# The Permeability of the Sodium Channel to Metal Cations in Myelinated Nerve

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ABSTRACT The relative permeability of sodium channels to eight metal cations is studied in myelinated nerve fibers. Ionic currents under voltage-clamp conditions are measured in Na-free solutions containing the test ion. Measured reversal potentials and the Goldman equation are used to calculate the permeability sequence: $Na^+ \approx Li^+ > Tl^+ > K^+$. The ratio $P_K/P_{Na}$ is 1/12. The permeabilities to $Rb^+$, $Cs^+$, $Ca^{++}$, and $Mg^{++}$ are too small to measure. The permeability ratios agree with observations on the squid giant axon and show that the reversal potential $E_{Na}$ differs significantly from the Nernst potential for $Na^+$ in normal axons. Opening and closing rates for sodium channels are relatively insensitive to the ionic composition of the bathing medium, implying that gating is a structural property of the channel rather than a result of the movement or accumulation of particular ions around the channel. A previously proposed pore model of the channel accommodates the permeant metal cations in a partly hydrated form. The observed sequence of permeabilities follows the order expected for binding to a high field strength anion in Eisenman's theory of ion exchange equilibria.

## INTRODUCTION

This paper continues the analysis of ionic selectivity in nerve membranes. A previous paper (Hille, 1971 *b*) showed that sodium channels are permeable to at least seven organic cations, the largest of which is aminoguanidine. Two organic cations, hydroxylamine and hydrazine, are almost as permeant as the $Na^+$ ion. The observations on organic cations led to the hypothesis that the channel is a short oxygen-lined pore with an opening approximately 3 × 5 A. Organic cations fit into the pore by forming hydrogen bonds with the oxygen atoms lining the narrow pore. Some of the organic ions are large enough to fill the pore completely as they pass through. This paper deals with the permeability of the sodium channel to eight metal cations, four of which

are measurably permeant. Most of these ions are small and can be accompanied by two to four waters of hydration in the model pore. The observed ionic selectivity sequence correlates well with a binding sequence predicted by Eisenman's (1962, 1969) theory of ion exchange equilibria.

## METHODS

The methods and some errors and limitations are described in a preceding paper (Hille, 1971 *b*). Briefly, single myelinated nerve fibers from the sciatic nerve are studied under voltage-clamp conditions at 5°C. The object of the experiments is to determine the reversal potential (zero current potential) for current in sodium channels while the nerve fiber is bathed in solutions containing the different metal cations to be studied. The change in reversal potential ($E_r$) on replacing all the external sodium with a test ion is used with the Goldman (1943; Hodgkin and Katz, 1949) equation to calculate the relative permeability of sodium channels to the test cation. For the experiments here, the change of $E_r$ on switching from the control Na Ringer to a sodium substitute ($S$) Ringer would be:

$$E_{r,s} - E_{r,\mathrm{Na}} = 55.2 \log_{10} \frac{P_S}{P_{\mathrm{Na}}} \frac{(S)}{(\mathrm{Na})}$$

where $P_S/P_{\mathrm{Na}}$ is the permeability ratio, parentheses refer to ionic activities in the external solution, and reversal potentials are in millivolts. Membrane currents are measured at 20 different depolarizing voltage steps, and corrected for capacity and leakage currents before the reversal potential is found. The currents in all figures have been corrected this way. As before, the voltages are corrected for junction potentials and the "attenuation artefact." The attenuation correction of 2–15% added to the measured voltages is made so that the corrected change of $E_r$ on diluting the external $Na^+$ eightfold equals the theoretical value, 49.8 mv. The ends of the nerve fiber are cut in isotonic KCl or CsF solutions in order to load the interior of the fiber with $K^+$ or $Cs^+$ ions and to reduce the axoplasmic $Na^+$ concentration.

*Solutions* The salts tested are LiCl, NaCl, KCl, RbCl, CsCl, $TlNO_3$, $CaCl_2$, and $MgCl_2$. The solutions follow the same format as that given in the previous paper (Hille, 1971 *b*) with either 110 mM NaCl or an osmotically equivalent amount of a test salt and 2 mM $CaCl_2$, 6 mM tetraethylammonium bromide, and 1 mM tris-(hydroxymethyl)aminomethane buffer at pH = 7.4. The solutions are called Li Ringer, Na Ringer, etc. The pH, osmotic pressure, and sodium and potassium content of all solutions were checked as before. Because of the insolubility of thallium halides, all components used in the thallium Ringer are nitrate salts. Tetramethylammonium Ringer is mixed with Na Ringer to give a low-sodium solution with 12.5% of the usual sodium concentration.

*Activity Coefficients* The correct use of the Goldman equation requires knowing the thermodynamic activity of the test cation in solution. In this paper the single ion activity coefficient for each monovalent cation is assumed to be equal to the

activity coefficient of its salt. Robinson and Stokes (1965) give the following molal activity coefficients for 0.1 molal solutions at 25°C: LiCl, 0.790; NaCl, 0.778; KCl, 0.770; RbCl, 0.764; CsCl, 0.756. The activity coefficient for 0.07 M $CaCl_2$ is 0.55 (Robinson and Stokes, 1965). By the Guggenheim convention (see Shatkay, 1968) the single ion activity coefficient for $Ca^{++}$ is equal to the square of this value or 0.30. The activity coefficient for $Mg^{++}$ has the same value. On this basis the cation activity coefficients for $Li^+$, $Na^+$, $K^+$, $Rb^+$, $Cs^+$, $Ca^{++}$, and $Mg^{++}$ are in the ratio 1.02:1.00:0.99:0.98:0.97:0.39:0.39. The activity coefficient of $TlNO_3$ is 0.702, but the salt is also only 85% dissociated in 110 mM $TlNO_3$ (Sillén and Martell, 1964). These numbers taken together give an activity coefficient of 0.65 for $Tl^+$ or 0.84 of the value for $Na^+$. These coefficients are used with the Goldman equation in computing the permeability ratios. Similar corrections were not made in the previous work on organic cations (Hille, 1971 *b*) because the appropriate coefficients were not available.

*Atomic Radii* All ionic crystal radii and van der Waals radii are taken from Pauling (1960). The Pauling radii agree well with cation-oxygen and cation-water interatomic distances in over 40 crystals listed by Wyckoff (1962). Other widely used ionic radii given by Goldschmidt (1926) and by Gourary and Adrian (1960) do not agree as well with the crystal structures.

## RESULTS

### *Lithium, Sodium, and Potassium Ions Are Permeant*

LITHIUM AND SODIUM Sodium channels are more permeable to lithium and sodium ions than to any other tested ion. Families of voltage-clamp currents for a node bathed in Na Ringer and Li Ringer are shown on the right side of Fig. 1, and the corresponding peak current-voltage relations for the same experiment are on the left of the figure. There is almost no change in the reversal potential $E_r$ for current in Na channels when all the external sodium is replaced by lithium. In eight experiments the change in $E_r$ was a decrease of 1.6 ± 1.5 mv (mean ± SD), which corresponds to a permeability ratio $P_{Li}/P_{Na}$ of 0.93 as calculated from the Goldman equation.

