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Ion permeability of the cytoplasmic membrane limits the maximum growth temperature of bacteria and archaea

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Summary

Protons and sodium ions are the most commonly used coupling ions in energy transduction in bacteria and archaea. At their growth temperature, the permeability of the cytoplasmic membrane of thermophilic bacteria to protons is high compared with that of sodium ions. In some thermophiles, sodium is the sole energy-coupling ion. To test whether sodium is the preferred coupling ion at high temperatures, the proton- and sodium permeability was determined in liposomes prepared from lipids isolated from various bacterial and archaeal species that differ in their optimal growth temperature. The proton permeability increased with the temperature and was comparable for most species at their respective growth temperatures. Liposomes of thermophilic bacteria are an exception in the sense that the proton permeability is already high at the growth temperature. In all liposomes, the sodium permeability was lower than the proton permeability and increased with the temperature. The results suggest that the proton permeability of the cytoplasmic membrane is an important parameter in determining the maximum growth temperature.

Introduction

Biological membranes consist of a bilayer or a monolayer of lipid molecules with their polar headgroups oriented toward the aqueous phase and their hydrophobic hydrocarbon chains forming the interior of the membrane. An important feature of biological membranes is that their structure is predominantly held together by non-covalent bonds such as Van der Waals and coulombic interactions, which make them highly impermeable to small ions. The low ion permeability of membranes results from the high energy requirement for the transfer of an ion from the aqueous phase into the apolar, hydrocarbon-like interior of the membrane. This barrier is important for the functioning of the cell membrane in processes such as energy transduction. Since this permeability usually reflects a simple diffusional process, the permeability of the membrane for ions will increase with the temperature. An intriguing feature of bacteria and archaea is that they can grow over a wide range of temperatures. Most prokaryotes are mesophiles and grow at temperatures between 20 and 55°C. A smaller group of prokaryotes can grow below −2°C, the freezing temperature of Arctic sea water (Baross and Morita, 1978). The highest temperature at which growth of bacterial species has been found is 90°C, while growth of archaeal species in marine volcanic areas has been observed at temperatures up to 110°C (Huber et al., 1986; Stetter et al., 1983; 1990).

Growth at the temperature extremes requires an optimal functioning of cellular metabolism, and a high stability of enzymes and other macromolecules (Adams, 1993). Many bacterial proteins or other macromolecules have been shown to be stable and active at temperatures that are often significantly above the maximal growth temperature (Marguet and Forterre, 1994). Minor changes, sometimes even of one single amino acid residue, can lead to a significant increase in thermostability (Yutani et al., 1977; Grütter et al., 1979; Goward et al., 1994; Cannio et al., 1994). The reasons why there is a maximum temperature of growth are, therefore, far from clear.

The cytoplasmic membrane has to retain its stability and functionality. To maintain the membrane in a liquid-crystalline state, cells vary the lipid composition when they are subjected to temperature changes (Russell and Fukunaga, 1990). This homeoviscous adaptation (Simonski, 1974) can be realized by adjusting the fatty acyl chain composition of the lipids, by varying the degree of acyl chain saturation, branching and/or cyclization. The headgroup composition of the lipids is hardly involved in this adaptation (Russell, 1984). Homeoviscous adaptation is thought to preserve membrane protein function, but its impact on the energy-transducing properties of the membranes has hardly received any attention. Metabolic energy can be...
obtained at essentially two levels: at substrate level by phosphorylation processes, and by energy-transducing processes in the cytoplasmic membrane. These processes are closely linked and together they determine the energy status of the cell. Energy transduction in the cytoplasmic membrane involves primary transport systems such as electron-transfer systems or ATPases. These systems translocate protons or sodium ions (Speelmans et al., 1993a) from the cytoplasm to the external medium and thereby generate an electrochemical gradient of protons (ΔµH+) or of sodium ions (ΔµNa+), respectively. The resulting forces can be used to drive membrane-bound processes such as ATP synthesis, uptake of solutes, flagellar rotation, etc. Obviously, this type of energy transduction can only operate if the membrane has a limited permeability for the coupling ions, protons or sodium ions. In previous studies on energy transduction in bacteria, we observed that the cytoplasmic membranes of the thermophiles *Clostridium fervidus* and *Bacillus stearothermophilus* at their respective growth temperatures are extremely permeable for protons, while the permeability for sodium ions remained low (Lolkema et al., 1994). To maintain a viable metabolic energy level, these organisms use two different strategies to cope with the increased proton permeability at elevated temperatures. *B. stearothermophilus* dramatically increases the rate of proton pumping by the respiratory chain (de Vrij et al., 1988), while *C. fervidus* uses sodium ions instead of protons as the sole energy-transducing coupling ion (Speelmans et al., 1993ab). A low sodium ion permeability versus the proton permeability would explain why *C. fervidus* can grow at much higher temperatures than *B. stearothermophilus*: at 80°C (Patel et al., 1987) versus 70°C, respectively (Table 1). The latter organism relies on protons in energy transduction.

