (according to the supplier, Sigma Chemicals). The linear pentadecapeptide gramicidin (Nutritional Biochemicals) is chiefly (about 72 percent) gramicidin A, the remainder being mainly a mixture of B and C which differ from A only in the L-amino acid at the No. 11 position (21). Conventional methods (7) were used to form membranes above 40°C. Below this temperature, care had to be taken to spread the lipid solution quickly, before its temperature reached that of the aqueous phase (otherwise, the lipid became too viscous to be spread), and below 35°C membranes could not be formed directly from freshly introduced lipid solutions. For these temperatures, membranes were actually formed at about 37°C and then cooled to the desired temperature. Unless stated otherwise, membranes were formed in the presence of antibiotics with continuous stirring of the aqueous solutions (22), and torus conductances were verified to be negligible. All conductances are reported per square centimeter of membrane. We accounted for variations in membrane area (as well as possible variations in membrane thickness) by using the ratio of the observed value of membrane capacitance (measured at 1000 hertz) to that of a membrane of known area at 45°C (0.43 μ farad/cm²). Since area corrections never exceeded a factor of 5, the interpretation of Figs. 1 to 3 is not sensitive to them.

Membranes were observed to be in the usual "liquid" state above 42°C, as judged by an appearance typical of conventional bilayers (19), by the free movement of lenses within them, and by their rapid bursting into droplets upon breaking. Below 40°C, they were judged to be "solid" by the loss of movement of lenses and inclusions and by the fact that the membrane, instead of bursting,

Freezing and Melting of Lipid Bilayers and the Mode of Action of Nonactin, Valinomycin, and Gramicidin

Abstract. *An abrupt loss of effectiveness of the presumed carriers, nonactin and valinomycin, in mediating ion conductance occurred at the same temperature as the membrane fluidity, judged visually, was lost. By contrast, the effects of the presumed channel-former, gramicidin, were the same on solid and liquid membranes. Taken together, these findings imply that freezing the membrane primarily reduces the mobility of these antibiotics with little effect on their solubility.*

Physicochemical studies have shown (1-3) that the interior of biological (as well as artificial) bilayer membranes is liquid-like. This is in accord with the well-known observations (4, 5) that neutral solutes diffuse across membranes and that their permeability is affected by changes in membrane fluidity.

We have found that we can reversibly solidify and liquefy membranes by "freezing" and "melting" a lipid bilayer of appropriate composition. This enables us to examine the effects of the state of the lipid on the ion permeation of bilayer membranes mediated by antibiotics thought to act by different mechanisms. The known effects and structures of macrocyclic antibiotics such as the macrotetralide actins (6-8) and valinomycin (9) are all consistent with the expectations for the action of a neu-

tral carrier (5, 6, 10) on membranes. By contrast, the known effects and structures of molecules such as gramicidin A (11-14), EIM (excitability-inducing material) (15, 16), alamethicin (15), and the polyene antibiotics (17) are consistent with their forming a conductive channel through the membrane. Since a channel-forming molecule need not diffuse back and forth in order to mediate ionic conductance across a membrane, a liquid-like interior is not necessary for the effectiveness of such a molecule. By contrast, a molecule that acts as a carrier of ions must be able to diffuse freely within the membrane interior in order to increase membrane conductance.

We report here that the effectiveness of nonactin and valinomycin (18) is discontinuously and dramatically obliterated upon taking the membrane

wrinkled and folded back on itself when broken. When a liquid membrane is frozen, the change of state is seen to occur very sharply at 41°C. When a solid membrane is melted, a temperature range of 40° to 42°C is encompassed during the transition. In the absence of added antibiotics, but in the presence of salt, the conductance of these membranes (indicated by the dashed line on all figures) was low and independent of whether the membranes were solid or liquid. This situation contrasts sharply with the effects of temperature on the actions of carriers and pore-formers as will now be described.