Currents in Li Ringer are always smaller than predicted from the independence principle for an ion with a permeability ratio of 0.93. The peak inward currents at −20 mv averaged 28% lower in Li Ringer than in Na Ringer. The solid current-voltage curve for Li Ringer in Fig. 1 is calculated on the assumption that the peak conductance is 20% lower than in Na Ringer at all potentials and that the membrane must be depolarized 3.3 mv more to open sodium channels in Li Ringer than in Na Ringer. Even the outward currents are reduced by lithium. Evidently lithium ions block sodium channels in addition to passing through. The block could arise because each lithium ion takes such a long time to pass through or dissociate from the channel that the permeation of other ions is delayed.

POTASSIUM Selection against potassium ions is crucial to the physiological function of sodium channels; nevertheless, potassium ions can pass through sodium channels. Fig. 2 shows families of voltage-clamp currents in Na Ringer and K Ringer. At several test voltages there are small transient inward currents in K Ringer. These are inward movements of potassium ions. In nine measurements, the reversal potential in K Ringer was 59.0 ± 4.7 mv lower than in Na Ringer, corresponding to a permeability ratio $P_K/P_{Na}$ of 0.086. The last frame of Fig. 2 shows that the potassium currents are abolished by the specific sodium-channel poison, tetrodotoxin. The current record in tetrodotoxin also serves to show that the errors introduced by the method for subtraction of leakage and capacity are negligible. 10 current traces are drawn

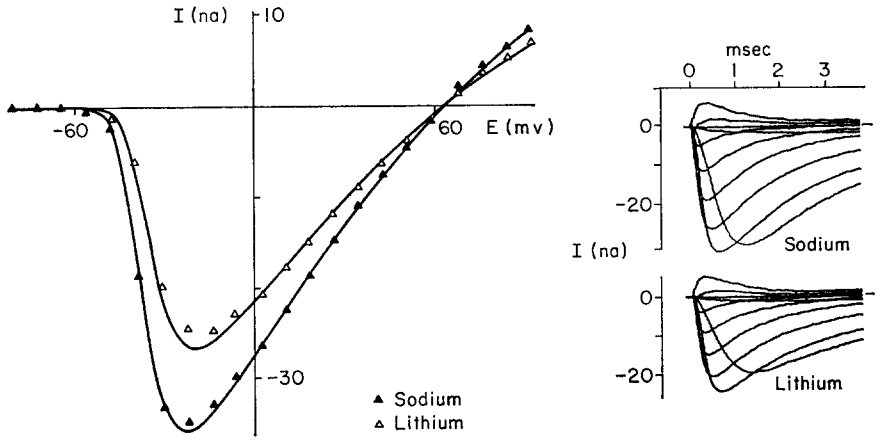


FIGURE 1. Sodium and lithium currents in the sodium channel. *Left:* peak current-voltage relations for a node bathed in Na and Li Ringer. The smooth curve through the sodium points is an arbitrary function. The smooth curve through the lithium points is derived from the sodium curve by assuming that the reversal potential is unchanged, that the conductance is 20% smaller, and that the membrane must be depolarized 3.3 mv more to open sodium channels. *Right:* families of voltage-clamp currents from the same experiment showing the currents at 10 different voltages spaced at 15-mv intervals. Ends of fiber were cut in KCl.

out in full by the plotting pen of the computer yet they superimpose on the zero current level as one line. The small inward potassium currents seen before tetrodotoxin is added are clearly significant by comparison.

The mechanisms for opening and closing sodium channels are not profoundly affected by replacing all external $Na^+$ ions with $K^+$ ions. The permeability changes to potassium fit the usual pattern of activation and inactivation for sodium channels. Fig. 3 shows the voltage dependence of the steady-state inactivation of sodium channels in Na Ringer and K Ringer. The graph on the left gives the relative size of inward Na or K currents during a fixed test pulse as a function of the voltage during a long conditioning prepulse.

The current traces on the right are some of the experimental records. There is no significant difference between steady-state inactivation in the two solutions. The time constant of inactivation $\tau_h$ can be measured from the decay of the late part (4–20 msec) of the inward current (not shown). Within the $\pm 30\%$ accuracy of the measurement with these small currents, $\tau_h$ is the same in Na and K Ringer. On the other hand, the inward currents in the traces of Fig. 3 rise to a peak more slowly in K Ringer, showing that the rate of activation of sodium channels is somewhat changed. The peak current-voltage relations (not shown) reveal that the membrane must be depolarized more to activate sodium channels in K Ringer. The changes in activation may be summarized by saying that the relationship between the activation parameters, $\tau_m$ and $m_\infty$, and voltage is shifted along the voltage axis by 8–10 mv on replacing Na Ringer by K Ringer.

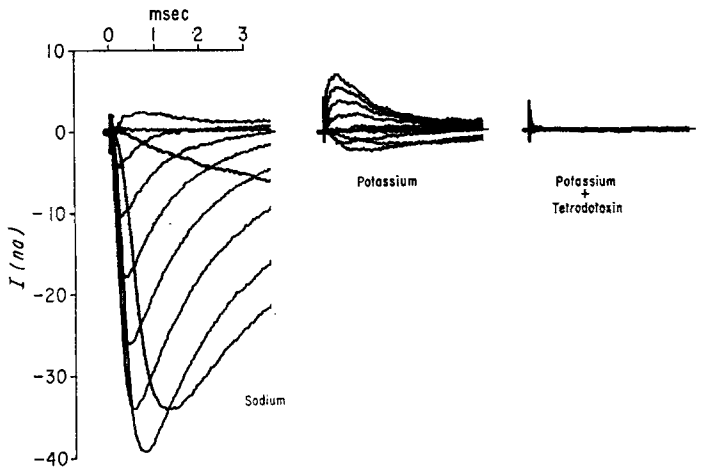


FIGURE 2. Block by tetrodotoxin of potassium currents in sodium channels. Families of voltage-clamp currents in Na Ringer, K Ringer, and K Ringer with 150 nM tetrodotoxin are shown. 10 voltage steps were spaced at 15-mv intervals. Ends of fiber were cut in CsF.

