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Biological Membranes in Extreme Conditions: Anionic Tetraether Lipid Membranes and their Interactions with Sodium and Potassium

Luis Felipe Pineda De Castro,^{†,‡,§} Mark Dopson,[¶] and Ran Friedman^{*,†,‡}

Computational Chemistry and Biochemistry research Group (CCBG), Department of Chemistry and Biomedical Sciences, Linnæus University, 391 82 Kalmar, Sweden, Centre of Excellence “Biomaterials Chemistry”, 391 82 Kalmar, Sweden, and Linnaeus University Centre for Ecology and Evolution in Microbial model Systems(EEMiS), 391 82 Kalmar, Sweden

E-mail: ran.friedman@lnu.se

*To whom correspondence should be addressed

[†]Computational Chemistry and Biochemistry research Group (CCBG), Department of Chemistry and Biomedical Sciences, Linnæus University, 391 82 Kalmar, Sweden

[‡]Centre of Excellence “Biomaterials Chemistry”, 391 82 Kalmar, Sweden

[¶]Linnaeus University Centre for Ecology and Evolution in Microbial model Systems(EEMiS), 391 82 Kalmar, Sweden

[§]Current address: Laboratory of Molecular Modeling, University of Gdansk, ul. Wita Stwosza 63, PL-80-308 Gdansk, Poland

Abstract

Archaea such as *Sulfolobus acidocaldarius* tolerate extreme temperatures and high acidity and can grow in the presence of toxic metals and low concentrations of Na^+ or K^+ . It is believed that their unique tetraether membranes protect them from harsh environments and allow their survival under such conditions. We used molecular dynamics simulations to study membranes comprised of glycerol dialkylnonitol tetraether lipids, which are the main component of *S. acidocaldarius* membranes, in solutions containing different concentrations of NaCl and KCl, or with Na^+ or K^+ counterions (trace cations, 0 M). Anionic binding sites on the membranes were almost 50% occupied in the presence of counter-ions. The free energy of cation-phosphate complexation and the residence times of ions near the membranes were found to be both ion- and concentration-dependent. Sodium ions had more favorable interactions with the membranes and a longer residence time, whereas higher cation concentrations led to shorter ion residence times. When only counter-ions were present in the solutions, large residence times suggested that the membrane may function as a cation attracting reservoir. The results suggested that the ions can be easily transferred to the cytoplasm as needed, explaining the growth curves of *S. acidocaldarius* under different salinities and pH.

Introduction

High temperatures, acidity and salinity are unsuitable for most forms of life. However, some organisms have evolved to survive under such conditions, that were prevalent when life emerged on Earth. The archaeon *Sulfolobus acidocaldarius* is a very robust extremophile, that can tolerate the presence of different metal ions, temperatures over 80°C and pH values as low as 1^{1,2}. The unique membranes of *S. acidocaldarius* and other extremophiles are the first barrier between the living organism and the harsh environment. *S. acidocaldarius* membranes are mostly comprised of durable tetra-ether

lipids, glycerol dialkylnonitol tetraethers (GDNT) (Figure 1). Therefore, understanding of the physical properties of GDNT membranes is important in the study of extremophiles. Moreover, archaeal membranes can have many uses in biotechnology.

Figure 1: The chemical structures of GDNT lipids.

Molecular dynamics (MD) simulations have become an indispensable tool in the simulation of membranes and membrane proteins,^{3–7} as they provide atomistic information on interactions involving such membranes and their immediate environment. This environment is comprised mostly of water and salt ions at physiological or higher concentrations. Thus, using established models of phospholipid bilayers, many studies have examined membrane-ion interactions. In most of these studies, MD simulations were used to study models of the common phospholipid membranes such as palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylglycerol (POPG), 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), and dipalmitoylphosphatidylcholine (DPPC). Ions affect such membranes in various ways. They may penetrate into the polar head-groups,^{8,9} tighten oppositely charged membranes,¹⁰ and reduce the diffusion coefficient of membranes.¹¹ Mechanistically, sodium ions form bridges between negatively charged groups which can explain many of the observed dynamic phenomena.¹² Interestingly, sodium has a high affinity to phospholipid membranes such as DOPC, whereas potassium has no particular affinity to the lipid head groups.^{13,14}

