Ion Selectivity of Temperature-Induced and Electric Field Induced Pores in Dipalmitoylphosphatidylcholine Vesicles†

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ABSTRACT: Temperature and electric field are known to alter the permeability of the bilayer membrane in phospholipid vesicles. A study of cation selectivity of these membrane pores is reported for multilamellar liposomes (MLV) and unilamellar large vesicles (ULV, 95 ± 5 nm diameter) of dipalmitoylphosphatidylcholine (DPPC). The permeability of ULV to Rb^+ was 1.0×10^{-6} μm/s at 22 °C and increased to 1.1×10^{-5} μm/s at the gel to liquid-crystalline transition temperature (T_m) of the bilayer, at 42 °C. The permeability of ULV to Rb^+ continued to increase beyond the T_m and reached 1.0×10^{-4} μm/s at 56 °C, a 100-fold increase over the permeability at 22 °C. In contrast, the permeability of ULV to Na^+ showed a local maximum of 6.0×10^{-6} μm/s at 42 °C and decreased at temperatures higher or lower than the T_m. For MLV, the permeability to both Rb^+ and Na^+ peaked dramatically at the phase transition temperature, 42 °C, and subsided at lower and higher temperatures. When ULV were exposed to an electric field, the permeability to Rb^+, Na^+, and sucrose surged at a field strength of 30 kV/cm; 30 kV/cm can induce a transmembrane potential of 210 mV. In ULV, the electrically perforated lipid bilayer exhibited selectivity for Rb^+ over Na^+ only at a narrow electric field range, between 31 and 33 kV/cm. For MLV, no well-defined breakdown voltage was recorded. The above results indicate that the fluidlike state of the DPPC bilayer is very permeable to Rb^+ but not to Na^+ and that the permeability characteristics of MLV to Na^+ and Rb^+ are completely different than those of ULV; the latter exhibit Rb^+ selectivity either through thermal fluctuations or through electric perforations, but the former do not.

Lipid vesicles mimic cell membranes in structures and in their ability to form a permeation barrier against molecules and ions. In an organism, regulated movements of metabolites and ions between the intra- and extracellular space, or the transport processes, are usually controlled by peptide channels or protein pumps and may require an energy source such as ATP, but there are other types of unregulated movements of ions and molecules, and understanding of the permeability characteristics of lipid bilayers appears just as important. In particular, it is now well documented that lipids in a cell membrane can undergo thermotropic phase separations or phase transitions, and these bilayer structure changes greatly influence the permeability of lipid bilayers. Ions or polar molecules, such as K^+, Na^+, 8-anilino-1-naphthalenesulfonate (ANS),[1] Tempocholine, sugars, apolipoproteins, etc., exhibit a maximum permeation or incorporation at the gel to liquid-crystal phase transition temperatures of lipids (Papahadjopoulos et al., 1973; Wu & McConnell, 1973; Marsh et al., 1976; Tsong, 1975; Tsong et al., 1977; Pownall et al., 1981). Other molecules, such as water, pyrene, etc., do not exhibit such an anomaly (Blok et al., 1976; Tsong, 1975), although they too show a characteristic dependence on the phase transition.

Most cells or organelles maintain a steady-state transmembrane potential, and this electric potential may also alter the dynamic property of the lipid bilayer and consequently play an active role in the regulation of membrane transport. Study of the bilayer selectivity of electrically induced pores to Na^+ and K^+ is especially relevant in view of the fact that nerve functions require rapid and alternating changes of membrane permeability to these two ions. Electric breakdown of lipid planar bilayers is known to occur between 100 and 300 mV of transmembrane potential (Tien, 1974; Jain & Wagner, 1980; Benz & Zimmerman, 1980; Benz et al., 1979). Previous work from this laboratory showed that a sucrose tracer loaded in ULV of DPPC also leaked out at this potential, i.e., 200 mV (Teissie & Tsong, 1981). Ion permeability of electrically induced pores in lipid vesicles has not been reported. In this paper, we compare effects of temperature and electric field on the permeability of ULV and MLV. We will focus on the bilayer selectivity of Rb and Na ions.

