

The Permeability of the Sodium Channel to Organic Cations in Myelinated Nerve

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ABSTRACT The relative permeability of sodium channels to 21 organic cations was studied in myelinated nerve fibers. Ionic currents under voltage-clamp conditions were measured in sodium-free solutions containing the test cation. The measured reversal potential and the Goldman equation were used to calculate relative permeabilities. The permeability sequence was: sodium \approx hydroxylamine $>$ hydrazine $>$ ammonium \approx formamidine \approx guanidine \approx hydroxyguanidine $>$ aminoguanididine \gg methylamine. The cations of the following compounds were not measurably permeant: *N*-methylhydroxylamine, methylhydrazine, methylamine, methylguanidine, acetamidine, dimethylamine, tetramethylammonium, tetraethylammonium, ethanolamine, choline, tris(hydroxymethyl)amino methane, imidazole, biguanide, and triaminoguanidine. Thus methyl and methylene groups render cations impermeant. The results can be explained on geometrical grounds by assuming that the sodium channel is an oxygen-lined pore about 3 Å by 5 Å in cross-section. One pair of oxygens is assumed to be an ionized carboxylic acid. Methyl and amino groups are wider than the 3 Å width of the channel. Nevertheless, cations containing amino groups can slide through the channel by making hydrogen bonds to the oxygens. However, methyl groups, being unable to form hydrogen bonds, are too wide to pass through.

This paper deals with the ionic selectivity of the excitability mechanism in nerve membranes. Previous work has shown that a variety of small organic cations can maintain the excitability of nerves in sodium-free solutions (Larramendi et al., 1956; Lorente de Nó et al., 1967; Lüttgau, 1958, 1961; Dodge, 1963; Tasaki et al., 1965, 1966; Tasaki and Singer, 1966; Watanabe et al., 1967). Voltage-clamp studies show that axon membranes become transiently permeable to these ions following a depolarization (Dodge, 1963; Tasaki et al., 1966; Binstock and Lecar, 1969). Like permeability changes to sodium, the permeability changes to sodium substitutes are abolished by tetrodotoxin and have a rapid activation and a slower inactivation following depolariza-

tion. These criteria show that organic sodium substitutes are effective because sodium channels are somewhat permeable to them.

The experiments in this paper were undertaken with the hope that a quantitative study of the permeability of sodium channels to organic ions would offer new insight into the ionic selectivity mechanism in the channel. Measurements with many ions might narrow the range of possible theories. I have attempted to measure the permeability of sodium channels to most monovalent cations small enough to be possibly permeable. Relative permeability is calculated from the change in the reversal potential for current in sodium channels when all the external sodium is replaced by the test cation. This paper concerns the permeability to organic ions. A geometrical and chemical hypothesis is proposed to explain the observed permeability sequence. Later work will concern alkali metal cations and mixtures of ions. Preliminary publications of some of this material have appeared (Hille, 1971 *a, b*).

MATERIALS AND METHODS

Nerve and Recording Single myelinated nerve fibers are studied under voltage-clamp conditions by the method of Dodge and Frankenhaeuser (1958). Most procedures have been described (Hille, 1967 *a*), but the equipment used is new and improved in many respects. Myelinated fibers averaging $16 \mu\text{m}$ in internodal diameter and $1500 \mu\text{m}$ in internodal length are dissected from the sciatic nerve of *Rana pipiens*. No effort is made to distinguish motor and sensory fibers. The nerve is mounted in an acrylic chamber constructed after the adjustable design of Nonner (1969) but with only four saline pools and three Vaseline (Chesebrough-Ponds Inc., New York) gaps (Fig. 1) as in the original Dodge and Frankenhaeuser (1958) method. The C and E

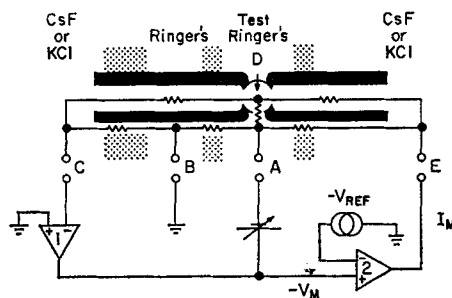


FIGURE 1. Schematic diagram of nerve and amplifiers. The myelinated fiber extends across three Vaseline gaps (stippling) with a node of Ranvier in pool A in test Ringer's solution. The cut ends of the fiber lie in pools C and E. The Vaseline gaps are (from left to right) $500 \mu\text{m}$, $200 \mu\text{m}$, and $200 \mu\text{m}$ wide. The A pool is $150 \mu\text{m}$ and the B pool $250 \mu\text{m}$ wide. Superimposed on the nerve is its DC equivalent circuit including resistances in the node, internode, and Vaseline seals. Four salt bridges connect the nerve chamber to the electronics (below). The two feedback amplifiers have a gain of 1000. Amplifier 1 permits potentiometric recording from the nerve. Amplifier 2 achieves the voltage clamp.