The question arises as to whether the proton permeability is a limiting factor for the maximal growth temperature of psychrophilic, mesophilic and thermophilic organisms. We now report on the temperature dependency of the proton and sodium ion permeability of liposomes prepared from lipids extracted from various subacteria and archaea that vary in their optimum growth temperature (Table 1). The results demonstrate that the proton permeability of these liposomes becomes extremely high at temperatures above the growth temperature of the organism the membranes are derived from. For all membranes, the permeability to sodium was found to be lower that that for protons. However, unlike the proton permeability, the increase of the sodium permeability with temperature was found to be lipid independent. The data suggest that the proton permeability of the cytoplasmic membrane is a major factor that determines the maximum growth temperature.

### Results

#### Isolation of lipids and formation of liposomes

Lipids were isolated from a psychrophilic bacterium and mesophilic and thermophilic bacteria and archaea (Table 1). With increasing growth temperature, bacteria reduce the number of unsaturated bonds or increase the degree of branching in their lipid acyl chains. Extreme thermophilic bacteria, like *Thermotoga maritima*, contain membrane-spanning lipid esters. Archaeal cytoplasmic membranes consist of isoprenoid ether chains that are

<table>
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<tr>
<th>Temperature (°C)</th>
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<th>maximumb</th>
<th>Acyl chain composition</th>
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<td><strong>Bacteria</strong></td>
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<td>21</td>
<td><em>Psychrobacter</em> sp.</td>
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a. The imposed growth temperature.

b. The maximum growth temperature.

c. T↑ denotes the alterations in the lipid composition when the growth temperature is increased.


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arranged in a bilayer in non-thermophiles, or form membrane-spanning tetraether lipids in extreme thermophiles. Lipids were isolated from the bacterial and archaeal species listed in Table 1, as described in the Experimental procedures. For comparison and quantitative purposes, it was necessary to have equally sized liposomes. From each of the lipid isolates, unilamellar liposomes were formed by freeze-thawing and extrusion through a 200 nm pore size filter. This procedure yields liposomes with an average diameter close to 200 nm (Elferink et al., 1994).

Each liposome preparation could sustain an artificially imposed electrical potential (ΔΨ) (data not shown) for at least several minutes, indicating the formation of sealed vesicular structures.

### Proton permeability

For proton permeability measurements, liposomes prepared with a high buffer capacity on the inside were equilibrated in a solution with low buffer capacity. Valinomycin, a potassium ionophore, was added to prevent the generation of a counteracting electrical potential as a result of electrolytic influx of protons. The external pH, as monitored with the fluorescent pH indicator pyranine, was lowered by an H⁺ pulse of 100 nmol H⁺ (Fig. 1A). The immediate fall in the external pH was followed by its slow increase as a result of the influx of protons into the liposomes. Rapid proton equilibration was obtained after the addition of the ionophore nigericin (Fig. 1A), which mediates an electroneutral exchange between potassium and protons. In the presence of valinomycin, nigericin causes a complete uncoupling of the membranes. The slow increase of pyranine fluorescence after the proton pulse was fitted to a first-order kinetic rate equation to yield the rate constant of H⁺ influx, kH⁺. The proton permeability of liposomes prepared from the lipids derived from the various organisms was measured over a wide range of temperatures: permeability increased with temperature (Fig. 2). The higher the growth temperature of the organisms from which the lipids were extracted, the higher the temperature was at which a certain proton permeability of their liposomes was reached. Liposomes prepared from lipids derived from the psychrophile Psychrobacter sp. are already highly permeable to protons at a low temperature. At around 30°C, the proton permeability was found to be at least fivefold higher than that observed for mesophiles. The proton permeability of liposomes derived from both mesophiles Methanosarcina barkeri and Escherichia coli exhibited a similar temperature dependency. Liposomes from B. stearothermophilus showed a slightly lower proton permeability than the liposomes from mesophiles. Liposomes derived from the extreme thermophilic bacterium T. maritima and the archaeon Sulfolobus acidocaldarius showed the lowest proton permeability at the higher temperatures. At the growth temperatures, the proton permeabilities of liposomes composed of T. maritima and B. stearothermophilus lipids were much higher than that for the other organisms. We noted that at temperatures far below the growth temperature, the proton permeability of most liposomes increased again. This was not the case for M. barkeri liposomes and could not be measured for Psychrobacter sp. The proton permeability of each of the liposome preparations was plotted in an Arrhenius plot, and the activation energy of the proton permeability (ΔG′H⁺) was calculated from the linear part of the graph (data not shown). The ΔG′H⁺ value was of the same order for each of the liposome samples, ranging from 40 to 55 kJ mol⁻¹. This suggests that the mechanism of proton permeability in these liposomes, which have vastly different lipid compositions, is identical. However, the temperature range wherein the proton permeability varies is unique for each of the liposomes and correlates more or less with
the growth temperature of the organism from which the lipids were isolated.