Simulations and experiments of membrane-ion interactions have led to a better understanding of how ions affect the membrane structure and dynamics, but have limited applicability to diether membranes. For this reason, Shinoda and co-workers used MD

simulations to study how zwitterionic diether membranes are influenced by NaCl.¹⁵ They observed ion-penetration into the lipid head-group region and salt bridges involving up to three lipid molecules. Several other models of tetraether membranes were developed.^{16–21} We studied di- and tetra-ether membranes by MD simulations, where the negative charge on tetraether membranes from *S. acidocaldarius* was neutralized by counterions.²² Analysis of the simulations revealed that the size of the counterions affected the physical properties of the membranes. It is thus interesting to examine if such membranes would interact differently with Na⁺ and K⁺ ions.

One of the suggested physiological roles of potassium ions in acidophiles is to aid in maintaining pH homeostasis by forming an inside positive membrane potential.²³ In addition, recent RNA transcript profiling revealed that this archaeon has different expression profiles depending on the cation and pH.²⁴ In this study we used MD simulations to analyze the interactions of GDNT membranes and Na⁺ or K⁺ solutions at different concentrations.

Computational Methods

Molecular dynamics simulations

Parameterization and equilibration of the membranes were performed as previously described.²² Briefly, initial membrane structures were taken from simulations performed by us previously.²² The systems were minimized, heated to the target temperature of 350 K, and equilibrated for 80 ns before data collection. Four independent 80 ns simulations of GDNT-4 membranes in the presence of Na⁺ or K⁺ for each salt concentration, i.e., 0 M (counter-ions only), 0.4 M, and 3 M, were executed using the Gromacs program^{25–27} (version 4.5.5). Cl⁻ was used as an anion (0.4 M and 3 M). To simulate the membrane in the presence of water and ions, the system was solvated at first by pre-equilibrated water,

and then a given number water molecules were randomly replaced by ions. The Lennard Jones parameters for the ions are given in Table 1. The SPC/E model²⁸ was used for the water. The neutralized only simulation boxes (0 M) contained 36 GDNT-4 lipids, 2088 water molecules, and 36 counter ions. Simulation boxes at 0.4 M contained 36 lipids, 2056 water molecules, 52 cations, and 16 anions. Simulation boxes at 3 M contained 36 lipids, 1892 water molecules, 134 cations, and 98 ions. One simulation for each system was elongated further to 200 ns. Analysis was carried out on the four 80 ns long simulations for each ion and concentration unless otherwise stated.

Membrane properties, i.e., area per lipid (APL), volume per lipid (VPL), membrane width (Lz), peak-to-peak distance of electron density profile (d_{PP}), and volume of the membrane unit (VMU) were calculated as described.²²

Table 1: σ and ϵ Lennard Jones parameters for ions

Ion	σ (nm)	ϵ (kJ mol ⁻¹)
Na ⁺	0.235019	0.543502
K ⁺	0.304866	0.418400
Cl ⁻	0.432000	0.418400

Ion complexation

The proportion of ion complexation was estimated by counting the number of Na⁺ (K⁺) ions located within $d_c = 0.32$ nm (0.34 nm) of a partially charged phosphate oxygen. The distances correspond to the first minimum of the phosphate-ion radial distribution function (RDF). The complexation free energy was calculated as:²⁹

$$\Delta G_{\text{complexation}} = -RT \ln(C_{\text{complexed}}/C_{\text{free}}) \quad (1)$$

where R is the gas constant, T is the temperature, $C_{\text{complexed}}$ is the equilibrium concentration of the ions that are complexed, and C_{free} is the equilibrium concentration of the ions that are not complexed.

Concentrations of the complexed and free ions were calculated as $N_{complexed}/V_{shell}$ and N_{free}/V_{bulk} . V_{shell} is the volume where ions could move while still in contact with the membrane, and is estimated as

$$V_{shell} = V_{box} - V_{mem} - L_z \times d_c \quad (2)$$

V_{box} and V_{mem} are the volumes of the simulation box and membrane, respectively, L_z is the membrane thickness, and d_c is the critical distance from the phosphate oxygens (see above). V_{bulk} is the volume of the box in which the ions can move outside of the membrane's shell:

$$V_{bulk} = V_{box} - V_{mem} - V_{shell} \quad (3)$$

To estimate the proportion of GDNT lipids that were complexed, the minimal distance between each of the GDNT lipids and all of the ions was calculated at every frame of the simulations. If that number was smaller than the cutoff distance then the lipid site was considered complexed. Note that the proportion of ions that are complexed and the proportion of lipids that are complexed need not be the same, even at maximum dilution, as a single ion can bind multiple lipids and vice versa.