Materials and Methods

Chemicals. Dipalmitoylphosphatidylcholine was purchased from Sigma (lot PO763). It was judged to be of sufficient purity by differential scanning calorimetry; the width of the main lipid phase transition was less than 0.30 °C for MLV. Thus, no further purification was done. $^{22}Na^+$ and $^{86}Rb^+$ as chloride salts and [^{14}C]sucrose were purchased from Amersham Corp. Sodium deoxycholate was prepared from deoxycholic acid supplied by Sigma. All other chemicals and reagents were of analytical grade.

Preparation of Lipid Vesicles. MLV was prepared as the following: 20 mg/mL DPPC was dissolved in glass redistilled optical-grade chloroform and then evaporated to complete dryness by blowing nitrogen in a glass tube. A suitable volume of buffer containing 25 mM NaCl and 5 mM HEPES at pH 7.2 was added, and the tube was slowly warmed to 55 °C. The

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[1] Abbreviations: ULV, unilamellar vesicle(s) with diameter of 95 ± 5 nm; MLV, multilamellar vesicle(s) with undefined size; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DOC, sodium deoxycholate; ANS, 8-anilino-1-naphthalenesulfonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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tube was then mechanically shaken for 10 min in a vortex. Afterward, the lipid suspension was incubated at 55 °C for another 10 min before being slowly cooled down for experiments.

Preparation of ULV followed the procedure of Enoch & Strittmatter (1979) with a slight modification (Dufour et al., 1981; Teissie & Tsong, 1981). In brief, DPPC at 20 mg/mL was dissolved in chloroform and evaporated to dryness by blowing nitrogen gas. The lipid was then resuspended in 25 mM NaCl and 5 mM HEPES at pH 7.2. The suspension was placed in a water-jacketed glass tube and sonicated at 55 °C, using a Biosonic IV sonicator with a standard titanium probe at 60-W power level, for 1 h. To avoid overheating of the suspension, an automatic switch was added which turned the sonicator on for 15 s and off for 15 s so that the actual sonication time was 30 min. The lipid dispersion was then centrifuged at 100000*g* in a Beckman TI-50 rotor for 1 h at 24 °C, and the supernatant containing the unilamellar small vesicles of 25-nm diameter was collected and warmed to 55 °C. A small aliquot of 125 mM deoxycholate was added and mixed to give the final mixture containing a DOC:DPPC mole ratio of 1:4. The mixture was incubated at 55 °C for another 30 min. Deoxycholate was removed by passing the mixture through a 60 × 2.5 cm column of Sephadex G-25 at an elution rate of 53 mL/h, at 55 °C. The fractions containing lipid were centrifuged for 30 min at 100000*g*. The pellet was resuspended, and the supernatant was discarded. Passage through a 1 × 50 cm Sepharose CL-2B column separated multilamellar vesicles from the unilamellar large vesicles (ULV). The final preparation of ULV has been characterized to be unilamellar, with a diameter of 95 ± 5 nm, containing 0.18 mol % of residual deoxycholate (Dufour et al., 1981; Teissie & Tsong, 1981).

The concentration of lipid in suspensions was determined by a phosphate assay using the Bartlett method (Bartlett, 1959). MLV and ULV were suspended in 25 mM NaCl and 5 mM HEPES at pH 7.2 plus $^{22}Na^+$ (200 μCi/mL) for Na^+ entry experiments, and in 25 mM NaCl, 5 mM RbCl, and 5 mM HEPES at pH 7.2 plus $^{86}Rb^+$ (1 mCi/mL) for Rb^+ entry experiments. In all experiments, the sample was kept at 55 °C for 30 min before addition of radioactive tracers at a desired temperature. Rb^+ entry into the vesicles was rapid at 55 °C, and this preincubation ensured that the ionic compositions in the external and the internal vesicle space were identical.

Temperature Dependence of Tracer Entry. MLV or ULV in an appropriate buffer in a glass tube was kept at a desired temperature for 30 min. Radioactive tracer was then added and time counting started. After an indicated time, a 50-μL aliquot was drawn, and the tracer not entrapped in vesicles was quickly removed by the centrifuge–column procedure (Penefsky, 1977). In brief, a disposable 1-mL plastic tuberculin syringe (Plastipak 5602; Becton-Dickenson & Co., Rutherford, NJ) was fitted with wool and then filled up with Sephadex G-50 presoaked with identical buffer. The column was then placed on a small culture tube (13 × 100 mm, VWR Scientific) and centrifuged in a table-top clinical centrifuge at 2000 rpm for 75 s. A 50-μL aliquot of sample was applied on top of the G-50 column and centrifuged into a new culture tube. Forty microliters of vesicle suspension came out at the bottom of the culture tube and was transferred to a 7-mL vial for radioactivity counting. Since the rate of permeation of Rb and Na ions at temperatures below 25 °C was insignificant compared to the rate at higher temperatures, the centrifuge–column procedure was found to be adequate for our purpose.