pools are filled with isotonic KCl or CsF solution and the B and A pools contain Ringer's solution. After the fiber is mounted in the chamber, the internodes are cut across in the C pool and in the E pool, leaving only the node in the A pool connected to half an internode on each side as shown in Fig. 1. The reversal potential for current in sodium channels was 55.2 ± 5.7 mv (mean \pm SD) in 5 uncut fibers, 58.5 ± 6.9 mv in 15 fibers cut in KCl, and 72.4 ± 8.4 mv in 18 fibers cut in CsF. These values are corrected for "attenuation" (see later) and are measured in the standard Ringer's solution about 30 min after the fiber is cut. The change of reversal potential arises from diffusion of cations across the cut, up the internode, and to the inside of the node as Koppenhöfer and Vogel (1969) have demonstrated for nerve fibers cut in NaCl and tetraethylammonium chloride. For reasons given later, a high value of the reversal potential is advantageous for measuring the permeability to relatively impermeant ions.

A large brass enclosure at a constant temperature of 5°C surrounds the nerve chamber. Four 1-M KCl-agar bridges connect the pools A, B, C, and E to 1-M KCl calomel electrodes. The experiment is begun after a one-half hour wait to allow the nerve, seals, temperature, and junction potentials to stabilize. The voltage clamp is turned on with the node at a holding potential of -80 mv (inside minus outside). A voltage-clamp series consists in measuring the membrane current of the node for 22 steps of voltage spanning the range from -80 to +77.5 mv in 7.5-mv steps (before correction for attenuation; see later). Resting inactivation of sodium channels is removed by a 50 msec hyperpolarizing prepulse to -125 mv which precedes each 20 msec test pulse. To record a complete voltage-clamp series takes about 25 sec. Then 20 vol of a new solution are rinsed through pool A and another voltage-clamp series is recorded. Test solutions are alternated with control solutions leaving the clamp on all the time. Membrane currents in five test solutions and six controls can be recorded in 30 min.

Recording The membrane current and voltage are recorded directly on-line in digital format with a Raytheon PB 440 digital computer (Raytheon Computer Operation, Santa Ana, Calif.). To permit an automated analysis of the experiments, two 9-bit analog-to-digital converters sample the current and voltage signals every 20 μ sec. The analog signals are filtered with a 5 kHz low-pass filter before conversion to reduce noise. Digital samples are taken for a 45 msec period, condensed (Hille, 1967 *a*), and stored on magnetic tape for later analysis.

Analysis The analysis obtains the peak current in sodium channels from the recorded membrane current and starts with the subtraction of capacity and leakage current. The first record of each voltage-clamp series measures capacity and leakage currents. The current during the prepulse step to -125 mv combined with Ohm's Law gives g_L , the leakage conductance. Only the last 2 msec of the 50 msec prepulse are used. The complete time-course of the current upon return to -80 mv gives the shape of the capacity transient. In many cases, the single exponential approximation previously used to fit capacity currents (Hille, 1967 *a*) is inadequate. The remaining 21 records of the voltage-clamp series are corrected for leakage and capacity currents assuming that leakage obeys a simple ohmic relation and that the amplitude of the capacity currents is linearly related to the voltage step. The new procedure for ca-

capacity currents has the disadvantage (for automated analysis) that when the measured capacity current is scaled up for the largest depolarizations, the noise on the original record at -80 mv is also scaled up. To reduce the noise, the original record is smoothed digitally with a variable time constant, low-pass filter. The filtering time constant increases from the beginning of the record to the end, so that the strongest smoothing is towards the end of the record.

After the leakage and capacity are subtracted, the current in sodium and potassium channels remains. For this analysis, negligible error is introduced by assuming that all the current is in sodium channels because there is 6 mM tetraethylammonium ion in all solutions and 95% of the potassium channels are blocked (Koppenhöfer, 1967; Hille, 1967 *a*). Thus it remains only to find the peak current in the corrected record. Because of noise, the lowest or highest single point in the record is not a good measure of the peak inward or outward current. Instead I use a third-order polynomial in time fitted by the method of least squares. The polynomial is fitted to 400- μ sec segments of the data and moved across the record until a negative minimum or a positive maximum is found. Subtracting leakage and capacity and finding the peak takes 1 or 2 sec per record. The records and fitted curves are checked on an oscilloscope at the computer console during the analysis.

Currents Outward membrane currents are defined as positive. The absolute magnitude of currents is calibrated by assuming that the resting resistance of the nodal membrane is 40 M Ω in normal sodium Ringer's solution (Tasaki, 1953, 1955). The magnitude of the maximum inward sodium current calibrated this way is 20–45 na, twice as high as in previous studies made in New York (Dodge, 1961, 1963; Hille, 1967 *a, b*, 1968 *a, b*). However, the methods used here would not distinguish between a higher sodium conductance or a higher resting resistance of the node. Only the ratios of quantities are actually determined. One of these is the ratio of the resting nodal resistance to the resistance R_{ED} (Fig. 1) of the current-passing path. For uncut fibers with KCl in the E pool the ratio was near 1.35 in the earlier work (Hille, 1967 *b*) and 1.05 in the present work. This measurement does not suggest a higher resting resistance in the present work. For cut fibers the same ratio varied from 2 to 10 depending on the position of the cut in the E pool.