Figure 1A illustrates a typical experiment performed to assess the effects of the fluidity of the membrane on the conductance mediated by nonactin in the presence of potassium and shows the abrupt onset of the K⁺-nonactin con-

ductance on melting. The lower portion of the figure shows the temperature of the aqueous phase, while the upper portion presents the membrane conductance measured simultaneously. The membrane was formed at 36°C while the temperature was being lowered to 32.5°C, was thinned completely at 35°C (indicated as zero time), and reached a steady-state temperature of 32.5°C in 4 minutes. During this time the membrane appeared solid, and the observed conductance ($7.3 \times 10^{-9} \text{ ohm}^{-1} \text{ cm}^{-2}$) was no different from that in the absence of nonactin. At 6 minutes, the temperature was raised to 38°C, and the membrane still appeared solid. (Note that the membrane conductance did not increase significantly, remaining the same as that in the absence of nonactin.) At 10 minutes, the temperature was raised to 44°C, and the membrane was seen to

melt in the 2-minute interval between 11 and 13 minutes, as indicated by the region labeled *Melts*. Concomitant with melting, a large enhancement (more than three orders of magnitude) of membrane conductance occurred which rapidly reached a steady-state value of $1.7 \times 10^{-5} \text{ ohm}^{-1} \text{ cm}^{-2}$. At 16 minutes, the temperature was again raised with no further visual change of state of the membrane and with a much smaller increase in conductance. This behavior is typical of 17 experiments done with nonactin in which invariably no detectable effect of nonactin was observed below the transition temperature of the membrane, whereas nonactin always produced its characteristic large increase of membrane conductance above the transition temperature.