Residence times

Residence times for the ions within 0.6 nm of the membrane phosphorus atoms were calculated as follows. First, the distribution of lifetimes for each individual ion in each individual simulation was calculated by the Gromacs utility `g_dist`. Then, the time to reach a contact number of 0.5 (indicating that an ion is within the given cutoff in 50% of the simulation frames) was taken as the residence time. Residence times were averaged over all ions and simulations at each concentration, yielding the average residence time, $\bar{\tau}$. The maximum residence time, that is, the longest time an ion was located within 0.6 nm

of the membrane phosphorus atoms, $\tau_{max,i}$ was also extracted. The maximum and median maximal residence time for each subset of simulations, τ_{max} and $\widetilde{\tau_{max}}$ were calculated as $Max(\tau_{max,i})$ and $Median(\tau_{max,i})$, respectively.

Electrostatic potential

The electrostatic potential along the membrane axis of the simulation box was calculated from the second half of the long (200 ns) simulation for each condition (ion and concentration). The potentials were calculated with reference to the center of the membrane, where the charge is close to zero. The charged phosphate groups are located on the left-hand side of the figure (negative values of the distance to the center along the z axis).

Results and discussion

Salt effects on the membrane structures

The area per lipid (APL), volume per lipid (VPL), membrane width (Lz), peak-to-peak distance of electron density profile (d_{pp}), and volume of the membrane unit (VMU) were calculated to examine how the different salts affect the structural properties of the GDNT-4 membranes (Table 2).

For NaCl, the APL, VPL and Lz decreased slightly when increasing the salt concentrations, whereas d_{pp} and VMU slightly increased. The most significant change was observed for the VPL, which shrunk by 2.5% at 3 M NaCl with reference to the volume with Na^+ counterions only. The changes were concentration-dependent, i.e., the effect of 3 M NaCl was larger than that of 0.4 M NaCl. Interestingly, this was not the case for potassium, where the changes were very subtle, except for the lateral diffusion coefficient which decreased with KCl concentration. This difference between the cations may be explained by their interactions with the phospholipids and the Cl^- ions. Sodium has a higher affinity

Table 2: Physical properties of the simulated membranes. Values are averages over four independent trajectories, with error estimates based on block averages over five blocks (APL, VPL, VMU, Lz and d_{PP}), and deviation within the 95% confidence interval (for the lateral diffusion coefficient, D_L). The data were calculated for Na^+ and K^+ solutions in three concentrations: 0 M (only counter-ions), 0.4 M and 3 M

Ion	C[M]	APL ($\text{\AA}^2$)	VPL ($\text{\AA}^3$)	Lz($\text{\AA}$)	d_{PP} ($\text{\AA}$)	VMU ($\text{\AA}^3$)	D_L ($\text{cm}^2 \text{s}^{-1}$)
Na^+	0	66.5(.2)	2813(14)	42.3(.3)	40.1(.2)	2668(.4)	0.31(.05)
	0.4	66.3(.2)	2799(17)	42.2(.4)	40.4(.3)	2678(.9)	0.36(.03)
	3	66.1(.2)	2743(14)	41.5(.3)	40.6(.9)	2686 (1)	0.23(.07)
K^+	0	69.5(.1)	2835(10)	40.8(.2)	38.1(.3)	2647(.5)	0.43(.06)
	0.4	68.5(.1)	2824(8)	41.2(.2)	39.5(.9)	2704(1)	0.29(.10)
	3	69.0(.1)	2805(9)	40.7(.2)	37.5(.6)	2588(.8)	0.23(.04)

to the phosphate moiety (Figure 2) whereas potassium has a higher affinity to the Cl^- ions in the solution. When the concentration of sodium ions increased, more ions were drawn to the membrane surface, affecting the membrane's physical properties. However, in concentrated KCl solutions, potassium ions were more likely to be attracted by the abundant chlorides. The higher lateral diffusion coefficient in the simulations with K^+ counterions only may be influenced by repulsion between the membrane's anionic lipids that attract Na^+ ions better than K^+ .

Figure 2: Radial distribution functions for the cations around (A) Cl^- ions (B) the charged oxygens of the phosphate group.