Potentials Membrane potentials are given as inside minus outside. The potentials reported in the Results section are corrected for two known systematic errors, junction potentials and the "attenuation artefact." The relevant junction potential is that between the agar bridge in the A pool and the A pool itself. This potential depends on the solution in pool A. It is measured with respect to a Beckman 39402 ceramic junction, saturated KCl, reference electrode (Beckman Instruments, Inc., Fullerton, Calif.). For most solutions the junction potential is within 1.5 mv of that in sodium Ringer's solutions. It does not depend significantly on time or on the order of testing the solutions.

The attenuation artefact is best understood by referring to the description by Dodge and Frankenhaeuser (1958, 1959). They show that if pool B is narrow (200 μ m), only 70% of the actual membrane potential changes are recorded. Evidently the requirement to keep point D, inside the node (see Fig. 1), at ground potential by the first feedback amplifier is not achieved. If pool B is wide (300 μ m) there is no attenuation,

but the clamp is poor. In my experiments the attenuation is determined from the change in reversal potential upon switching from a Ringer's solution with a normal [Na] to a Ringer's with $\frac{7}{8}$ of the sodium chloride replaced by tetramethylammonium bromide. The theoretical change at 5°C is 49.8 mv. The observed changes in nine nodes and with a 250 μm B pool averaged 44.5 mv or 10.6% less than the theoretical value. In this paper the averaged attenuation (10.6%) is used to correct experiments where attenuation is not measured directly. In retrospect an attenuation correction should have been used in a previous paper where effects of divalent ions and pH on voltage-dependent properties were measured (Hille, 1968 *b*). The membrane potential and the recorded potential are (arbitrarily) assumed to be equal at the holding potential of -80 mv in sodium Ringer's solution.

Drift Since the goal of these experiments is to measure the reversal potential for current in Na channels, particular attention was paid to reducing DC drift inherent in the method. The Frankenhaeuser (1957) method of potentiometric recording has the same sensitivity as the parent Huxley-Stämpfli (1951) method to junction potentials, electrode differences, and amplifier drift. Changes in these parameters are magnified by the feedback. The problem arises because the recording pool C is not perfectly insulated from ground. In the method as used by Dodge and Frankenhaeuser (1958) the shunt to ground through the BC Vaseline seal (Fig. 1) is 5 $\text{M}\Omega$ and the recording resistance R_{CD} from the inside of the node to pool C is about 40 $\text{M}\Omega$. With these values, a 9 mv error signal at D, inside the node, appears at the recording electrode in C as a 1 mv signal. Conversely a 1 mv error or drift in the balance of the amplifier means a 9 mv error inside the node. Also a 200 pa amplifier input current, or a 1 mv difference in electrode potentials between the B and C electrodes, or a 1 mv junction potential in the fluid paths in the BC Vaseline seal, have the same effect.

The drift was reduced by increasing the seal resistance R_{BC} , by decreasing the recording resistance R_{CD} , and by building a new amplifier. The seal resistance was increased to about 15 $\text{M}\Omega$ by widening the BC partition from 150 to 500 μm (Fig. 1). R_{CD} was decreased to about 20 $\text{M}\Omega$ by cutting the fiber in the C pool. The new transistor amplifier used an FM3954A dual field effect transistor at the input with drift of less than 4 $\mu\text{v}/^\circ\text{C}$ and gate current of less than 10 pa. With the improvements in method, a 1 mv error in pool C would lead to an error of only 2.2 mv inside the node. The amplifier did not need to be rebalanced during the experiment, and the drift of a stable parameter like the sodium reversal potential was often less than 3 mv in a 45 min experiment. The same factors which reduce the sensitivity to drift at the input also reduce the influence of noise at the input.

Solutions The control solution (called Na Ringer's) contains 110 mM NaCl, 2 mM CaCl_2 , 6 mM tetraethylammonium bromide, and 1 mM tris(hydroxymethyl)amino methane buffer (Tris), pH = 7.4. The test solutions (called for simplicity guanidine Ringer's, etc.) contain the same salts except that all of the NaCl is replaced by an osmotically equivalent quantity of the test salt. The following salts have been tested: guanidine·HCl, hydroxyguanidine· $\frac{1}{2}\text{H}_2\text{SO}_4$, aminoguanidine· HNO_3 , triamino-guanidine·HCl, methylguanidine· $\frac{1}{2}\text{H}_2\text{SO}_4$, formamidine acetate, acetamidine acetate, hydrazine·2HCl mixed with hydrazine, methylhydrazine (+HCl), dimethylamine·HCl, tetramethylammonium bromide, tetraethylammonium bromide,