The changes in $\tau_m$ and $m_\infty$ are in the same direction as would be expected if there were a resistance in series with the nodal membrane giving a current-dependent error in the clamp potential. This artefact is, however, not the origin of the observed shifts, for if it were, the shift in $\tau_m$ and $m_\infty$ could be duplicated by any method of reducing the size of the Na currents. An experiment was done using a 7:1 mixture of tetramethylammonium chloride Ringer with Na Ringer. The inward Na currents are less than 12% as large as in Na Ringer, but still there is an 8 mv difference between the voltage dependence of $\tau_m$ and $m_\infty$ measured in this low-sodium solution and that measured in K Ringer. Another indication of the relative unimportance of series resistance lies in the current traces of the original experiment (Fig. 3,

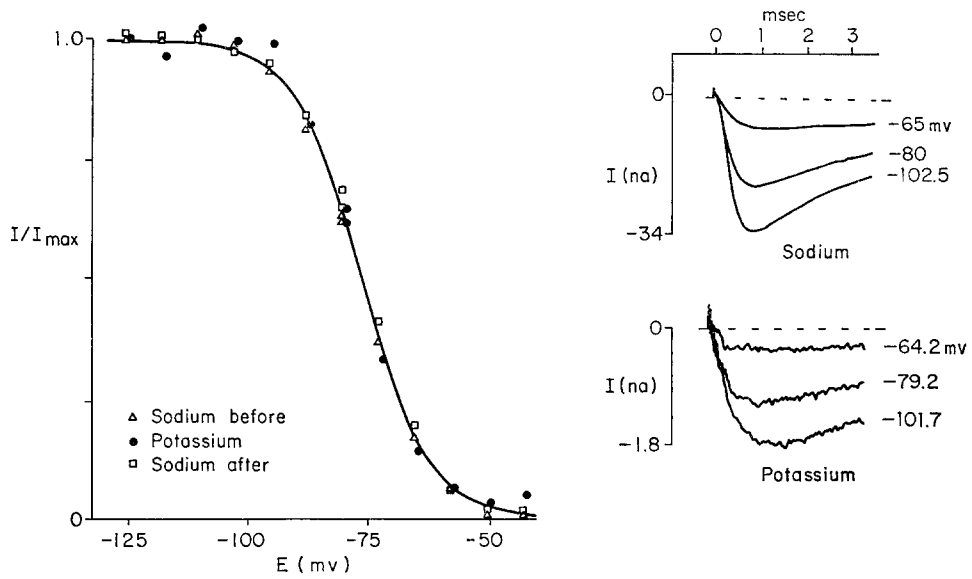


FIGURE 3. Steady-state inactivation for sodium and potassium currents in sodium channels. *Left:* relative amplitudes of inward currents measured at −30 mv following 50-msec conditioning prepulses to the indicated voltages. The curve is drawn from the expression $1/(1 + \exp(E + 76)/7)$. *Right:* current traces from the same experiment with the voltage of the prepulse marked. Note that the currents in K Ringer are shown at much higher amplification than the currents in Na Ringer. Records here and in Fig. 2 are from the same node.

right). When the inward Na current is significantly reduced by a depolarizing conditioning pulse, the kinetics of the rise of the current are slowed, but not by more than the equivalent of a 2 mv decrease in membrane depolarization.

*Rubidium and Cesium Ions Are Not Measurably Permeant*

No inward currents in sodium channels are detected in Rb or Cs Ringer. Voltage-clamp currents in 12.5% Na Ringer and Cs Ringer are compared in Fig. 4. The change in $E_r$ on changing from Na Ringer to 12.5% Na Ringer is by definition 49.8 mv (see Methods). The change in $E_r$ on changing from Na Ringer to Cs Ringer cannot be measured because there are no discernable inward Cs currents; however, the change is more than 104.0 mv, corresponding to a permeability ratio $P_{Cs}/P_{Na}$ smaller than 0.013. There is no indication of how close to this limit the actual permeability ratio is. Similar experiments with Rb Ringer give a permeability ratio $P_{Rb}/P_{Na}$ smaller than 0.012.

*Thallium Is Permeant*

Thallium is a metal lying between mercury and lead on the periodic table and having an atomic weight of 204.4. It occurs in two common oxidation states: thallous, $Tl^{+1}$, and thallic, $Tl^{+3}$. Like silver ions, thallous ions are very

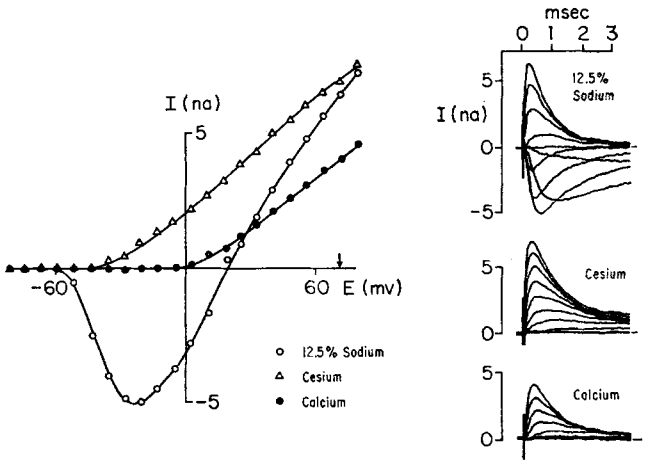


FIGURE 4. Voltage-clamp currents in low-sodium, cesium, and calcium Ringer. *Left:* peak current-voltage relations. The arrow indicates the reversal potential in the control Na Ringer. *Right:* time-course of the currents in the same experiment. Ends of fiber were cut in CsF.

polarizable and form complexes involving *d* orbitals. As with silver salts, the thallous halides (TlCl, TlBr, TlI) are too insoluble to work with, but $TlNO_3$ is moderately soluble, and thallous acetate is very soluble. All of these salts are extremely poisonous. Control experiments showed that sodium currents are 20% smaller in sodium acetate than in $NaNO_3$ and NaCl, so $TlNO_3$ was selected for testing.

Families of voltage-clamp currents in Na Ringer, 12.5% Na Ringer, and Tl($TlNO_3$) Ringer are compared in Fig. 5. There are clear inward Tl currents in Tl Ringer. Although the maximum inward Tl currents are smaller than the Na currents in 12.5% Na Ringer, the reversal potential in Tl is 20.8 mv more positive than in 12.5% Na. In five measurements the decrease in reversal potential on changing from 100% Na Ringer to Tl Ringer was 30.3 ± 7.4 mv, corresponding to a permeability ratio $P_{Tl}/P_{Na}$ of 0.33. The inward thallium currents are only one-third of the size expected from the independence principle. The measurements are difficult to make for several reasons. The nodal membrane is unstable in Tl Ringer, and may suddenly develop a very low resistance and be ruined by the ensuing large voltage-clamp currents. Even if this disaster does not occur, the Na currents rarely recover to 50% of their original size after the node has been exposed to thallium for only 60 sec. Thallous ions are directly toxic to the membrane and to sodium channels.