Specific membrane ion-interactions

GDNT membranes are asymmetric, with an anionic myo-inositolphosphate on one side and calditol attached to D-glucopyranose on the other (Figure 1). To follow up on cation binding, we first calculated radial distribution functions (RDFs) between the solvent-exposed polar or negative ions and the cations (RDFs have the same shape as plain distance distributions for small ions^{29,30}). The cations were strongly attached to the charged phosphate oxygens (Figure 2B), which have a higher affinity for Na⁺. Other polar oxygens do not have a particular affinity to the alkali ions. A double layer (chloride ions concentrated around the cations) was not observed.

Each lipid in the GDNT membranes carries a single negative charge. To find out if the binding to the membrane is favorable to the solvation of ions, we calculated the percentage of the ions that form a closed sphere complex (i.e., without any intervening water molecules) with any of the phosphate oxygen atoms and the free energy associated with such complexation events, $\Delta G_{\text{complexation}}$. When only Na⁺ counter ions were present, 51% of these were complexed with the membranes, which resulted in a complexation free energy that was negative (Table 3, Table S1). The higher the concentration, the higher the number of ions that form such complexes, but this was purely due to their increased overall concentration. The share of the ions that were complexed steadily decreased with increasing of salt concentration, such that only 25% of the sodium ions were complexed with the membrane at the highest concentration. The number and proportion of complexed ions was smaller for K⁺ relative to Na⁺. When it comes to $\Delta G_{\text{complexation}}$, it was always less favorable for the potassium ions, which have a higher tendency to be located in the solvent.

It is also interesting to examine the proportion of lipids whose charges were neutralized by the counterions at the various concentrations. Apparently, addition of counter ions leads to binding of Na⁺ (K⁺) to 54% (44%) of the lipids. These values only changed by a few percent in 0.4 M salt, whereas as many as many as 78% (60%) of the lipids were

complexed by Na⁺ (K⁺) ions at the highest concentration.

It is also worthwhile to assess if any sort of binding cooperativity can be observed in the simulations. Binding of ions to the membrane neutralizes some of the membrane's negative charges. Thus, one could expect that the more ions that bind the phosphate, the less likely it is for further ions to bind (negative cooperativity). In that case, the distributions of the number of occupied ion binding sites (negatively charged lipids) will be left-skewed. The calculated distributions are displayed in Figure 3. The distributions were symmetric for the systems with counter-ions only and those simulated with in 0.4 M solutions. Negative cooperativity was observed in simulations of membranes in 3 M salt, and was more pronounced for Na⁺ ions, where the systems had finite (though small) probability to be fully occupied by ions.

Table 3: Inner-sphere complexes of Na⁺ and K⁺ ions with the membranes. The percentage of the ions (of all ions in the simulation box) that were within a distance of 0.32 nm (Na⁺) or for (K⁺) 0.34 nm from any charged phosphate oxygen is shown. The complexation energy was calculated from the proportion between the bound and free ions (see the Methods section). Values in parentheses are standard deviations.

Ion	Concentration	% Complexed	$\Delta G_{\text{complexation}}$ (kcal/mol)	% sites occupied
Na ⁺	0	51(7)	-1.4	54(7)
	0.4	55(5)	-1.1	58(8)
	3	25(3)	-0.6	78(6)
K ⁺	0	41(8)	-1.1	44(8)
	0.4	31(5)	-0.8	47(8)
	3	17(2)	-0.2	60(8)

The charge density and electrostatic potential in the simulation box

Density profiles show the distribution of different chemical groups in the simulation box. The density profiles for phosphorous atoms and the ions are shown in Figure 4. The cations were slightly further from the membrane center than the phosphate moiety, as was the case also for DOPC membranes.¹⁴ However, the density profiles reveal some

Figure 3: The distribution of the number of occupied sites (lipid molecules that bind counterions). Results for simulations with Na^+ and K^+ ions are shown in black and blue, respectively. Pure water, solid lines with circles; 0.4 M solutions, dashed lines with squares; 3 M solutions, points with triangles.

differences between the negatively charged archaeal membranes and zwitterionic DOPC phospholipid. The most pronounced difference is that the profiles of both Na^+ and K^+ ions had a peak in the density at a distance of slightly over 2 nm from the membrane center, whereas only Na^+ ions had such peak in simulations with DOPC.¹⁴ Another difference is that while there was a trough in the number distribution for Na^+ ions near DOPC, no such trough was observed here - their density decayed smoothly toward the bulk density. The local minimum in the density profile of Na^+ near DOPC is likely due to repulsion to choline, which is not a part of the GDNT archaeal membranes.