tris(hydroxymethyl)amino methane (+HCl), choline chloride (all from Eastman Organic Chemicals, Rochester, N. Y.); hydroxylamine·HCl, NH_4Cl , methylamine·HCl, imidazole (+HCl) (from J. T. Baker Chemical Co., Phillipsburg, N. J.); biguanide· $\frac{1}{2}\text{H}_2\text{SO}_4$ (Aldrich Chemical Co., Milwaukee, Wis.); *N*-methylhydroxylamine·HCl (K & K Laboratories Inc., Plainview, N. Y.); and 2-aminoethanol (+HCl) (Matheson, Coleman, and Bell, Cincinnati, Ohio). Throughout, cations with a dissociable proton are referred to by the simpler name of the neutral basic form rather than by the more correct name of the cation, e.g., guanidine, rather than guanidinium. The compounds have not been further purified or analyzed except for washing triaminoguanidine·HCl with ethanol to remove a trace of yellow impurity. All solutions are tested for pH, osmolarity, and sodium and potassium content. With the exceptions mentioned below, the pH is between 6.9 and 7.4. Osmolarities agree within 3% of the control solution, and sodium and potassium content are below 150 μM . Solutions of sodium substitutes are kept frozen until used.

Six of the substances tested have pK_a 's below 9.0, so that at pH 7.4 they are not fully ionized. To increase the concentration of cation, these compounds are tested at a lower pH in the absence of added buffer. Tetramethylammonium hydroxide or HCl are used to adjust the pH. The compounds followed by their pK_a and the pH of the Ringer's solution at 25°C are as follows: Tris 8.07, 6.40; hydrazine 7.97, 5.99; methylhydrazine 7.87, 6.10; imidazole 7.00, 6.40; hydroxylamine 5.95, 5.84; *N*-methylhydroxylamine 5.90, 5.37. The concentration of test cation is calculated from the known pK_a and the measured pH.

RESULTS

Na Channels are Permeable to Hydroxylamine and Hydrazine

Fig. 2 shows families of voltage-clamp currents for a node bathed in Na Ringer's, hydrazine (NH_2NH_3^+) Ringer's, and hydroxylamine (OHNH_3^+) Ringer's. The curves in Na Ringer's illustrate the normal features of Na current in sodium channels. For most depolarizing steps the current is inward (negative), and for steps beyond the reversal potential the current is outward. During the depolarizing step, the sodium permeability P_{Na} rises from a

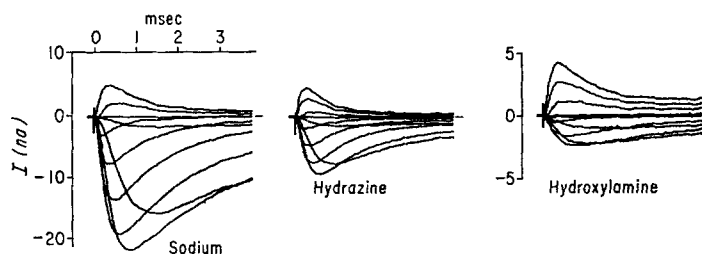


FIGURE 2. Families of voltage-clamp currents for a node bathed in Na, hydrazine, and hydroxylamine Ringer's. The time-courses of the ionic current in sodium channels at 10 different voltages spaced at 15-mv intervals are superimposed. The clamp voltages span the range from -65 to $+70$ mv (before correction for attenuation). Ends of fiber cut in KCl.

negligible initial value to a peak and then falls again more slowly to a low value. Very small depolarizations increase P_{Na} less rapidly and less completely than large depolarizations. In hydrazine and hydroxylamine Ringer's (Fig. 2) the voltage-clamp currents are similar to Na currents except smaller. The inward currents in these sodium-free solutions must be carried by inward movements of hydrazine and hydroxylamine ions. The permeabilities to hydrazine and to hydroxylamine rise and fall with time-courses like the time-course of P_{Na} . Apparently sodium channels are quite permeable to these organic cations.

Two methods are commonly used to calculate ionic selectivity from voltage-clamp measurements. The first is to compare the amplitude of currents or conductance in a test and a control solution. The computation uses the "independence principle" of Hodgkin and Huxley (1952). To be valid this method requires that the number of open channels be the same in each solution and that there is no saturation or block of open channels by the control or test ion. The second method uses the reversal potential, E_r (zero current potential), and the Goldman (1943; Hodgkin and Katz, 1949) equation. This equation was originally derived for the condition of a constant field through the membrane, but is now known to be much more broadly applicable (see Cole, 1968). The result does not depend on the number of conducting channels. For the experiments here, the change of E_r on switching from the control Na Ringer's to a sodium-substitute (S) Ringer's would be:

$$E_{r,s} - E_{r,Na} = 2.303 \frac{RT}{F} \log_{10} \frac{P_s}{P_{Na}} \frac{[S]}{[Na]}$$

where $2.303 RT/F$ is 55.2 mv at 5°C and the square brackets refer to cation concentrations (strictly activities) in the different external solutions. The ratio of permeabilities P_s/P_{Na} is a measure of the S -Na selectivity and is referred to hereafter as the permeability ratio for S , where it is understood that Na is the reference ion. The Goldman equation is used to calculate permeability ratios in this paper. Tacit assumptions are also made that selectivity is not changed by changing the bathing medium and that selectivity is not dependent on voltage. The error in measuring the change in reversal potentials is probably less than ± 5 mv, corresponding to an error in the permeability ratios of less than $\pm 20\%$.