*Divalent Ions Are Not Measurably Permeant*

Voltage-clamp currents in Ca Ringer containing 75 mM $Ca^{++}$ are shown in Fig. 4. Below a membrane potential of 0 mv no current in sodium channels

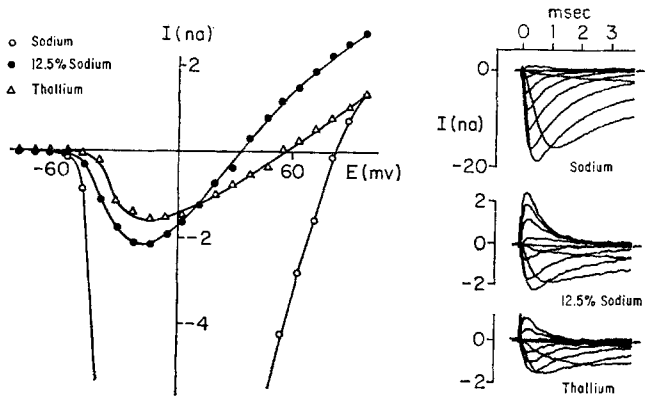


FIGURE 5. Sodium and thallium currents in the sodium channel. *Left:* peak current-voltage relations. *Right:* families of voltage-clamp currents in the same experiment. Note the difference in current scales. Ends of fiber were cut in KCl. The holding potential was −85 mv.

is detected, and above 0 mv the current is clearly outward. In general, the permeability ratio for a divalent ion compared with a monovalent ion is not uniquely determined by this type of information because the calculation depends on such factors as the number of microscopic rate-limiting barriers, on whether there is a constant field, and on which definition of permeability is used. In the example at hand, the situation can be simplified by assuming that at 0 mv there is no electric field, so only the relative mobilities of $Na^+$ and $Ca^{++}$ need be considered. In this experiment a 13.8 mM $Na^+$ solution (12.5% sodium) gave a reversal potential of 19.7 mv, and a 6.5 mM $Na^+$ solution would have given 0 mv. Thus, at 0 mv, 75 mM $Ca^{++}$ is no better at carrying current than 6.5 mM $Na^+$. Assigning ions of equal particle mobility (not equivalent mobility) an equal permeability makes the ratio $P_{Ca}/P_{Na}$ less than 0.043 (6.5 × 0.5/75). After correction for activity coefficients the limit on the permeability ratio becomes 0.11. A similar experiment with 80 mM $Mg^{++}$ Ringer established an upper limit for the ratio $P_{Mg}/P_{Na}$ of 0.10. Measurements of the reversal potential for $Ca^{++}$ and $Mg^{++}$ are severely limited by the "threshold"-raising property of divalent ions (Frankenhaeuser and Hodgkin, 1957; Hille, 1968 *b*): the more divalent ions there are in the solution, the more the membrane must be depolarized to open sodium channels.

## DISCUSSION

### *Comparison with Previous Results*

MONOVALENT METAL IONS My measurements offer few surprises. As early as 1902, Overton showed that lithium is a sodium-substitute for func-

tioning of a nerve-muscle preparation. J. W. Moore (1958) verified that $Li^+$ ions give inward lithium currents resembling sodium currents in the voltage clamp. Frankenhaeuser and L. E. Moore (1963) discovered inward potassium currents in sodium channels, and Chandler and Meves (1965) measured the permeability ratios for all alkali metal cations. There are now numerous reports of the permeability of sodium channels to monovalent metal ions (Table I) with some scatter of values but little fundamental disagreement. Frankenhaeuser and Moore's value of $P_K/P_{Na}$ in *Xenopus* was calculated using the independence principle (including an activity coefficient correction) from the amplitude of transient inward K currents in very hypertonic potassium solutions. However, K currents turn out to be smaller than predicted by the independence principle, so the value obtained is an underestimate. All other values in Table I were obtained using the Goldman equation and reversal potentials, as in this paper. The measurement of $P_{Rb}/P_{Na}$ by Moore et al. (1966) is placed in parentheses because the original records seem unconvincing with a large capacitative transient and a large inward delayed Rb current in potassium channels obscuring the putative transient inward Rb current in sodium channels.

TABLE I
PERMEABILITY RATIOS FOR METAL CATIONS IN SODIUM CHANNELS

| Reference | Genus | Permeability relative to Na | | | | |
|---|---|---|---|---|---|---|
| | | Li | K | Rb | Cs | Tl |
| Frankenhaeuser and Moore, 1963 | *Xenopus* | — | 0.057 | — | — | — |
| Chandler and Meves, 1965 | *Loligo* | 1.1 | 0.083 | 0.025 | 0.017 | — |
| Moore et al., 1966 | *Loligo* | 1.0 | 0.083 | (0.083) | <0.05 | — |
| Rojas and Atwater, 1967 | *Dosidicus* | — | 0.04 | — | — | — |
| Atwater et al., 1969 | *Dosidicus* | — | 0.10 | — | — | — |
| Binstock and Lecar, 1969 | *Loligo* | — | 0.11 | — | — | — |
| Adelman, 1971 | *Loligo* | — | 0.10 | — | — | — |
| This paper | *Rana* | 0.93 | 0.086 | <0.012 | <0.013 | 0.33 |

Goldman and Binstock (1969) have claimed that the value of $P_K/P_{Na}$ from Chandler and Meves (1965) is "much too" high to explain measured reversal potentials in *Myxicola* giant axons. However, Goldman and Binstock's measurement of reversal potential was made without correction for leak or capacity, without correction for the unknown junction potential between axoplasm and electrode, and without reduction of the very large currents in potassium channels. Finally, their calculation requires knowing the unknown $Na^+$ and $K^+$ activities in *Myxicola* axoplasm. Considering these difficulties their claim seems ambiguous.

Some general conclusions may be drawn from the observations. The mean

values for $P_{Li}/P_{Na}$ and $P_K/P_{Na}$ in Table I are 1.01 and 0.086 (omitting Frankenhaeuser and Moore's value). There is no reason to doubt that these averages are close to the correct value and that $P_{Rb}/P_{Na}$ and $P_{Cs}/P_{Na}$ are both below 0.03. It also seems certain that the transient Li currents and K currents are carried in sodium channels because of the similarity in the kinetics of the permeability changes (Chandler and Meves, 1965; Moore et al., 1966) and because of the complete block of these currents by the specific poisons tetrodotoxin and saxitoxin (Moore et al., 1967; Rojas and Atwater, 1967; Hille, 1967, 1968 *a*). Thus, the calculated permeability ratios are reliably ascribable to sodium channels. The similarity between the permeability ratios of sodium channels in squids and amphibians (Table I and Hille, 1971 *b*) implies that the selectivity filter has changed little during over $5 \times 10^8$ yr of independent evolution.

DIVALENT IONS The permeability of the sodium channel to divalent ions is a pivotal point in many theories of excitation, but relatively little direct experimental evidence is available. From a strictly steric viewpoint, it is quite probable that $Ca^{++}$ and $Mg^{++}$ can pass through the sodium channel since their crystal diameters are almost the same as those of $Na^+$ and $Li^+$. The question is whether the rate of passage is negligible or significant. Tracer fluxes of $^{45}Ca$ associated with normal action potentials at 19°C have been measured in squid giant axons. Hodgkin and Keynes (1957) found an extra calcium influx proportional to the external calcium concentration and amounting to, for example, 0.08 pmole/cm$^2$ per impulse in a solution with 112 mM $Ca^{++}$. Tasaki et al. (1967) found similar influxes. The observed extra $Ca^{++}$ influx per millimole of $Ca^{++}$ is about 2% as large as the observed extra $Na^+$ influx per millimole of $Na^+$ during the action potential. However, these observations do not determine whether sodium channels are the pathway for this entry of $Ca^{++}$ ions.