**Sodium ion permeability**

The sodium permeability of the liposomes was measured by analysing the efflux of $^{22}$Na. Liposomes were loaded with 1 mM $^{22}$Na and diluted 100-fold in a buffer of identical composition except that the $^{22}$Na was replaced by the non-radioactive $^{23}$Na. Under these conditions, there is no concentration gradient of sodium across the membrane, and only equilibration resulting from passive diffusion is monitored. The release of $^{22}$Na by the liposomes was measured by a filtration assay. The release of $^{22}$Na was extremely slow and followed first-order rate kinetics, as shown for liposomes composed of *S. acidocaldarius* lipids (Fig. 1B). Instantaneous equilibration of the $^{22}$Na was effected by the addition of gramicidin (data not shown). The temperature dependency of the sodium permeability of the membrane was measured for the different liposomes, and the first-order rate constant $k_{\text{Na}}$ was used for comparison: $k_{\text{Na}}$ increased with the temperature (Fig. 3). The sodium permeability remained virtually identical for the different lipids when plotted as a function of the temperature. The activation energy of the sodium permeability ($\Delta G_{\text{Na}}$) was 47 kJ mol$^{-1}$, which is of the same order as proton permeability. These data suggest that the lipid composition of the membrane has little effect on the membrane permeability for sodium. Moreover, the permeability of the membrane for sodium ions is much lower than for protons.

**Discussion**

In this study, we compared the proton and sodium ion permeability of liposomes composed of lipids derived from various bacteria and archaea, thereby covering nearly the entire range of temperatures at which these organisms can grow. For non-electrolytes, it has been shown that the passive permeability characteristics of membranes are essentially the same as those of liposomes prepared from extracted lipids (de Gier *et al.*, 1971). Liposomes, therefore, represent a good model system for a comparative study of the permeability characteristics of biological membranes. We used the first-order rate constant $k$ to describe the rate by which protons and sodium ions equilibrate across the membrane. The use of this parameter as a measure of the ion permeability is valid as liposomes that are obtained by extrusion are of equal average diameter (Ellerink *et al.*, 1994). The value of $k$ is independent of the amount of lipids in the sample (Ellerink *et al.*, 1994). The permeability coefficient, $P_T$ (in $\text{cm s}^{-1}$), can be derived from the first-order rate constant as described by Yamauchi *et al.* (1993). For protons, the values for $P_T$ measured in this study range from $10^{-13}$ to $10^{-5}$ cm s$^{-1}$ depending on temperature. Values of $P_T$ reported in the literature range from $10^{-10}$ to $10^{-4}$ cm s$^{-1}$ depending on the lipids employed in the study (Deamer and Bramhall, 1986). For sodium, the experimentally determined value for $P_T$ is much lower, i.e. of the order of $10^{-13}$ to $10^{-11}$ cm s$^{-1}$. These values are close to those reported in the literature (Gutknecht and Walter, 1981; Nozaki and Tanford, 1981). Therefore, the observed difference in proton and sodium ion permeability is $10^5$ to $10^7$.