The reversal potential is obtained from plots of peak current *versus* voltage. Fig. 3 gives current-voltage relations on two different scales for the experiment of Fig. 2. In Na Ringer's E_r is +58.5 mv. Replacing Na Ringer's with hydrazine Ringer's reduces E_r by 13.0 mv. Hydroxylamine Ringer's reduces E_r by 22.8 mv. In eight experiments with hydrazine, E_r changed by 13.0 ± 2.0 mv (mean \pm sd), and in four experiments with hydroxylamine, E_r

changed by 20.5 ± 2.8 mv. By the Goldman equation, the permeability ratio for hydrazine cations is 0.59 and the permeability ratio for hydroxylamine cations is 0.94; the sodium channel is nearly as permeable to these two organic cations as to Na ions.

Despite a high calculated permeability, the inward currents of hydroxylamine in Fig. 2 are very small relative to the control. Several factors contribute to the small size. First, the test Ringer's contains only 50 mM hydroxylamine cations to carry inward current, in contrast to 110 mM Na⁺ in the control Ringer's. Second, the test Ringer's has a pH low enough (pH 5.8) so that 20% of the sodium channels are blocked by protons (Hille, 1968 *b*). Even after taking the effects of pH, cation concentration, and permeability ratio into account, the largest inward current is less than half the size expected

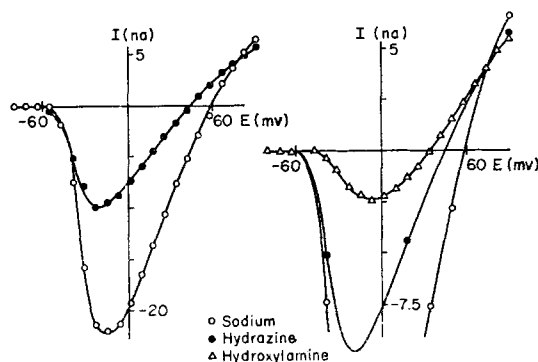


FIGURE 3. Peak current-voltage relations in Na, hydrazine, and hydroxylamine Ringer's. The peak current in sodium channels is plotted against clamp voltage for the experiment of Fig. 2. For clarity the curves are drawn twice, on different scales, but most of the points defining each curve are given only once. Voltage axis corrected for attenuation.

from the independence principle. The depression of currents can be described as a combination of a tetrodotoxin effect and a high calcium effect, because in addition to reduced currents the depolarization needed to activate sodium channels is increased. Many compounds studied in this paper have similar "pharmacological" effects. Some of these compounds also promote electrical breakdown of the nerve membrane and even cause visible wrinkling of the myelin sheath. It is because the Goldman-Hodgkin-Katz equation is insensitive to pharmacological alterations of conductance that it is used here to measure selectivity. An experimental analysis of the application of the independence principle to sodium channels will be given in a future paper.

Na Channels are Permeable to Many Organic Cations

Five other cations are measurably permeant, although their permeability ratios are well below those of hydroxylamine and hydrazine. Of the five,

ammonium (NH_4^+) and guanidine ($\text{NH}_2\text{:C}(\text{NH}_2)_2^+$) are the most familiar. They reduced E_r by 43.4 ± 5.0 mv and 48.4 ± 3.2 mv, corresponding to permeability ratios of 0.16 and 0.13. Voltage-clamp currents and peak current-voltage relations in ammonium and guanidine Ringer's are given in Figs. 4 and 5 together with records in impermeant tetramethylammonium (TMA) Ringer's. The currents in ammonium and TMA Ringer's fit the predictions of the independence principle satisfactorily while the currents in guanidine Ringer's are 30–50% smaller than the predictions.