Squid giant axons can produce action potentials in external solutions containing no other salt than 200 mM $CaCl_2$ (Watanabe et al., 1967). The axon must be internally perfused with a low ionic strength solution like 25 mM CsF. Because under some conditions these action potentials are blocked by tetrodotoxin, they may arise in part from an influx of $Ca^{++}$ ions through sodium channels. The membrane conductance at the peak of the calcium action potential is only 2% of the value during a normal sodium action potential, indicating quite a low relative conductance to $Ca^{++}$.

Calcium fluxes have also been studied using the light emission of the protein aequorin injected into a squid giant axon (Baker et al., 1971). Under voltage-clamp conditions in artificial seawater there is a tetrodotoxin-sensitive component of calcium influx the final amplitude of which is related to the integral of the measured sodium current. If it could be shown that the time-course of the calcium flux parallels the time-course of sodium permeability, there

would be no further doubt that sodium channels are permeable to $Ca^{++}$ ions. Baker et al. (1971) assume that there is a calcium current in sodium channels obeying Ohm's law and, from the estimated ratio of "calcium conductance" to sodium conductance, calculate a permeability ratio $P_{Ca}/P_{Na}$ of 0.01. The calculation involves a number of assumptions and uses the tracer flux measurements of Hodgkin and Keynes (1957) for an absolute calibration. Aequorin also reveals other components of calcium permeability. In conclusion, there is evidence that $Ca^{++}$ ions can pass through open sodium channels, although with a very low permeability. The calculation of $P_{Ca}/P_{Na}$ by Baker et al. (1971) is based on the relative amplitudes of currents, a method which often gives smaller results than the calculations based on reversal potentials used in this paper. My measurements place an upper limit of 0.11 on the permeability ratio $P_{Ca}/P_{Na}$. Further work is needed.

*The Sodium Equilibrium Potential*

The sodium channel was originally shown to be sodium selective from studies with different external $Na^+$ ion activities (Hodgkin and Huxley, 1952 *a*). When external NaCl is replaced by choline chloride or sucrose, the reversal potential changes in accordance with the equation for a sodium electrode. Hence, the reversal potential is called the "sodium potential" or "sodium equilibrium potential" symbolized by $E_{Na}$. In the absence of much external $K^+$ ion and in the absence of direct measurements of internal cation activities, the experiment shows that $Na^+$ ions are more permeant than $K^+$ ions, but not that the channel is *perfectly* sodium selective. In other words, the experiment does not show that $E_{Na}$ is the Nernst equilibrium potential nor that the measured $E_{Na}$ can be used with the Nernst potential to predict the internal $Na^+$ activity.

In fact, with a permeability ratio $P_K/P_{Na}$ as large as 1/12, the internal $K^+$ ion activity may often be more important than the internal $Na^+$ ion activity in determining the measured value of $E_{Na}$. For instance, in a hypothetical axon bathed in a K-free solution with $Na^+$ activity of 110 and internally perfused with an Na-free solution with $K^+$ activity of 120, the reversal potential $E_r$ for sodium channels should be 57.4 mv at 5°C. This example might correspond to a node bathed in Na Ringer (110 mM $Na^+$) with ends cut in 120 mM KCl where the measured $E_r$ is 58.5 $\pm$ 6.9 mv (Hille, 1971 *b*). Cutting in KCl probably permits much of the diffusible sodium in the axoplasm to leave in exchange for potassium in the end pools. Before the internode is cut in KCl, $E_r$ is 55.2 $\pm$ 5.7 mv, showing that there is little contribution from internal sodium to $E_{Na}$ in the uncut fiber. If the internode is cut in CsF, $E_r$ rises to 72.4 mv, presumably because relatively impermeant $Cs^+$ ions replace some of the $K^+$ ions in the axoplasm. Because of the likelihood of systematic errors in measuring the absolute value of potential with single myelinated nerve fibers,

the difference between the measurements is probably more significant than the absolute value. Evidently, even a cell with absolutely no sodium inside would have a fairly low limit on $E_r$ set by its imperfect sodium channel. Further increases of $E_r$ after removing the internal $Na^+$ would require lowering the internal $K^+$ activity.

In summary, the outward currents seen in sodium channels with large depolarizations may often be K currents, and the "sodium equilibrium potential" as usually measured is a mixed Na-K diffusion potential. Although these facts were not explicitly stated in the original quantitative theory of Hodgkin and Huxley (1952 *b*), they do not detract from or in any way jeopardize the theory. Indeed there should be no reason to abandon the common usage of the terms "sodium potential," "sodium equilibrium potential," "$E_{Na}$," "$g_{Na}$," and "$I_{Na}$" so long as their limitations are recognized and so long as an appropriate, more cumbersome, but more correct terminology is used when fine distinctions are important. The terms "Nernst potential for sodium" and "reversal potential for sodium channels" could be used to distinguish the thermodynamic from the observed potential when necessary.

The distinction between reversal potential and Nernst potential must be made in discussing whether or not sodium ions are partly bound in the axoplasm. In the past the measured reversal potential and the Nernst equation have been used to estimate the internal sodium ion activity (for example Hodgkin and Huxley, 1952 *a*). The calculated activity often agrees with the value expected if almost all the axoplasmic sodium (measured chemically) were free. Because of the contribution of axoplasmic potassium ions to the reversal potential, such estimates of sodium activity are actually too high and more of the internal sodium may be bound than was originally supposed.

### *The Gating Mechanism*

MONOVALENT IONS Many experiments in this paper on metal cations and in the earlier paper on organic cations (Hille, 1971 *b*) show that the opening and closing of sodium channels are relatively independent of the monovalent cation in the external solution. To be more precise, most sodium substitutes do have a small effect on gating: namely the membrane often must be depolarized a few millivolts more to activate sodium channels, a calcium-like action. This small effect and the deviations from the independence principle also reported with these ions are examined further in a later paper. Nevertheless, the conductance changes always have the familiar "$m^3h$" kinetics of the Hodgkin-Huxley (1952 *b*) theory and the gating mechanism operates with little regard for what cation happens to be flowing through the channel or for whether the net cation flux is inward or outward. Thus the opening and closing of gates cannot be attributed to local accumulation or depletion of specific monovalent cations at special controlling sites. For example, the

inactivation of sodium channels could not be due to accumulation of sodium at the inner membrane surface or of potassium at the outer surface during a depolarization, as has been suggested in some theories (Tobias, 1964; Tasaki, 1968; Weiss, 1969; Adelman and Palti, 1969). The striking changes in the voltage dependence of sodium inactivation seen by Adelman and Palti (1969) in potassium-depolarized squid giant axons is probably due more to the long *depolarization* in KCl than to a specific effect of external potassium *ions*. A different theory, that sodium channels are opened by an accumulation of hydrogen ions at the outer surface of the membrane (Stephens, 1969), is directly contradicted by experiments in which the external and internal pH is deliberately changed (Hille, 1968 *b*; Ehrenstein and Fishman, 1971).