An important finding of this study is that the temperature limit within which $k_T$ dramatically increases is different for each of the liposomes. The relevant range coincides with the growth temperature of the organism from which the lipid was isolated. In contrast, this correlation is not observed for $k_{\text{Na}}$. Rather, the lipid composition of the membrane has only a minor effect on the membrane permeability for sodium, and the rate of permeation seems only to be influenced by the temperature. These data support the view that protons and sodium ions permeate the membrane by distinct mechanisms (Deamer and Nichols, 1989). Various mechanisms have been proposed to explain this basal permeation of protons across the lipid bilayer. Based on theoretical considerations, it seems likely that transient water pores are involved across which the protons can permeate fast via a wire-like conductance mechanism (Nagle and Morowitz, 1978). Entry of protons into the water pore must be extremely fast to account for the experimental values for proton permeation (Marrink, 1994). The high proton permeation rates have also been attributed to weakly acidic contaminants, which act as proton carriers (Gutknecht and Walter, 1981; Gutknecht, 1987). Lipid hydrolysis and oxidation are considered to be possible origins for weak-acid protonophores.
In contrast, sodium permeation is likely to occur via the solubility–diffusion mechanism, in which the transport rates of the permeant are proportional to the product of the solubility coefficient and the diffusion constant of the permeant in the membrane. The low value of the activation energy of the permeation of sodium across the membrane (about 50 kJ mol⁻¹) suggests that the permeating entity is the hydrated sodium ion (about 80 kJ mol⁻¹) rather than the monovalent ion (about 160 kJ mol⁻¹) (Georgallas et al., 1987). The measured rates of sodium permeation are several orders of magnitude higher than predicted according to the solubility–diffusion model (Hauser et al., 1973). To lower the energy barrier for ions to permeate the membrane, transient defects in the membrane have been implicated that would allow strands of water to penetrate into the bilayer. Although the lipid compositions of the various liposomes are different, the activation energy for proton permeation was found to be in the same range, i.e., 40 to 55 kJ mol⁻¹. This must imply that the basal mechanism of proton permeation is similar, but that the temperature range within which a steep increase in permeability is observed is dictated by other attributes of the membrane. One of these attributes may be the fluidity of the membrane. It should be emphasized, however, that in the case of *B. stearothermophilus* the maximum growth temperature is not determined by the upper boundary of the phase transition of the membrane lipids (McElhaney and Souza, 1976).

More specific factors related to lipids have been implicated to explain the low proton permeability of liposomes composed of archaeal lipids. Unlike ester lipids such as egg yolk phosphatidylcholine, membranes composed of the archaeal model lipid diphytanyl-sn-glycero-3-phosphocholine are highly resistant to proton permeation. This has been attributed to the inability of the bulky isoprenoid chains to form hydrogen-bonding chains of water between the phytanyl chains of the membrane lipids (Deamer and Nichols, 1989). The membrane of *S. acidocaldarius* contains branched (isoprenoid) tetraether chains, whereas lipids from *T. maritima* are composed from ester-bound acyl chains with few branches (Table 1). Both organisms were grown at the same temperature, but the proton permeability of *T. maritima* is higher than that of *S. acidocaldarius* (Fig. 2). However, this discrepancy is not apparent when liposomes composed of *M. barkeri* and *E. coli* are compared. Both organisms live at the same temperature, but the diether lipids of *M. barkeri* contain isoprenoid chains like those of the *S. acidocaldarius* lipids. The presence of bulky isoprenoid chains alone is clearly not sufficient to account for the observed differences in proton permeability. It may well be that the continuum of the tetraether lipids that results in a monolayer organization of the membrane presents an additional energy barrier.

What is the physiological consequence of the organism-specific temperature-dependent proton permeability? The value of \( k_{H^+} \) of the liposomes at the respective growth temperatures of most of the organisms used in this study is of the order of 0.02 to 0.2. The temperature dependency of \( k_{H^+} \) appears to be related to the growth temperature of the microorganism. The value of \( k_{H^+} \) in *B. stearothermophilus* liposomes is, however, exceptionally high at its growth temperature. This has been noted before in membrane vesicles of *B. stearothermophilus* (de Vrij et al., 1988), and it has been suggested that in this organism the high proton permeability of the membrane is compensated for by a high respiration rate. Nevertheless, with increasing temperature, a point will be reached at which the microorganisms are unable to compensate for the increase in proton permeation. Our data, therefore, suggest that the proton permeability is an important growth-limiting factor at the upper boundary of the growth temperature.