The other three permeant cations used are derivatives of guanidine. Formamidine ($\text{NH}_2\text{:CHNH}_2^+$) is obtained by replacing an $-\text{NH}_2$ group by a hydrogen. Hydroxyguanidine and aminoguanidine are obtained by adding an $-\text{OH}$ or an $-\text{NH}_2$ group to one of the nitrogens of guanidine. The per-

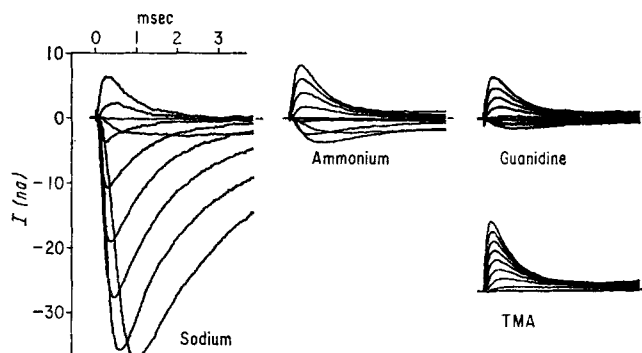


FIGURE 4. Families of voltage-clamp currents for a node bathed in Na, tetramethylammonium (TMA), ammonium, and guanidine Ringer's. Current traces as in Fig. 2. Ends of fiber cut in KCl.

meability ratios for these compounds range from 0.06 to 0.14 and are summarized in Table I. Current-voltage relations for a node bathed in hydroxyguanidine, aminoguanidine, and methylguanidine Ringer's are given in Fig. 6. As with guanidine, the guanidine derivatives (including formamidine) reduce currents to below the size expected from their permeability ratios. The depression of current by guanidine derivatives is especially apparent for the outward currents of Fig. 6 which are smaller than the outward currents in 12.5% Na Ringer's despite the higher reversal potential in the sodium-containing solution.

Methylated Cations are Impermeant

One of the current-voltage curves in Fig. 6 is for methylguanidine Ringer's. In contrast to the results with hydroxyguanidine and aminoguanidine, no inward methylguanidine currents are detected; the cation is relatively impermeant. In the absence of inward currents it is not possible to measure a

reversal potential, but E_r is certainly more negative than the most negative voltage which gives a clear outward current. For this experiment, E_r is less than -32.0 mv in methylguanidine Ringer's and equal to $+72.6$ mv in Na Ringer's. Hence the change in E_r is larger than 104.6 mv, corresponding to a permeability ratio less than 0.01 .

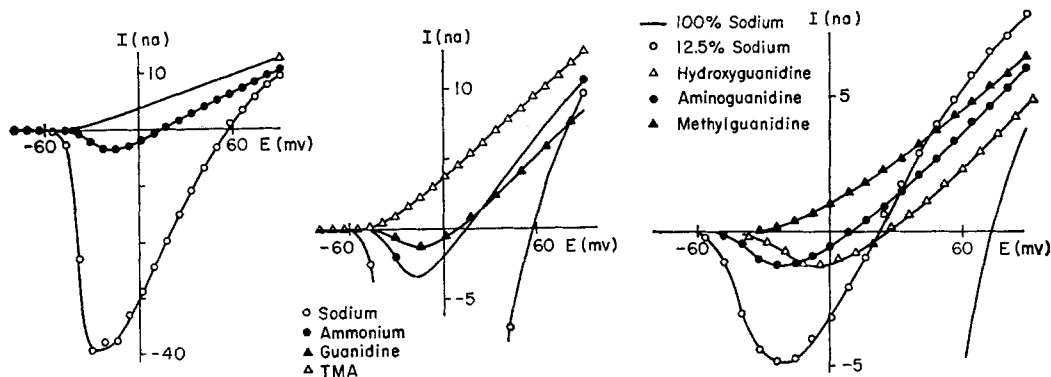


FIGURE 5

FIGURE 5. Peak current-voltage relations in Na, tetramethylammonium (TMA), ammonium, and guanidine Ringer's. Experiment of Fig. 4.

FIGURE 6

FIGURE 6. Peak current-voltage relations with guanidine derivatives. The low-sodium solution is one part Na Ringer's in seven parts tetramethylammonium Ringer's. Ends of fiber cut in CsF.

TABLE I
PERMEABILITY RATIOS FOR
PERMEANT CATIONS AND THEIR METHYL DERIVATIVES

P_X/P_{Na}	X	P_{MeX}/P_{Na}	Methyl-X	P_X/P_{MeX}
0.94	Hydroxylamine (4)	<0.056	N-Methylhydroxylamine (3)	>17
0.59	Hydrazine (8)	<0.025	Methylhydrazine (2)	>25
0.16	Ammonium (5)	<0.007	Methylamine (4)	>23
0.14	Formamidine (3)	<0.008	Acetamidine (2)	>14
0.13	Guanidine (10)	<0.010	Methylguanidine (3)	>13
0.12	Hydroxyguanidine (3)			
0.06	Aminoguanidine (3)			

Number of measurements in parentheses.

The impermeability of sodium channels to methylguanidine extends to the methyl derivatives of four other permeant cations. The left-hand side of Table I lists the seven permeant organic cations and their permeability ratios. The right-hand side gives the permeability ratios for the methyl derivatives of five of these ions calculated as explained for methylguanidine. None of the methylated ions is measurably permeant. Methylation reduces the permeability ratios at least 12–25-fold, and possibly very much more.

This fact is central to the structural hypothesis for sodium channels to be proposed.