Electric Field Induced Ion and Sucrose Entry. We have used an instrument originally designed for the temperature jump kinetic study (Eigen & De Maeyer, 1963). Previously, this instrument was used for voltage induction of transient pores in erythrocyte membranes and lipid bilayers (Kinosita & Tsong, 1977, 1979; Teissie & Tsong, 1981; Tsong, 1983). A capacitor of 0.05 μF was charged a voltage of up to 40 kV. The electric energy stored in the capacitor was then discharged into a suspension of lipid vesicles. The discharge chamber consisted of two platinum electrodes, 1 cm apart, and a cylindrical Kel-F enclosure that had an inside diameter of 0.7 cm. There was some space which was not directly under the electrodes, and only one-third of the suspension was exposed to the electric field each time. The discharge of electric energy followed an exponential decay, with a time constant of RC/2 (Eigen & De Maeyer, 1963; Teissie & Tsong, 1981). With the capacitor used and the salt concentration in the suspension, the decay time constant was measured to be 60 μs. The electric field strength was recorded as the initial charged voltage of the capacitor. Associated with each voltage pulse was a Joule heating temperature jump. The temperature jump could be as large as 10–15 °C, depending on the initial applied voltage. Then the discharge chamber was water circulated at 10 °C, and a 30-s interval was applied between each voltage discharge. The temperature of the chamber returned to its initial value within 10 s. As discussed in detail in Kinosita & Tsong (1977) and Teissie & Tsong (1981), all the phenomena we report here using this electric pulse instrument were due to effects of the electric field and were unrelated to Joule heating.

Results

Temperature Dependence of Ion Permeability. Experimentally we measured the uptake of radioactive tracer under a zero concentration gradient condition. The exchange follows:

$$C_o \underset{k_{oi}}{\overset{k_{io}}{\rightleftharpoons}} C_i$$

where C_o and C_i are respectively the concentration of ion outside and inside the vesicle and k_{io} and k_{oi} denote respectively the rate constant for exchange into the vesicle and for exchange out to the external medium. For our purpose, we obtained only the initial rate of tracer uptake and indicate this rate constant by k. k measures influx in the absence of efflux and is valid only at time zero. It has units of s^{-1}. C_o and C_i were identical in our experimental conditions, and we shall use C to indicate the concentration of the ion. The number of moles of ion exchanged is expressed as

$$n_x = CV$$

where V is the volume of a vesicle. The initial flux across the bilayer, J (in moles per second), is

$$J = kn_x$$

J is by definition

$$J = PAC$$

where P is the permeability (in micrometers per second) and A is the surface area of a vesicle. After rearrangement, one obtains

$$P = kV/A$$

We used $A = 2.8 \times 10^{-10}$ cm^2 and $V = 4.4 \times 10^{-16}$ cm^3 for ULV in the calculation. For MLV, we have used the same quantity assuming it to have identical structure. This means the values given here are not the true permeability of MLV but are useful just for the comparison.