DIVALENT IONS Another widely discussed mechanism of gating involves movement of calcium ions in the electric field of the membrane (Gordon and Welsh, 1948; Hodgkin et al., 1949; Frankenhaeuser and Hodgkin, 1957; Lettvin et al., 1964; Goldman, 1964; Fishman et al., 1971). The original motivation for the theory came from the well-known threshold-shifting effects of calcium ions: in low-calcium solutions a nerve needs a smaller depolarization to reach firing threshold than in high-calcium solutions. In a modern form the theory postulates that sodium channels at rest are closed because calcium ions physically plug them up. The calcium ions are drawn into the channels by the electric field of the resting membrane. Because sodium channels will not let calcium ions pass all the way through, the calcium ions accumulate in a blocking position. Calcium ions leave the blocking position when the field pulling them in is reduced by a depolarization.

In terms of the Hodgkin-Huxley model, the calcium theory gives a qualitative account both for the activation of the "$m^3$ gate" with depolarization and for the way that the voltage dependence of $m_\infty$ is shifted by calcium. However, quantitative arguments show that the predicted shift of $m_\infty$ is always too large (Frankenhaeuser and Hodgkin, 1957; Fishman et al., 1971). Furthermore, the theory could never give an account for the inactivation gate ("$h$ gate") which *closes* channels during a long depolarization. This failure is significant because the voltage dependence of $h_\infty$ is shifted by calcium in the same way as the voltage dependence of $m_\infty$ (Frankenhaeuser and Hodgkin, 1957; Hille, 1968 *b*), suggesting a common mechanism. A different theory involving the surface potential of an ionic double layer at the membrane explains the similar effects of calcium concentration on $m_\infty$ and $h_\infty$ more satisfactorily but gives no account for the actual gating mechanism (Frankenhaeuser and Hodgkin, 1957; Hille, 1968 *b*; Gilbert and Ehrenstein, 1969).

The calcium theory of gating also faces contradiction from the still incomplete studies on the permeability of sodium channels to calcium ions. The calcium theory requires that if sodium channels are at all permeable to cal-

cium ions, the flux of calcium ions should be larger in the resting state than when channels are open. This is because in the resting state there are supposed to be several calcium ions in the channels, while in the activated state there are none. Thus, the theory will fail if the following proposition is conclusively established: sodium channels are permeable to calcium ions and more calcium ions pass through open sodium channels than through closed ones. As has already been discussed, preliminary evidence points to the correctness of the crucial proposition, so the calcium theory of the $m^3$ gate may soon be definitively refuted. The same kind of reasoning argues against a calcium gate theory to explain the voltage dependence and the shift of activation by calcium in bona fide calcium channels (Hagiwara and Naka, 1964). In conclusion, it is more likely that the voltage-sensing and gating functions of the sodium channel are achieved by inherent structural parts of the channel than by movements of freely diffusible ions in the internal or external medium.

*Selectivity and the Pore Hypothesis*

THE PORE A previous paper (Hille, 1971*b*) reports permeability measurements for many organic cations in sodium channels. The paper also describes a hypothetical pore structure designed to accept all permeant organic cations and to reject all impermeant organic cations. The pore has a rectangular hole 3.1 × 5.1 A formed by a ring of oxygen atoms. This hole is both the pathway for ion flow and the selectivity filter which determines which ions can flow. The narrow part of the pore is further supposed to be very short and to bear a single negative charge. The charge attracts cations and repels anions. The previous paper (Hille, 1971 *b*) explains selectivity against impermeant organic cations on the simplest steric grounds: the impermeant ions are too large to enter the pore, while the permeant organic ions are small enough to fit. However, the paper offers little explanation for the *sequence* of selectivity among those ions which can fit into the pore. The same problem of sequence arises for the metal cations studied here, for almost all of them satisfy the steric requirements for entry, and no theory for the Na channel could be complete unless it explains why $Na^+$ ions are preferred over $K^+$ ions. The origin of the selectivity sequence is now considered. First the environment of the ions in the pore is described and then energetic factors favoring $Na^+$ ions in the pore are discussed, but a complete explanation of selectivity is not reached.

WATER AND IONS IN THE PORE With an opening 3.1 × 5.1 A, the pore is large enough to accommodate several water molecules (equivalent diameter 2.80 A). The oxygen atoms forming the pore are hydrogen bond acceptors which would stabilize perhaps three or four water molecules inside. In this sense the pore is an "aqueous pore," although its water molecules

would have fewer ways to move than in bulk water. As the scale drawings at the top of Fig. 6 show, alkali metal ions are roughly the size of a water molecule. Hence, on entering the pore they might take the place of one water molecule. The lower two rows of drawings in Fig. 6 are an attempt to show the packing of water molecules and ions into the pore. The water molecules are represented in two ways, either as a heart-shaped side view or as a circle of 2.80 A diameter with the label *W*. The circle means a water molecule of

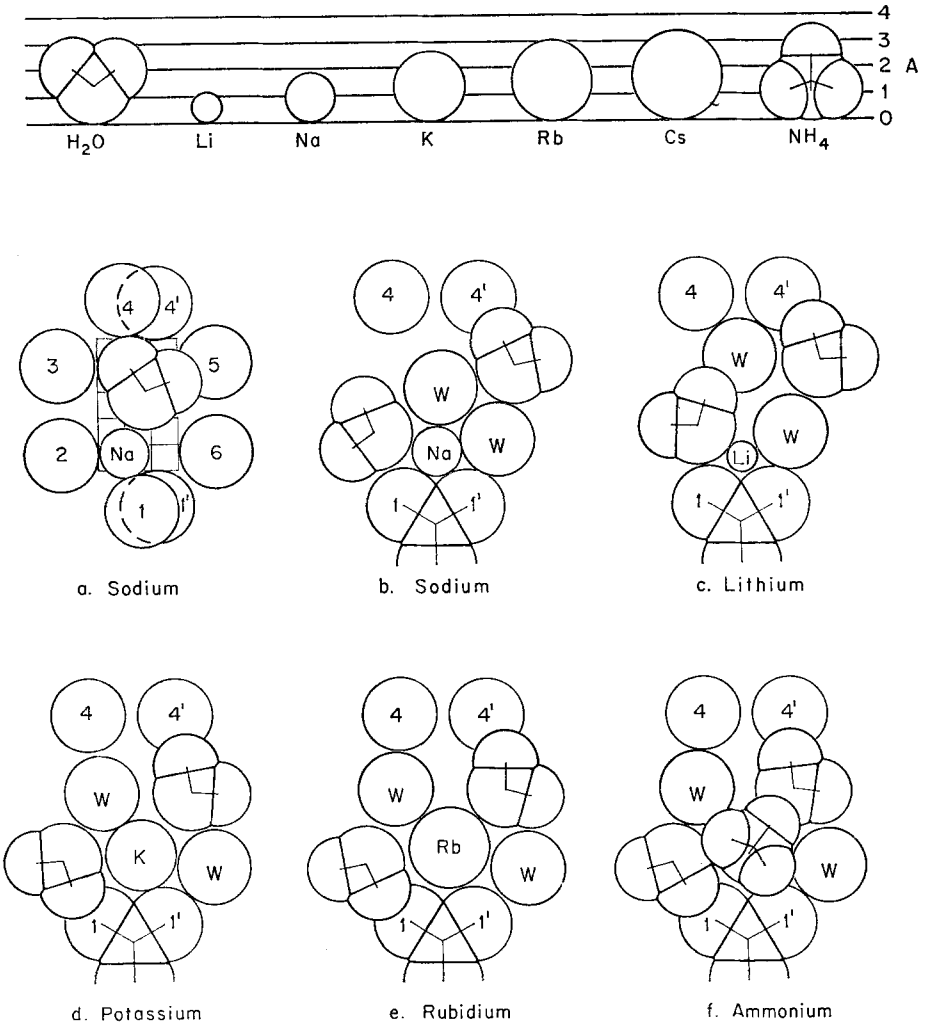


FIGURE 6. Scale drawings of water molecules and small ions in the model pore. The top row shows water, the alkali metal ions, and ammonium against a scale in Ångstrom units. Frames *a-f* show ions and water molecules surrounded by the numbered oxygen atoms of the pore. Frame *a* is a face view of the pore and *b–f* are longitudinal sections cutting through oxygens O1, O1′, O4, and O4′. Some water molecules are labeled *W*.