The widely used sodium substitutes, choline, TMA, and Tris, give no inward currents. Voltage-clamp currents with these and several other impermeant cations are given in Fig. 7. The permeability ratios for eight such ions are summarized in Table II. Except for triaminoguanidine, imidazole,

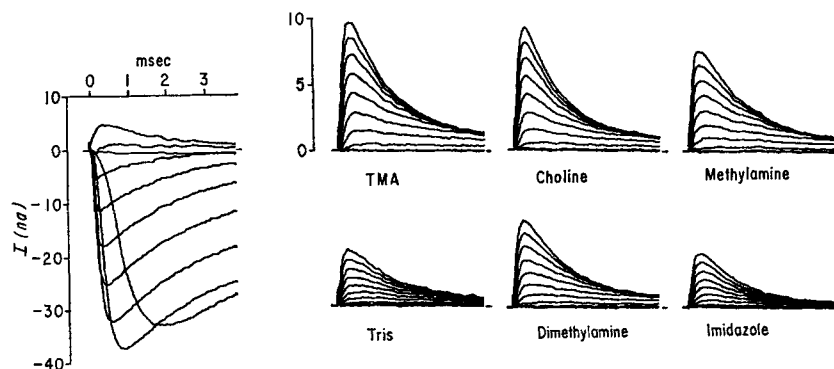


FIGURE 7. Voltage-clamp currents with impermeant cations: tetramethylammonium (TMA), choline, methylamine, tris(hydroxymethyl)amino methane (Tris), dimethylamine, and imidazole. Ends of fiber cut in CsF.

TABLE II
PERMEABILITY RATIOS FOR OTHER IMPERMEANT CATIONS

P_X/P_{Na}	X
<0.007	Dimethylamine (3)
<0.005	Tetramethylammonium (12)
<0.008	Tetraethylammonium (2)
<0.014	Ethanolamine (2)
<0.007	Choline (2)
<0.007	Tris(hydroxymethyl)amino methane (1)
<0.008	Imidazole (2)
<0.010	Biguanide (1)
<0.013	Triaminoguanidine (2)

Number of measurements in parentheses.

and biguanide, these impermeant cations have two or more $-\text{CH}_3$ or $-\text{CH}_2-$ groups.

The measurement of small permeability ratios is limited by several factors. With relatively impermeant ions, the currents are very small and may be obscured by noise. The currents are reduced further if the cation has anesthetic pharmacological properties, if the measurement has to be made at low pH, and if the test substance is not fully ionized. Another factor reducing the currents at negative potentials is the incomplete activation of sodium channels by small depolarizations. The calcium-like actions of low pH and of some of

the test substances further decreases the activation. For most sodium substitutes neither inward nor outward current is resolved below -50 mv, and in Na Ringer's E_r is not higher than $+75$ mv. Thus, in the best circumstances the largest measurable change in E_r is 125 mv ($50 + 75$), corresponding to a permeability ratio of 0.005.

DISCUSSION

Comparison with Previous Results

Very few published experiments give permeability ratios for organic cations in the sodium channel. Ammonium is the most studied ion. With frog nodes of Ranvier, Dodge (1963) reported a ratio of 0.21. If his calculation is repeated, but assuming an attenuation of 11% in the voltage record, the ratio becomes 0.17, very close to the value of 0.16 in the present experiments. In the squid giant axon the ratio for ammonium is 0.27 (Binstock and Lecar, 1969). Finally, in the squid giant axon the permeability ratio for guanidine is very roughly 0.25 (Chandler and Meves, unpublished; see Meves, 1970). Permeability ratios for all organic cations may possibly be higher in squid giant axons than in myelinated fibers, but more experiments would be needed to decide.

Many qualitative experiments show that organic cations are permeant. Action potentials in myelinated nerves and giant axons can be elicited in sodium-free solutions containing hydroxylamine, hydrazine, ammonium, guanidine, and aminoguanidine (Larramendi et al., 1956; Lorente de Nó et al., 1957; Lüttgau, 1958, 1961; Dodge, 1963; Tasaki et al., 1965, 1966; Tasaki and Singer, 1966; Watanabe et al., 1967). The action potentials are similar in shape and time-course to sodium action potentials, except smaller, and they are blocked by tetrodotoxin. Frog sartorius muscle and dorsal root cells also give tetrodotoxin-sensitive action potentials in hydrazine solutions (Koketsu and Nishi, 1966). In frog sciatic nerve the influx of hydrazine per impulse in hydrazine Ringer's is almost as large as the influx of Na per impulse in Na Ringer's (Cheng, 1962). Stimulation of squid axons in sea water raises the efflux of guanidine which has been injected into the axoplasm (Tasaki and Spyropoulos, 1962). Quantitative interpretation of experiments with action potentials requires at least two assumptions: first that the test ion obeys the independence relation in the sodium channel, and second that neither the test ion nor the Na ion use or affect the leakage, potassium, or other channels to a significant degree. Unfortunately, the assumptions do not hold for any of the permeant ions I have tested.