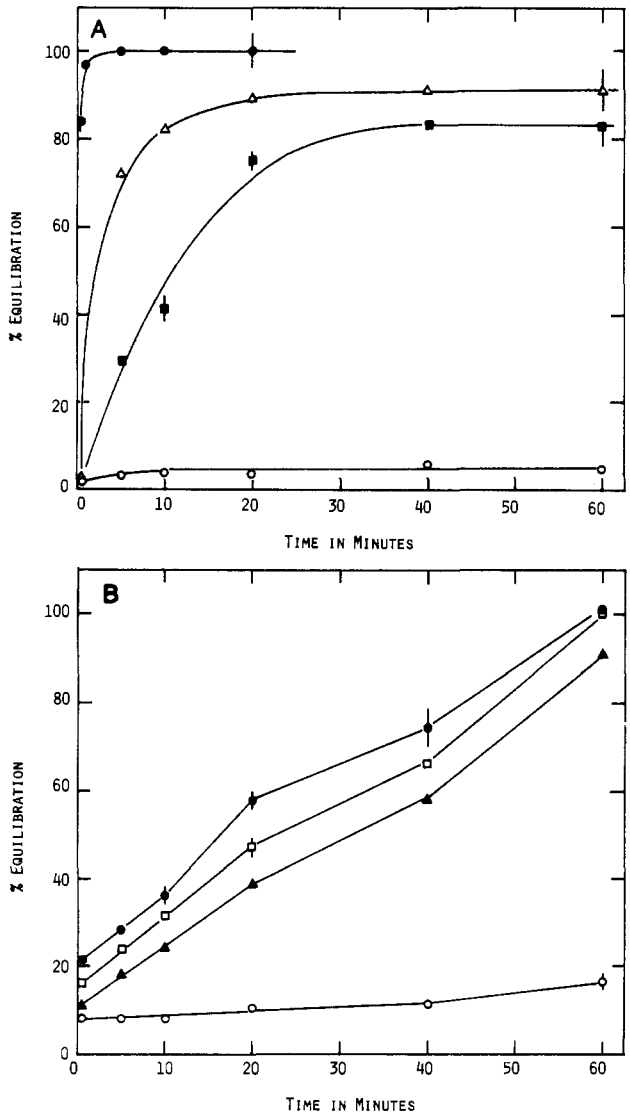

FIGURE 1: Time-dependent entrance of radioactive tracers into unilamellar large lipid vesicles (ULV). Ordinates indicate percent reached to final equilibrium. Experimental details are given under Materials and Methods. (A) Permeation of $^{86}Rb^+$ at temperatures of 22 (○), 42 (■), 45 (△), and 50 °C (●). (B) Permeation of $^{22}Na^+$ at temperatures of 22 (○), 41.5 (□), 49.5 (▲), and 55 °C (●). Each data point is the mean of two experiments, each with triplicate measurements. Some error bars are given.

The permeability of DPPC bilayers, either in MLV or in ULV, to both Na and Rb ions was very small at temperatures below 35 °C. Figure 1A gives the time-dependent $^{86}Rb^+$ entry into ULV at selected temperature, and Figure 1B gives $^{22}Na^+$ entry into the same ULV sample. The background level for Na^+ appeared, in this case, slightly higher than that for Rb^+, 10% compared to 2%, although the permeability was 1 order of magnitude higher for Rb^+ than for Na^+ at temperatures below 35 °C. At 22 °C, the permeability of DPPC ULV for Rb^+ was 1.0×10^{-6} μm/s, and it was 1.5×10^{-7} μm/s for Na^+. These values increased to 1.1×10^{-5} μm/cm and 6.2×10^{-6} μm/s, respectively, for Rb^+ and Na^+ at 42 °C, the gel to liquid-crystalline phase transition temperature of the ULV. The most obvious difference for the two ions occurred at temperatures above the T_m of the phase transition. This is shown in Figure 2. The rate of Na^+ permeation decreased again after the T_m, although this rate continued to soar for Rb^+. At 55 °C, the permeability for Rb^+ was 1.0×10^{-4} μm/cm, a 100-fold increase over the permeability at 22 °C, and was more than 20-fold higher than that for Na^+.

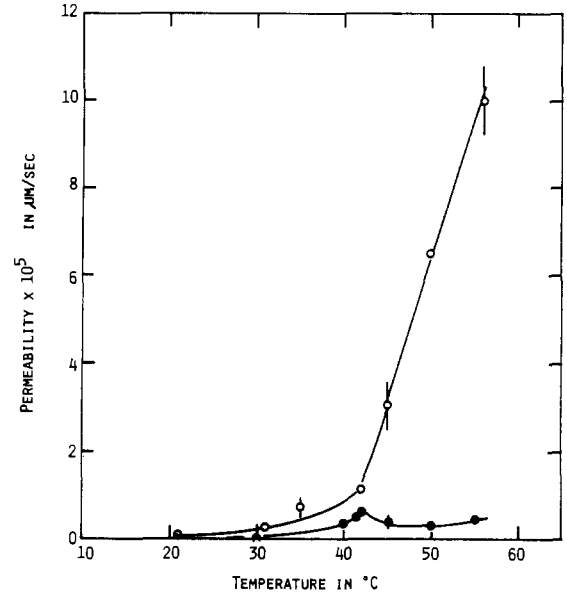

FIGURE 2: Permeability (in micrometers per second) of ULV to Rb^+ (○) and Na^+ (●) at different temperatures. The initial rate of tracer entry into ULV was used to calculate the permeability according to the text. Each data point is the mean of two experiments, each with triplicate measurements.