unspecified orientation. In general the negative end of the water dipole is turned towards the cation and each water molecule makes hydrogen bonds. The water molecules associated with the cation in the pore serve as waters of hydration. In solution a cation is completely surrounded by the oxygens of waters of hydration, and in the pore it is still surrounded by oxygens, some of which are mobile water oxygens and some, fixed oxygens of the pore. The drawings are now considered individually.

Frame *a* of Fig. 6 is like a figure previously presented (Hille, 1971 *a*, *b*) showing a face view of the pore with an $Na^+$ ion and a water molecule inside. Six oxygen atoms, designated O1–O6, lie in the plane of the membrane. They form the opening of the pore. Two more oxygens, O1′ and O4′, lie behind O1 and O4. All of these oxygens are somehow connected and belong to the permanent structure of the pore. The single negative charge of the pore is distributed between O1 and O1′ which are part of an ionized carboxylic acid group ($COO^-$) in the model. The $Na^+$ ion lies against the acid group and O2. The 0.9 A overlap of a water hydrogen over O5 indicates the formation of a hydrogen bond with water as the donor and O5 as the acceptor. Fig. 6 *b* represents the same pore-$Na^+$-water complex in longitudinal section. The carboxylic acid group is drawn in full below and O4 and O4′ are seen above. The total length of the narrow pore as drawn is only 4–5 A. Four water molecules are in the pore, three of them touching the $Na^+$ ion. The circular profile near O4 is the water molecule already seen in Fig. 6 *a*. Altogether, six oxygens, three from the channel and three from water, touch the ion.

The remaining frames of Fig. 6 give packing drawings for $Li^+$, $K^+$, $Rb^+$, and $NH_4^+$ ions in the pore. The arrangements are quite similar to the one for $Na^+$. Lithium and potassium have plenty of room in the channel. Rubidium with a diameter of 2.96 A just fits into the 3.1 A gap between O2 and O6, while cesium, diameter 3.38 A, is physically too large to enter this part of the pore. Ammonium with an unbonded diameter of more than 3.50 A fits only because hydrogen bonding can reduce its effective diameter to about 2.90 A. The fourth hydrogen on $NH_4^+$ projects away from the viewer to bond with O2 of the channel. Indeed, the permeability to ammonium and to ammonium derivatives (hydrazine and hydroxylamine) was one of the important arguments for lining the model pore with oxygen atoms (Hille, 1971 *b*). The drawings show that as the radius of the ion increases, the number of contacts with oxygens, whether from water or from the pore, increases. Thus, counting oxygens within 0.3 A of the cation, the coordination is $Li^+$, 5; $Na^+$, 6; $K^+$, 7; $Rb^+$, 8; and $NH_4^+$, 8. A packing drawing for $Ca^{++}$ (diameter 1.98 A) would be the same as that for $Na^+$ (diameter 1.90 A) and a drawing for $Tl^+$ (diameter 2.80 A) would be intermediate between that for $K^+$ (diameter 2.66 A) and $Rb^+$ (diameter 2.96 A). The drawings of Fig. 6 are perhaps disappointing in that they do not yield an immediate explanation of why $K^+$ passes through sodium channels with more difficulty than $Na^+$.

FIELD STRENGTH AND EQUILIBRIUM BINDING The full selectivity sequence for the sodium channel given in Table II is evidently still unexplained. A completely steric theory for selectivity starting with the premise that small ions negotiate the hole in the channel more easily might seem reasonable for the alkali ions, but the theory fails entirely to explain why the larger hydroxylamine cation ($OH\text{-}NH_3^+$) is as permeant as $Li^+$ and much more so than ammonium ($NH_4^+$) or why $Tl^+$ is so much more permeant than $K^+$. Evidently, chemical or electrical forces must be invoked in addition to geometric arguments. Indeed, an existing electrostatic theory for ion exchange provides an excellent foundation for explaining the observations.

TABLE II
PERMEABILITY RATIOS FOR ALL MEASURABLY PERMEANT MONOVALENT CATIONS IN SODIUM CHANNEL OF FROG NODE

| $P_{Ion}/P_{Na}$ | Ion |
|---|---|
| 1.0 | Sodium |
| 0.94 | Hydroxylamine |
| 0.93 | Lithium |
| 0.59 | Hydrazine |
| 0.33 | Thallium |
| 0.16 | Ammonium |
| 0.14 | Formamidine |
| 0.13 | Guanidine |
| 0.12 | Hydroxyguanidine |
| 0.086 | Potassium |
| 0.06 | Aminoguanidine |

Eisenman (1962, 1969) has developed an electrostatic model for cation selectivity applicable to a very wide range of ion exchange equilibria. Coulombic forces of attraction between cation and a binding site and between cation and water are assumed to determine the free energy change associated with moving the cation from water to the site. The calculations show that the free energy change depends on the radius of the cation, so the theory predicts discrimination between ions of different sizes. The free energy change also depends on the electrical properties of the binding site (charge, dipole moment, and radius or distance of closest approach). In fact, changing the site can change the entire *sequence* of selectivity towards, for example, the alkali metal cations. Eisenman summarizes the electrical properties of the binding site by the concepts of field strength and dipole strength which are measures of charge-radius or dipole-radius products. As the field strength or dipole strength of a site increases, the predicted selectivity for the five alkali metal cations changes through 11 different sequences. Sites of very low field strength give one extreme binding sequence $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$,

called sequence I, and sites of very high field strength give the reverse sequence $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$, called sequence XI. Intermediate field strengths give "transitional" sequences which are not monotonic with the radius of the cation. In these terms the permeability sequence for sodium channels is a high field strength sequence on the borderline between sequence X ($Na^+ > Li^+$) and sequence XI ($Li^+ > Na^+$).