The dependence on temperature of the ability of nonactin to mediate bi-

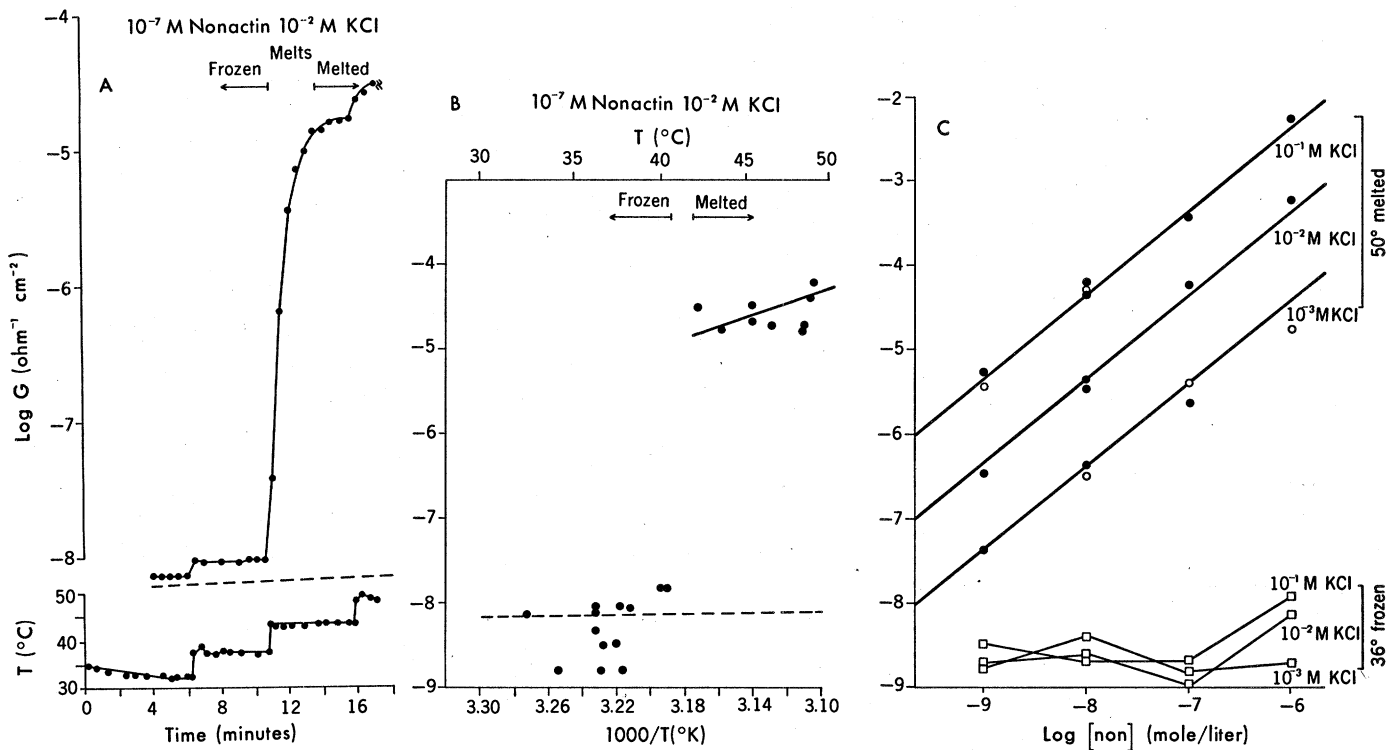


Fig. 1. The effect of temperature on nonactin-mediated conductance. (A) The effect of melting on nonactin-mediated conductance in a single membrane. The bilayer membrane was formed in the presence of 10^{-7} M nonactin and 10^{-2} M KCl at 36°C and cooled to 32.5°C. Zero time is taken as the time at which the membrane became optically black. The temperature was then raised in steps, as indicated by the thermistor record in the lower portion of the figure. The membrane broke (as indicated by the termination of the plot) before reaching a steady state at the highest temperature. The designations *Frozen*, *Melts*, and *Melted* refer to the visual appearance of the membrane. The dashed line represents the average conductance observed for membranes in the absence of nonactin (the range being $10^{-7.5}$ to $10^{-9} \text{ ohm}^{-1} \text{ cm}^{-2}$). (B) The discontinuous effect of temperature on nonactin-mediated membrane conductance. Conductances from 12 different membranes are plotted as a function of reciprocal temperature. The slope of the line drawn above the transition temperature corresponds to a temperature coefficient of 29 kcal/mole which we calculate from experiments done on single membranes at two different steady-state temperatures above the transition temperature [for example, as in (A)]. The dashed line represents the average of conductance observed in the absence of nonactin. (C) Nonactin-mediated conductances in melted (circles) and frozen (squares) membranes as a function of antibiotic and salt concentrations. Solid membranes were formed at about 36°C in the presence of the indicated concentrations of KCl and nonactin. After the conductances reached the steady-state values, the bath was heated to about 50°C. (Solid circles) Membranes melted and reached a steady-state conductance at 50°C without breaking. (Open circles) Membranes formed at 50°C. Note that the results are identical. The aqueous phase was not stirred. The heavy lines are drawn with a slope of 1. G , conductance.

layer conductance is summarized in Fig. 1B, where the logarithms of the steady-state membrane conductances in the presence of nonactin and potassium are plotted as a function of the reciprocal steady-state temperature. This figure illustrates that there is a large discontinuity in the effectiveness of nonactin on bilayers which correlates with the visually observed freezing and melting of the membranes. When the membranes are solid, nonactin never has a detectable effect. When the membranes are liquid, nonactin always induces the large increase in conductance characteristic of its action on the usual "liquid" membranes (7, 23). The strict proportionality between membrane conductance and concentrations of nonactin and potassium, illustrated in Fig. 1C for GD membranes in their liquid state (at 50°C), is exactly as reported for the commonly characterized membranes that are liquid at room temperature. Indeed, the