A comparison between the density profiles of Na^+ and K^+ reveals that the latter were slightly broader at the maximum and that the maximum for K^+ was lower, confirming their tendency to be associated with the bulk water more than with the membrane. This was more pronounced in 0.4 M solution, where screening effects were weaker. At the high concentration, there was even a small peak for Cl^- ions 2.7 nm away from the membrane

center, that was due to interactions with the cations near the membrane. However, at 3M NaCl or KCl solution the increase in the number density is too small to consider formation of a double layer.

Figure 4: Number densities of lipid phosphorous atoms (violet), Cl^- (red), and Na^+ (black) or K^+ red. The membrane center is at 0. The x-axis shows distance from the membrane in nm. Calculations were performed for simulations of GDNT-4 membranes in 0.4 M NaCl (top left), 3 M NaCl (top right), 0.4 M KCl (bottom left), and 3 M KCl (bottom right).

The electrostatic potential across the membranes is shown in Figure 5. The phosphate moiety made the potential negative on the left-hand side of the figure, with a small peak due to binding of the ions that was more pronounced for Na^+ than K^+ and for systems with more cations (0.4 M and 3 M relative to counter-ions only). The right-hand side also showed a negative potential, which was more pronounced for simulations with salts (NaCl / KCl). This is due to the chloride ions that avoided the negatively charged phosphates. Overall, the electrostatic potential was quite similar in shape for all GDNT-4 membrane systems. Only small differences were observed between the systems simulated in the presence of 0.4 M and 3 M salts, whereas the difference between systems with 0 M ions (counter-ions only) and 0.4 M salt was more pronounced. This is explained

by the coverage of the membrane by cations already where only counterions are present (Table 3). The different ions did not seem to influence the dipole of the membrane, which was around 100 D per lipid (Table S2).

Figure 5: The electric potential across the simulation box. The potential is shown over the z axis for each ion and concentration. The charged phosphate groups are located on the left-hand side of the figure (lower values of z). Potentials are calculated from the second half of a single 200 ns long simulation for each ion and concentration. The membranes are centered at $x=0$ nm. Note that the (average) box length is not exactly the same due to the simulation conditions (constant pressure, variable volume).

Residence times of the ions near the membrane surface

The negative potential that surrounds the phosphate edge of the membrane generates an electric field to which the positive ions are attracted, an effect that is also seen with other biomolecular solutes.²⁹ To quantify the effect of the membrane potential on the ions dynamics, we evaluated the residence time of each ion for simulations with different ion concentrations. Following Vácha and co-workers,¹⁴ the ions are considered to reside by the membrane when the ion to phosphorus atom distance is 0.6 nm or smaller. The mean residence times for the alkali ions (Table 4) were concentration- and ion-dependent. Higher

concentrations resulted in lower residence times, which can be explained by screening of charges. Sodium ions have considerably longer residence times compared with potassium ions, which can be reasoned by their higher affinity to the membrane and by their lower tendency to form a hydrated complex with the chloride anions. Of note, the residence times calculated here from four 80 ns simulations are somewhat underestimated. Calculations from 200 ns simulations (one for each system) resulted in larger residence time for the ions (Table S3).

The residence times were considerably larger than those calculated by Váchá and co-workers for zwitterionic DOPC membranes (0.35 and 0.08 ns for Na⁺ and K⁺ ions, respectively, at 1 M concentrations¹⁴). Since the force-field was the same in both simulations, variations can be attributed to the anionic nature of the archaeal membranes. In both cases (DOPC and GDNT-4), the maximal residence time of the sodium ions can be as large as the simulation time. However, the median value of the maximum residence time (over all ions and simulations) was considerably smaller and a better representative of a large residence time of a typical ion in the simulations, as it traveled from binding site to binding site along the membrane. The values for Na⁺ and K⁺ ions (Table 4) were in the ns range and were ion- and concentration-dependent.