The thermoacidophile *S. acidocaldarius*, which grows at pH 2.5 and 85°C, has to maintain a steep proton gradient in order to keep the internal pH near to neutrality, i.e., pH 6.5. This can only be realised with a proton-resistant membrane and a highly efficient respiratory chain that expels the protons from the cytosol (Schäfer et al., 1990). Energy transduction in this organism is proton coupled, and our data demonstrate that, even at high temperatures, the membranes are highly resistant to proton permeation. For *S. acidocaldarius*, the ratio of \( k_{H^+} \) to \( k_{Na^+} \) is only 10-fold, and, under these conditions, it is not likely that sodium would be a preferable coupling ion. For the other organisms tested, the ratio of \( k_{H^+} \) to \( k_{Na^+} \) becomes higher, and conditions may prevail that make it more advantageous to use sodium ions instead of protons. The rate of permeation is proportional to the concentration of the permeating ion and its diffusion constant. In addition, the proton permeability of model membranes steeply depends on the magnitude of \( \Delta \psi \) (O'Shea et al., 1984; Krishnamoorthy and Hinkle, 1984). Since the sodium permeability may be limited by its membrane solubility, it is likely that \( \Delta \psi \) has less of an effect on the rate of sodium permeation. In the presence of a \( \Delta \psi \), the ratio of \( k_{H^+} \) to \( k_{Na^+} \) may, therefore, be even higher. In this respect, the thermophile *C. fervidus* exclusively uses sodium as the coupling ion (Speelmans et al., 1993a). The membrane of this organism is endowed with a high proton permeability, and the payoff for *C. fervidus* is that it is unable to maintain its intracellular pH so it is confined to growth in an environment of neutral pH. Many microorganisms with membranes with a restricted proton permeability, however, are able to use both sodium ions and protons as coupling ions. The existence of both the proton and sodium cycles makes the bioenergetic system more versatile and allows the organisms to rapidly adopt to changing environmental conditions (Skulachev, 1994).
Our data suggest that proton permeability is an important growth-limiting factor at the upper boundary of the growth temperature. Microorganisms have developed different mechanisms to cope with the increased permeability of the cytoplasmic membrane at higher temperatures. They may maintain the proton motive force either by increasing the rate of proton pumping or by dramatically altering the membrane composition so that the membrane becomes less permeable to ions. Possibly as a last resort, cells may entirely change their energy-transducing mechanism by coupling these processes to an ion that is less permeable than protons, such as sodium ions.

**Experimental procedures**

**Strains and purification of lipids**

*S. acidocaldarius* DSM 639 was grown aerobically in a 501 fermenter at 80°C in Brock’s medium supplemented with 5.8 mM L-glutamic acid, 50 mM K₂SO₄, and 5.8 mM sucrose (Brock et al., 1972). *M. barkeri* DSM 805 (Kandler and Hippe, 1977), grown at 30°C, was kindly provided by V. Müller. *T. maritima* MS8, DSM 3109 (Huber et al., 1986), grown at 80°C was kindly provided by R. Huber. *B. stearothermophilus* ATCC 7954 cells were grown aerobically in a 101 fermenter at 60°C in 2x Luria broth. *Psychrobacter* sp. (Micrococcus cryophilus ATCC 15174) (McLean et al., 1951) cells were grown in 8 g l⁻¹ BBL Nutrient broth (Becton and Dickinson) at 4°C. Freeze-dried cells of *S. acidocaldarius*, *M. barkeri* and *T. maritima* were extracted with soxhlet and fractionated essentially as described by Lo and Chang (1990). Lipids were finally suspended in chloroform:methanol:water (65:25:4 by volume), and stored at -20°C. Freeze-dried cells of *B. stearothermophilus* and *Psychrobacter* lipids were isolated essentially as described by Ames (1968) with some modifications (Vitanen et al., 1986) and were purified by acetone:ether extraction (Kagawa and Racker, 1971). E. coli phospholipid (E. coli L-α-phosphatidyl ethanolamine type IX, Sigma) was purified by acetone:ether extraction. Lipids were stored in chloroform at -20°C.