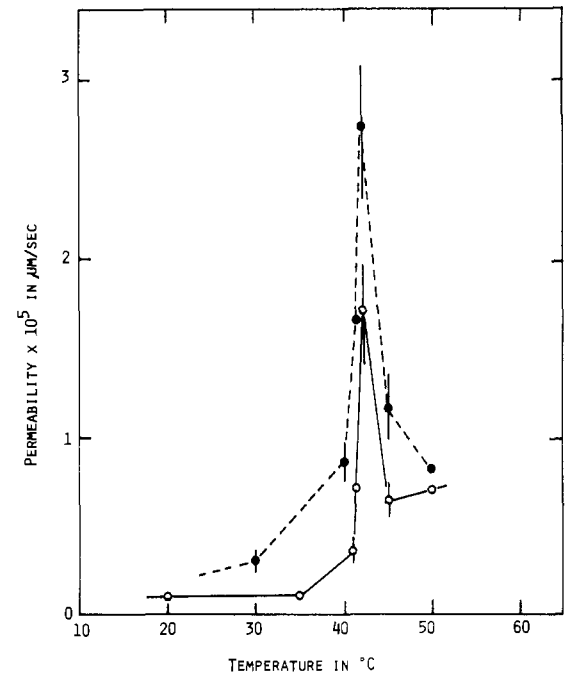

FIGURE 3: Permeability of MLV to Rb^+ (○) and Na^+ (●) at different temperatures. MLV has an ill-defined structure and size, and we have used the initial rate of tracer uptake to calculate the permeability, assuming that vesicles were in the ULV form. This is for the purpose of comparison only. Notice that the ordinate in this figure is expanded compared to Figure 2. Both ions exhibit a maximum rate of permeation at 42 °C, the T_m of the gel to liquid-crystalline phase transition temperature of the MLV.

For MLV, the permeability to Rb^+ and Na^+ was generally smaller. The shape and the size of MLV were ill-defined, and no permeability in a normal sense can be assigned. Instead, we will express experimental results only as a relative rate to ULV. The temperature dependence of Rb^+ and Na^+ permeation into MLV is given in Figure 3. As can be seen, the permeability of MLV bilayers to both ions peaked at the phase transition temperature and subsided again beyond T_m. The difference in permeability for the two ions was less significant

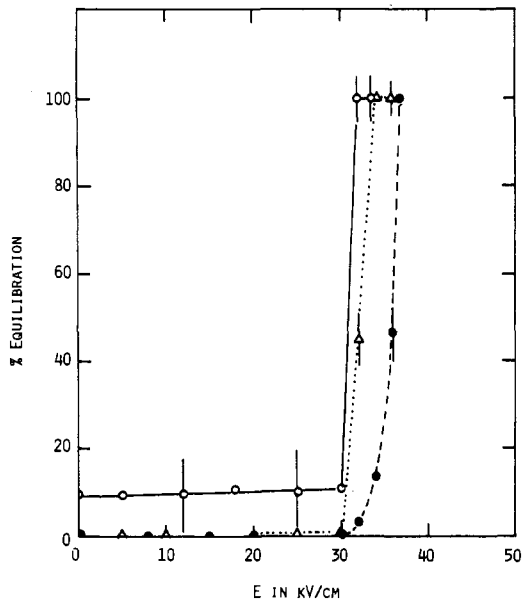

FIGURE 4: Electric breakdown of ULV. Vesicles in suspension were exposed to five electric pulses, with a 30-s interval between each pulse, of indicated field strength. The electric field had a decay constant of 60 μs. The initial temperature of the sample was 10 °C, and vesicles were never exposed to a temperature higher than 25 °C for more than 15 s. Thus, the uptake shown here was due to effects of the electric field, not the Joule heating (see text for details). $^{86}Rb^+$ uptake (○), $^{22}Na^+$ uptake (●), and [^{14}C]sucrose uptake (△) are shown. The ordinate indicates the percent equilibration with the external medium. Electrically induced permeability changes are transient (Teissie & Tsong, 1981).