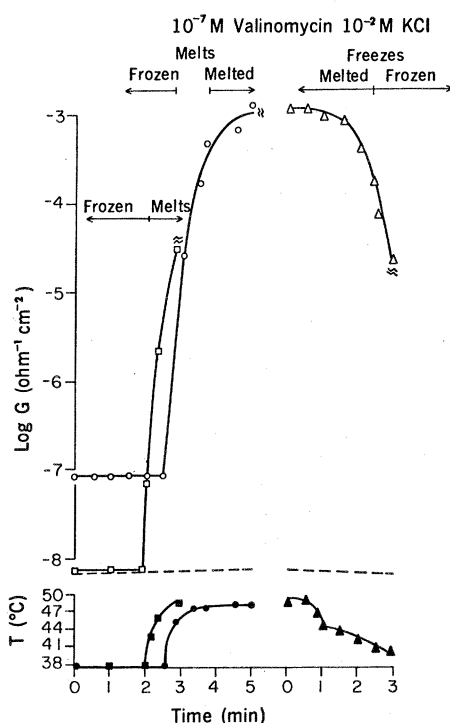


Fig. 2. The effect of melting and freezing on valinomycin-mediated conductance. Membranes were formed in the presence of $10^{-7}M$ valinomycin and $10^{-2}M$ KCl at the temperatures indicated. Zero represents the time at which thinned membranes reached a steady-state conductance. Squares and circles represent membranes formed at 37.5°C and heated to 47.5°C; one membrane (squares) broke while melting. The third membrane (triangles) was formed at 48°C, and the temperature was then lowered to 39.5°C. The dashed line represents the average conductance observed for the membranes in the absence of valinomycin.

nonactin-induced conductance of this neutral lipid at 50°C agrees quantitatively with that of the neutral lipid phosphatidyl ethanolamine from *Escherichia coli* (23), which is liquid at room temperature, when extrapolated to the temperature of the present experiment with a temperature coefficient of 29 kcal/mole (24). Note by contrast the complete lack of nonactin effects on GD membranes in the solid state (36°C).

Observations analogous to those of Fig. 1, A and B, were obtained for valinomycin on each of three membranes studied. Typical data are presented in Fig. 2, which in addition shows for valinomycin a typical example of how freezing the membrane shuts off the action of both nonactin and valinomycin.

Although the experiments on nonactin and valinomycin have demonstrated that freezing the membrane obliterates their effects on mediating membrane conductance while melting restores these effects, these experiments alone do not distinguish whether the effect of freezing is to exclude these molecules from the membrane (that is, decrease their solubility) or to impair their ability to diffuse through the membrane (that is, decrease their mobility). The following experiment on gramicidin, however, is germane to this distinction.

The dependence on temperature of the ability of gramicidin to mediate ion conductance is illustrated in Fig. 3, which is to be contrasted with Fig. 1B. Both solid and liquid black membranes were formed in $10^{-2}M$ KCl at the temperatures indicated. Two kinds of data are presented, both giving the same results. The first (open circles) represents steady-state values of conductances of membranes to which gramicidin was added to a concentration of $10^{-9}M$ after their formation. The second (closed circles) represents steady-state values of conductances of membranes formed in the presence of $10^{-9}M$ gramicidin from the lipid mixture remaining in the chamber. This figure illustrates that above the transition temperature, the level of gramicidin-mediated conductance for this lipid is comparable to that seen (12) on a widely used neutral lipid, phosphatidyl choline, at room temperature when extrapolated to temperatures above 42°C with a temperature coefficient near 0 kcal/mole (a value consistent with the data of this figure) (26). In sharp contrast with the con-

ductance behavior of membranes observed in the presence of nonactin and valinomycin, freezing the membrane per se does not lower the conductance mediated by gramicidin, as seen by the absence of a discontinuity in this figure. Indeed, this supposed channel-former has a large effect on conductance over the entire temperature range studied, and the only effect of freezing appears to be a change in the temperature coefficient.

In order to interpret the observation that solidifying the membrane obliterates the conductance mediated by nonactin and valinomycin whereas that mediated by gramicidin appears remarkably unaffected, we must first examine what are the structural differences between liquid and solid membranes (27). The interior of bilayer membranes above the phase transition temperature is thought to have considerable fluidity due to thermal agitation of the hydrocarbon tails (3). This fluidity provides a means by which molecules can