Table 4: Residence times (ns) of Na⁺ and K⁺ ions within 0.6 nm of the phosphorus atoms of the membranes. $\bar{\tau}$ is the average residence time per ion, calculated as the average time at which the ion has a 50% probability to detach the membrane if bound. $\widetilde{\tau}_{max}$ is the median maximal time that an ion spends at contact distance and τ_{max} is the maximal residence time over all ions and simulations.

Ion	Concentration	$\bar{\tau}$	$\widetilde{\tau}_{max}$	τ_{max}
Na ⁺	0	9.2	12	80
	0.4	4.8	8.2	80
	3	1.9	4.6	80
K ⁺	0	1.7	4.0	62
	0.4	0.6	2.8	47
	3	0.3	1.7	49

Attraction and detainment of the ions by GDNT membranes (antenna effect)

Archaeal growth experiments revealed that *S. acidocaldarius* could grow in low Na^+/K^+ medium (16 μM potassium and 4 μM sodium).²⁴ To be able to sustain growth under such conditions, these organisms must have evolved an efficient mechanism to pump ions into their cytoplasm. Cations transporters are essential for this, but these proteins must be able to gather the scarce ions from the extracellular environment. The simulations of archaeal GDNT membrane suggest that the membranes themselves may make ion transport more efficient by adsorbing ions at their surface, thereby generating local ion reservoirs. Thus, there is an elevation of the effective concentration of cations at the membrane surface and hence also near any cation transporter which is located in the membrane. As noted earlier, trace amounts of sodium and potassium ions were sufficient to occupy about half of the membrane anionic sites (Table 3, 0 M). Taking this, and the fairly long residence times of the ions near the membrane into account, it is plausible that the anionic GDNT-rich archaeal membrane functions as a cation attracting reservoir. The cations in turn only loosely bind to any specific binding site on the membrane, and are transported into the cytoplasm as needed by cation transporters. This is analogous to the antenna effect that is operative for protons at physiological concentration,^{31,32} where proton pumps include residues at their surface whose role is to attract protons from the surrounding solution, and hold them long enough for them to be transferred to the orifice of the channel.

The proposition of the GDNT membrane acting as cation antenna is further strengthened by the finding that growth of the archaea is somewhat impaired when they are in an environment that is both low on salt and highly acidic ($\text{pH}=1.6$).²⁴ Under highly acidic pH , many of the membrane binding sites are neutralized by protons, making it more difficult for the archaea to scavenge for alkali cations that are necessary for their growth.³³ Thus, the

antenna effect is non-operative or less efficient at high acidity, where the concentration of protons is higher than that of alkali cations.

Conclusions

Simulations of GDNT-4 membranes, which represent archaeal anionic membranes were carried out in the presence of Na^+ counter-ions, K^+ counter-ions, 0.4 M NaCl and KCl solutions and 3 M NaCl and KCl solutions. Similar to phospholipid membranes (see e.g.,¹⁴), and despite their different structure, the archaeal membranes adsorbed Na^+ ions much better than K^+ . This preference to Na^+ had not been hitherto reported. Some interesting findings were observed in the simulations, that point to specific ion effects that depend on the specific interactions between the components of the simulated system. Even when only counter-ions were present in solution, about half of the membrane anionic sites were occupied by cations. This leads to contraction of the membranes, which increases with the salt concentration for NaCl. The situation is more complex for KCl though, as the membrane's biophysical properties are almost the same in 0.4 M and 3 M solutions. The Na^+ and K^+ cations bind to the negative phosphate groups but do not seem to significantly interact with the polar, non-charged fraction of the membranes. The affinity of the cations to the membrane strongly depended on the salt concentration. Negative cooperativity of cation binding to anionic sites on the membrane (a reduction in the probability of a free site to become occupied due to cation-cation repulsion) was weak and could only be observed at 3 M. A double layer (membrane-cations-chlorides) was not observed at all.

The residence times of the ions near the membrane surface were in the order of several ns, and were concentration and ion-dependent (the higher the concentration, the lower the residence time). The combination of fairly low affinity for any given cation site and long residence time at low ionic concentration suggested that an antenna effect was in place, i.e., the membrane attracts the ions and forms a reservoir that ion channels can

then transport to the cytoplasm. This antenna effect was presumably significantly weaker in low-pH, which may explain why archaea could proliferate under low-pH medium or medium that was almost devoid of K^+ and Na^+ , but their growth was limited when they were kept at low-pH and in a low Na^+/K^+ medium.

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Graphical TOC Entry