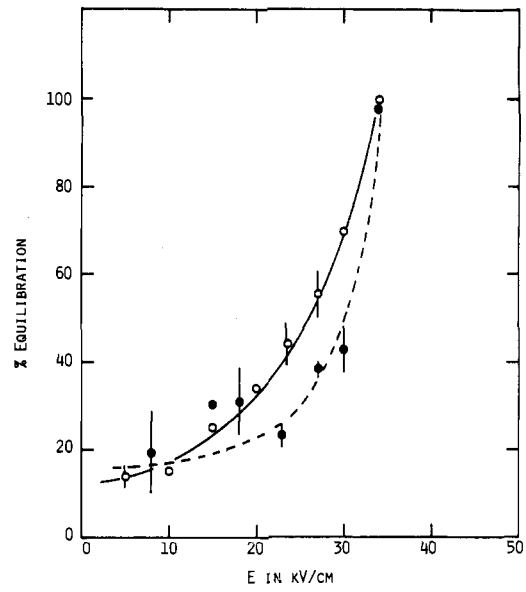

FIGURE 5: Electric breakdown of MLV. This experiment was similar to the one shown in Figure 6, except that MLV was used. Only $^{86}Rb^+$ (○) uptake and $^{22}Na^+$ (●) uptake were followed in this case.

for MLV in the entire temperature range studied (20–50 °C).

Electric Field Induced Transient Permeability Change. As mentioned under Materials and Methods, only one-third of a sample suspension was exposed to the electric field for each applied electric pulse. To ensure that more than 99% of the vesicles will be exposed to the electric field at least once, we treated samples with five pulses. The electric-induced uptake of Rb^+, Na^+, and [^{14}C]sucrose by ULV is shown in Figure 4. At a low electric field, the membrane was quite impermeable to all three species (temperatures below 25 °C), consistent with the above observation. When the electric field reached 30 kV/cm, the permeation barrier of the ULV was disrupted, and all three species permeated into the vesicles, with equal efficiency. The change in the permeability was abrupt, and there was no differentiation of these three species by the electric field induced membrane pores above 35 kV/cm.

The effect of electric field on the permeation barrier of MLV was similar but not identical. The rupture of MLV structures appeared to be more gradual. The result is shown in Figure 5. There was no threshold voltage, although at a field strength of 30 kV/cm there was a general breakdown of the MLV structure.

DISCUSSION

Effect of Temperature on Bilayer Permeability. The phenomenon that many ionic or hydrophilic species exhibit a maximum rate of permeation into, or across, the lipid bilayer has been attributed to the increased structure fluctuation in the vicinity of the T_m [for theoretical work, see, e.g., Nagle (1980) and references cited therein]. Many experimentalists favor a simple concept that lipid in the bilayer can exist in microclusters composed of molecules either in the gel state or in the liquid-crystalline state (Lee, 1977; Freire & Biltonen, 1978; Snyder & Freire, 1980; Kanehisa & Tsong, 1978, Tsong & Kanehisa, 1977; Tsong et al., 1977). These clusters are not stable domains. Instead, they can grow or shrink in the time scale of microseconds for the ULV and milliseconds for the MLV (Holzwarth et al., 1982; Eck & Holzwarth, 1983; Tsong, 1974; Tsong & Kanehisa, 1977; Kanehisa & Tsong, 1978). Lattice defects in the boundary regions of these microclusters, or aqueous pores (Huang & Thompson, 1966; Tien, 1974), grow as the temperature reaches the midpoint of the phase transition but subside again after the transition is over. As a result, ion permeability also peaks at the T_m. The observation that the permeability of ULV to Rb^+, but not to Na^+, is greatly enhanced in the liquid-crystalline state is rather unexpected, and it points to two significant facts. First, the fluctuating pores of the lipid bilayer in the fluid state can discriminate between Rb^+ and Na^+. Second, the bilayer structure of DPPC is different for MLV and ULV.

Ion selectivity of biological membranes and lipid bilayers incorporated with ionophores is known to be inherent in the specificity of ionic channels. How the thermal fluctuation pores of the lipid bilayer confer to this selectivity is of considerable interest. The crystal ionic radius is 1.47 Å for Rb^+ and 0.97 Å for Na^+, but the hydration radius is 2.4 Å for Na^+ and roughly 1.7 Å for Rb^+ (Moore, 1972). Thus, if pore size determines the permeation rate, the hydration radius of ions may explain at least part of the difference. Ion permeability of ionophores usually depends on two factors, the complexation of ions to ionophores and the translocation of ions. Rb^+ and Na^+ may also bind to the lipid bilayer before the translocation. The translocation step is especially determined by the chemistry, such as hydration, size, polarity, charge density, etc., of the permeant.