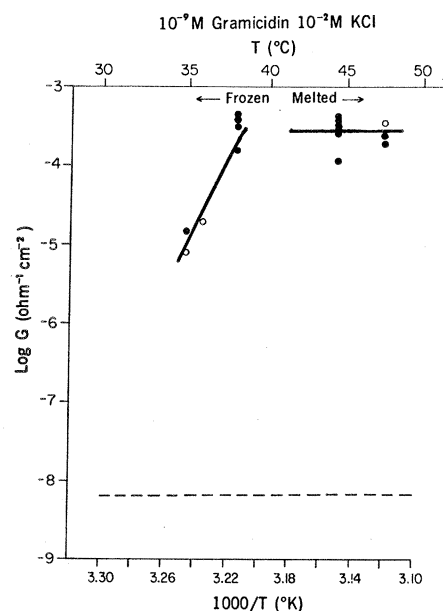


Fig. 3. The absence of a discontinuous effect of temperature on the conductance mediated by gramicidin. Membranes were formed in $10^{-2}M$ KCl at the temperatures indicated. Open circles show conductances observed for membranes to which gramicidin was added to a concentration of $10^{-9}M$ after their formation. Closed circles represent conductances for membranes formed in the presence of $10^{-9}M$ gramicidin from lipid already in the chamber. Note that both procedures gave identical results. The slope of the line drawn above the transition temperature corresponds to a temperature coefficient of 0 kcal/mole. The dashed line represents the average conductance in the absence of gramicidin.

diffuse across the hydrocarbon membrane interior much in the way they diffuse in a liquid hydrocarbon solvent such as hexane or decane [compare Träuble (28) for theory]. By contrast, when a membrane is taken below its transition temperature the hydrocarbon tails are thought to align themselves into a closely packed hexagonal array (2). This packing would hinder the mobility of those foreign molecules (such as ion carriers) which are large compared to the interstices between hydrocarbon chains, since it is more difficult to diffuse across a solid interior, just as it should be more difficult for a lipid-soluble molecule to move through a solid paraffin block than through liquid paraffin at the same temperature. Furthermore, foreign solutes might be excluded from the interior of such a regularly arranged membrane in a manner analogous to the exclusion of some of the salt from ice when brine is frozen. Such a process would reduce the concentration of foreign molecules present in the membrane interior, and the two parameters necessary for the effectiveness of a carrier—mobility and solubility—might therefore both be expected to be reduced by freezing. By contrast, reduction in mobility should have little influence upon the effectiveness of a molecule which mediates ion permeation by providing a channel that bridges the membrane, since the diffusion of an ion through a channel should remain unaltered even if movement through the hydrocarbon interior is reduced. Only a decrease in the solubility of the channel-former, resulting in a smaller number of transmembrane channels, would be expected to impair the effectiveness of such a molecule.

We therefore believe that the most parsimonious interpretation of the striking observation that gramicidin is effective in mediating ionic conductance in solid membranes, whereas nonactin and valinomycin are not, is that freezing the membranes primarily affects the mobility of these molecules while having little effect on their solubility. Thus, for gramicidin to be as effective on a solid membrane as on a liquid membrane, it must still be able to penetrate (that is, be soluble in) the solid membrane. Since a channel need not diffuse back and forth to be effective, even if it took gramicidin a factor of 10^4 longer to enter (that is, "diffuse" into) the membrane, it would still reach its equilib-

rium concentration within the time of our observations and would therefore lead to the same ionic conductance whether added to a liquid or a solid membrane. By contrast, since a carrier has to diffuse freely in the membrane, a decrease in the mobility of nonactin or valinomycin by a factor of 10^4 would be sufficient to account for the observed effect of freezing without having to postulate that the molecule has been excluded from the membrane (29). The qualitative differences observed here for molecules that are believed to be carriers of ions as opposed to molecules that are thought to form ion-conducting channels not only support the notion that the primary effect of solidifying the membrane is on the mobility of the species within the membrane (rather than on the solubility) but also strongly support the notion that the supposed carriers, nonactin and valinomycin, do indeed act as carriers and that the supposed channel-former, gramicidin, does indeed act by forming channels.

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26. Note that a temperature coefficient of 0 kcal/mole has been observed (12) for the action of gramicidin on phosphatidyl choline membranes.
27. Whereas we refer throughout to the condition of GD membranes below 40°C as "solid" or "frozen," it must be understood that until physicochemical studies are done on the mixture of which our lipids are composed, this will remain an assumption. When such methods are applied to glyceryl 1,2-dipalmitate and glyceryl 1,2-distearate in a presumably anhydrous state, transition temperatures of 50° and 60°C, respectively, are obtained (30); these values are compatible with ours when the hydrated condition of our membranes is considered. However, for the matters at issue in this report, the precise structure of the cooled membranes is not critical; it is important only that the fluidity of the membranes decreases, and our visual observations show clearly that this does occur, sharply, at 41°C.
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