What sequence is predicted for binding of organic cations to a high field strength anion? Even neglecting all but coulombic forces, this question is more complicated than for alkali cations, because each substituent group on the cation modifies the total distribution of charge. The quantity which must be known can be called the field strength of the cation at the cationic group. In ammonium derivatives with a constant radius at the cationic $—NH_3^+$ moiety, the sequence of field strength is governed by the electron withdrawing effects (electronegativities) of the substituents. On this basis the sequence of positive field strength is hydroxylamine ($OH—NH_3^+$) > hydrazine ($NH_2—NH_3^+$) > $NH_4^+$. The $pK_a$'s for these compounds, 6.0, 7.9, and 9.2, respectively, demonstrate the same trend. In guanidine derivatives the positive charge is delocalized, and the sequence $NH_4$ > formamidine ($NH_2{:}CHNH_2^+$) > guanidine ($NH_2C{:}(NH_2)_2^+$) is predicted as the positive charge is progressively spread over 1, 2, and 3 $—NH_2$ groups. Guanidine has a $pK_a$ of 13.6. However, because their $\pi$ orbitals are very polarizable, guanidine derivatives might bind to a high field strength anion almost as well as $NH_4^+$ does. To predict the position of all the organic cations within the sequence for alkali metal cations would require molecular orbital calculations of charge distribution. The field strength of $NH_4^+$ might lie between $K^+$ and $Rb^+$ because the sequence of effective radii is $K^+ < NH_4^+ < Cs^+$, but of course the metal cations lack the tetrahedrally oriented hydrogen bond "valences" of $NH_4^+$.

Carboxylic acid groups with a $pK_a$ near 5.0 are high field strength anions and exhibit binding sequence XI, favoring small cations. For example, the thermodynamic association constant for lithium acetate in water is 2.5 times the value for sodium acetate (Sillén and Martell, 1971). From its crystal radius, $Tl^+$ would be expected to fall between $K^+$ and $Rb^+$ in a high field strength binding sequence, but because of its very highly polarizable outer electron shells, $Tl^+$ often binds more strongly even than $Li^+$. The reported stability sequence is $H^+ > Tl^+ > Li^+ > Na^+ > K^+$ for complexes with the following anions: acetate (Sillén and Martell, 1964, 1971), ethylenediamine-*N*,*N*,*N*′*N*′-tetraacetate ($EDTA^{4-}$), uramildiacetate ($Ur^{3-}$), and many related polyacetates (Irving and da Silva, 1963 *a*, *b*). The $Tl^+$ ion is also favored by anions of lower field strength like $NO_3^-$ (Sillén and Martell, 1964) and by neutral compounds like some macrocyclic polyethers (Christensen, et al., 1971). The ratio of association constants $K_{Na}/K_K$ for the above high field strength anions ranges from 2.5 to 45. I have not found measurements of

stability constants for complexes between a series of ammonium compounds and acetate or some other model anion. A particularly well-studied high field strength glass, NAS 11–18F, gives the permeability sequence $Na^+ >$ hydrazine $> Li^+ >$ hydroxylamine $> K^+ \approx$ guanidine $> NH_4^+ > Rb^+ > Tl^+ > Cs^+ > Ca^{++}$ (Eisenman, 1965). With the special exception of $Tl^+$ the sequence is close to the one for the sodium channel; however, the permeability ratio $P_{Na}/P_K$ is around 1000, so the glass is considerably more selective than the channel or organic high field strength anions in water. According to an argument of Eisenman (1962, 1965), the higher Na/K selectivity in glasses is understandable from the more complete dehydration of the cations when bound to sites in glass.

THE SODIUM CHANNEL AS A HIGH FIELD STRENGTH SITE Chandler and Meves (1965) suggested that the sodium channel behaves like a high field strength system, possibly accepting partially hydrated ions. Indeed the predictions of the electrostatic theory of ion binding agree well with the permeability sequence for the sodium channel. As Tables I and II show, the sodium channel follows the high field sequences $Li^+ \approx Na^+ > K^+ > Rb^+ > Cs^+$ and hydroxylamine > hydrazine > ammonium > formamidine > guanidine. The high position of $Tl^+$ in the measured sequence has already been rationalized from known association constants of other thallous complexes. Furthermore, there already is a reason to suppose that the sodium channel contains a high field strength anion, because titrations with acid produce a block of sodium permeability which is 50% complete at pH 5.2 (Hille, 1968 *b*). The block follows exactly the theoretical titration curve of a single acid group. The high apparent $pK_a$ shows that $H^+$ binds much more strongly to the channel than any of the alkali cations. In the model the $H^+$ binding site is a carboxylic acid.

A surprising result of the field strength theory is that it offers an explanation which seems to make little use of the postulated geometrical properties of the model pore. Nevertheless, the geometry of the pore is still needed to explain the impermeability to all methylated cations (Hille, 1971 *b*), many of which should be quite permeant according to their effective cationic field strength. The model pore is simply too narrow to accept methyl groups. The narrowness and "chemistry" of the pore also guarantee that several waters around the ion are replaced by oxygens of different field strength or dipole strength in the pore, an essential prerequisite of the field strength theory. The narrowness of the pore may have the added effect of making it harder for $K^+$ and $Rb^+$ ions to enter because they are probably coordinated with fewer oxygen atoms in the model pore than in aqueous solution. The coordination numbers in the model pore are 5, 6, 7, and 8 for $Li^+$, $Na^+$, $K^+$, and $Rb^+$ (Fig. 6), while Pauling (1960) suggests 4, 6, 8, and 10 as typical values (for crystals). As each

oxygen contact may add between 5 and 30 kcal/mol of calculated stabilization energy (Eisenman, 1962, 1969), a reduction of coordination can be very important. Finally, the narrowness of the pore excludes $Cs^+$ ions from lying next to the negatively charged acid group. Parenthetically it should be noted that the pore as shown in Fig. 6 *a* is not a rectangular 3.1 × 5.1 A hole, because it is bounded by spheres (oxygen atoms) 2.8 A in diameter. Indeed the pore is much wider than 3.1 A in the region between O2 and O3, O5, and O6, and, if the drawing is taken literally, would be large enough to pass a $Cs^+$ ion and even a methyl group exactly centered in the pore. This discrepancy probably could be overcome by some readjustment of the positions of the oxygens and is even more easily eliminated by recognizing that there must be a whole framework of other atoms supporting the postulated oxygens. The framework would fill many of the gaps left by representing oxygens alone.

Despite an apparent success in predicting sequences, there remains a major gap in the application of the field strength theory to the permeability of the sodium channel. The theory deals with *equilibrium* binding while permeability is a *kinetic* property requiring a rate theory. As yet there is no analogous field strength theory for the individual binding and unbinding rates ("mobilities") in ion exchange. The agreement between the binding theory and the permeability sequences of the channel implies that the desired microscopic kinetic model of the channel should contain rates or equilibria which depend explicitly on field strength. This paper gives permeability ratios derived from the Goldman equation, but these numbers do not suffice to specify a rate theory model. Also needed is a fuller description of the deviations from the independence principle already mentioned for several ions, including $Tl^+$, $Li^+$, and hydroxylamine, in this and the previous paper (Hille, 1971 *b*). These measurements are reserved for a future paper. They offer some information on which ions bind long enough in the channel to block the passage of other ions and help to sort out rate effects from equilibrium effects in the selectivity sequence. To speculate further on the physical origin of selectivity in the sodium channel would not be fruitful until this additional information is assessed.

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