That the MLV was unable to disoriminate Rb^+ and Na^+ even at the liquid-crystalline state is a surprise. The enhancement of permeation rate at the T_m of the phase transition was also much more dramatic for MLV than it was for ULV. There are several noted differences in physical properties of the two vesicle forms, and these factors may contribute to the observed behaviors of the bilayer permeability. First, the phase transition is highly cooperative for the MLV compared to that for the ULV. The cooperative unit, i.e., the ratio between the van't Hoff ΔH and the calorimetric ΔH, was 200–1000 for the MLV, but it was only 22 for the ULV. Second, the cluster relaxation times were 15 and 150 ms for the MLV and 15 and

150 μs for the ULV (unpublished results). Third, in MLV, interlayer interaction exists; e.g., in the case of DMPC, the T_m is consistently lower for MLV than for ULV; 23.8 °C for MLV and 24.6 °C for ULV. In DPPC, this trend is reversed, and the T_m values are 42.2 °C for MLV and 41.6 °C for ULV (unpublished results).

Reversible Electric Breakdown of Bilayer. As was discussed in greater detail in previous communications (Kinosita & Tsong, 1977; Teissie & Tsong, 1981; Tsong, 1983), a spherical lipid vesicle exposed to an electric field E will experience a maximum potential drop across the membrane of $1.5aE$, where a is the outer radius of the vesicle. Assuming that the thickness of the bilayer is 50 Å and a is 450 Å, the electric field across the bilayer is $14E$; i.e., it is 14 times greater than the applied electric field. It was hoped that this greatly amplified electric field might facilitate formation of pores that would discriminate Rb^+ and Na^+ at certain electric field strengths as some theoretical studies would suggest [see, e.g., Sugar (1981) and references cited therein]. This turned out to be the case. The results in Figure 4 indicate that there was a threshold potential at which the bilayer ruptured, and before this breakdown voltage was reached, the permeability of the bilayer to Rb^+, Na^+, and sucrose remained at the background level. Thus, short electric pulses (60 μs) of intensity lower than 25 kV/cm (which would generate a transmembrane electric field of 375 kV/cm or a transmembrane potential of 190 mV) did not facilitate formation of pores. A sudden uptake of Rb^+, Na^+, and sucrose took place at 30 kV/cm; 30 kV/cm can generate 210 mV of transmembrane potential, and it is known that at this potential electric breakdown of lipid bilayer occurs (Tien, 1974; Teissie & Tsong, 1981). Data in Figure 4 also indicate that between the applied electric field of 31 and 33 kV/cm, i.e., the induced transmembrane potential of 220–235 mV, the bilayer in the ULV became very conductive to Rb^+ but only slightly conductive to Na^+. The window for discriminating the two ions was very narrow. Whether this property of the bilayer plays a role in biological functioning of cell membranes is not clear.

The lipid vesicles used, either MLV or ULV, were impermeable to sucrose at all temperatures studied here [see also Teissie & Tsong (1981)]. At temperatures above the T_m, ULV was very permeable to Rb^+ but only moderately permeable to Na^+. At temperatures below the T_m, both ULV and MLV were quite impermeable to Rb^+ and Na^+. On the other hand, electrically induced pores in ULV became very permeable to the two ions above a field strength of 35 kV/cm regardless of temperature (under 25 °C for the conditions used here).

The effect of applied electric fields on the permeability of MLV to Rb^+ and Na^+ was similar to that observed for the ULV (Figure 5). There was a gradual uptake at low electric fields, and only at a field strength greater than 30 kV/cm was a surged uptake observed. The magnitude of the induced transmembrane potential is linearly proportional to the radius of the vesicle, and larger vesicles are expected to rupture at a lower field strength. Thus, the result in Figure 5 is consistent with the electric breakdown result of ULV. Although one expects a multilayered membrane to break at a higher transmembrane potential than 210 mV, our result does not allow us to draw quantitative information in this respect.

Registry No. DPPC, 2644-64-6; Rb, 7440-17-7; Na, 7440-23-5.

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