**Preparation of liposomes**

Lipids were dried by vacuum rotary evaporation and hydrated in 50 mM morpholinopropane sulphate (MOPS) pH 7.0, 75 mM KCl and 25 mM dithiothreitol to a final concentration of 40 mg ml⁻¹. Liposomes were obtained by five consecutive freezing and thawing steps, followed by extrusion through 200 nm polycarbonate filters (Avestin) using the Liposofast™ (Basic, Avestin) extrusion apparatus. These liposomes (LUVETS) are unilamellar with an average size that is close to the pore size of the filter used (Efferink et al., 1994).

**Measurement of proton and sodium ion permeability**

Proton permeability was measured essentially as described by Nichols and Deamer (1980). The external buffer of the liposomes was replaced with 0.5 mM MOPS pH 7.0, 75 mM KCl and 75 mM sucrose (buffer A) by chromatography over a Sephadex G-25 M PD-10 (Pharmacia) column to obtain a low buffering capacity on the outside. Liposomes were diluted to 1.5 mg ml⁻¹ in 2 ml buffer A. The potassium ionophore valinomycin (1 nmol mg⁻¹ lipid) was added to prevent the formation of a reversed transmembrane \( \Delta \Psi \). The fluorescent pH probe pyranine (10 μM) was added to the medium to monitor changes in the external pH. Excitation and emission wavelengths were 450 and 508 nm, respectively. After equilibration, 100 nmol H⁺ (from a 50 mM H₂SO₄ stock solution) was added to lower the external pH. Influx of protons into the liposomes was monitored by following the partial recovery of the internal pH, as measured by an increase in pyranine fluorescence. Nericin (1 nmol per mg lipid) was finally added to equilibrate the proton gradient across the membrane. Samples of 100 nmol OH⁻ and 50 nmol H⁺ were used for calibration purposes (Efferink et al., 1994). Fluorescence measurements were performed on a Perkin-Elmer LS-50B or an SLM Amino 4800C fluorimeter, using a thermostated magnetically stirred sample compartment. Fluorimeter data were fitted to the first-order kinetic equation:

\[
f(t) = a \left(1 - e^{-kH^+ t}\right) + c
\]

in which \( a \) is the amplitude of the fluorescence signal, \( k_H^+ \) is the first-order rate constant of proton influx and \( c \) is the offset. The proton pulse was imposed at \( t = 0 \), and \( k_H^+ \) was used to compare the proton permeability of the different liposomes.

The sodium permeability of the liposomes was analysed by the efflux of 22Na. Liposomes were prepared in 50 mM MOPS KOH pH 7.0, 100 mM KCl and 0.1 mM NaCl. To this suspension (550–650 μl final volume), 100 μl NaCl (approximately 60 × 10⁻⁶ C.p.m.; specific activity > 2200 Ci mol⁻¹, Amersham, UK) was added to yield a final concentration of 1 mM. Liposomes were equilibrated with 22Na by incubation for 18–46 h at room temperature, or 40°C for thermophiles. To initiate 22Na efflux, 100 μl of the liposomal suspension was diluted in 10 ml of 50 mM MOPS KOH pH 7.0, 100 mM KCl and 1 mM NaCl. Since the permeability of sodium is low compared with that of protons (Speelmanns et al., 1993b; Gutknecht and Walter, 1981), it was not necessary to include valinomycin to prevent the generation of a \( \Delta \Psi \). Samples of 1 ml were taken and filtered over a 0.2 μm BA83 (Schleicher and Schuell) nitrocellulose filter. Filters were rinsed with 2 ml 200 mM KCl, and the amount of label retained on the filter was counted with a liquid scintillation counter. To determine the amount of 22Na bound to the filters, liposomes were permethanated for sodium ions by the addition of 1 mM Gammantic D (in dimethyl sulphoxide). Sodium efflux data were fitted to equation (1), as described for proton influx, to yield \( k_{Na^-} \).

**Other analytical techniques**

The integrity of the liposomes was tested by their ability to maintain an imposed potassium diffusion gradient in the presence of valinomycin. Values of \( \Delta \Psi \) (inside negative) were measured with the fluorescent probe 3,3'-diethylthiacarbocyanine iodide (DiSC₃(5)) as described (Singh et al., 1985).

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References