Tasaki and coworkers (Tasaki et al., 1965, 1966; Tasaki and Singer, 1966; Watanabe et al., 1967) have ranked nearly 40 organic cations in order of "favorability" as an external medium for squid giant axons. Favorability

was measured by the percentage of axons giving large, small, or no graded responses. On this basis methylamine was more favorable than TMA, and TMA was more favorable than tetrabutylammonium, etc. Most of these measurements probably bear little relation to the permeability of sodium channels and pertain more to which cations favor calcium action potentials. The external bathing solutions contained at least 200 mM CaCl_2 and the internal perfusion solution, glycerol and CsF, conditions which give calcium action potentials without external monovalent ions (Watanabe et al., 1967). The most "favorable" cations may be a combination of those which do not block calcium-permeable channels and those which do block other channels.

A Proposal for the Structure of the Selectivity Filter

The purpose of this section is to develop a molecular model of the sodium channel to explain ionic selectivity. My measurements with organic cations add new constraints on possible models. Probably the most difficult fact to explain is the impermeability to small methylated cations. Any proposal for the selectivity mechanism must also be consistent with other properties of the transport process in the channel: the very high ionic flux per channel, the near fit to the independence principle, and the temperature coefficient of the permeability. On this basis, I assume that the sodium channel is a pore through which sodium ions move by their own thermal motion, encountering barriers little larger than those in ordinary aqueous diffusion. A more detailed discussion of the arguments for a pore is reserved for a later paper concerning the independence principle.

FREE-SOLUTION MOBILITY According to Lecar et al. (1971) the permeability of the pore formed by "excitability inducing material, EIM" in black lipid membranes follows the order of free-solution mobilities for alkali cations. This means that, for example, the permeability ratio for potassium relative to sodium is about 1.5. Presumably the pore is so wide that it does not interfere with hydration shells of the ions as they pass through. The pore might have to have a diameter of more than 15 Å to give this result. If the sodium channel were similar, it would give the following permeability ratios (ratios of limiting equivalent conductivity, Robinson and Stokes, 1965; Scudder, 1914): ammonium 1.49, potassium 1.47, calcium 1.19, methylamine 1.17, dimethylamine 1.03, guanidine 1.01, sodium 1.00, hydroxylamine 1.0, tetramethylammonium 0.90, and lithium 0.77. The measurements in this paper are very different from the known free-solution mobilities, and hence the sodium channel must be relatively narrow at some point to achieve a different selectivity.

VAN DER WAALS SIZE Unhydrated size might be an important factor

in determining the ability of an ion to pass through a narrow pore. If the ion is too large, it cannot pass. In the following discussion, ions are characterized by the smallest rectangular box which can contain them. The size of the ion is determined from drawings like those in Fig. 8 constructed with conventional bond angles, bond lengths, and van der Waals radii (Pauling, 1960) and checked against actual crystal structures (Wyckoff, 1962). The largest permeant ion is aminoguanidine, a planar molecule about $3.7 \times 5.9 \times 7.6$ Å. The van der Waals surfaces of an entire guanidine molecule and of the major atoms (C, O, N) of the other permeant ions would fit into a box only 3.1 Å deep because the major atoms are planar or linear (Okaya and Pepinsky, 1957). The nonplanar hydrogens on hydroxyl, amino, and methyl groups

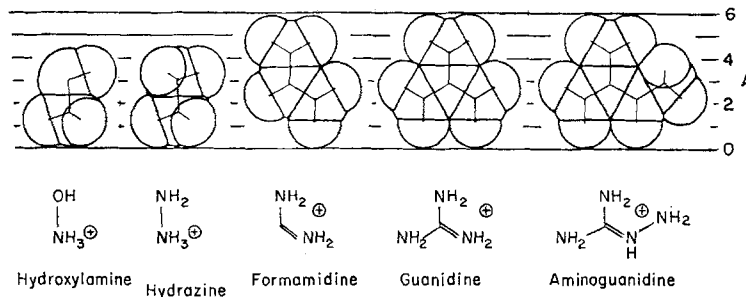


FIGURE 8. Scale drawings of hydroxylamine, hydrazine, formamidine, guanidine, and aminoguanidine. The outline gives the van der Waals surface and the thin lines inside represent the internal bonds. A third proton on the ammonium end (bottom) of hydroxylamine and hydrazine is hidden behind the proton which points at an angle out of the page. The scale is indicated at the right. Methylamine would look the same as hydrazine with the understanding that the third methyl hydrogen is hidden in the picture.

give a little more thickness bringing the total up to 3.7–3.8 Å. As can be seen in Fig. 8, the next larger dimension of even the largest permeant ions does not exceed 6.0 Å. Thus, all the permeant ions could fit in a pore as small as 3.8×6.0 Å.

Van der Waals size alone cannot account for selectivity because many impermeant ions are also small. Indeed, all the methylated cations in Table I could fit through a 3.8×6.0 Å pore. The difficulty is most striking with methylamine. Except for the progressive addition of a hydrogen atom, hydroxyl, amino, and methyl groups are almost isosteric so that hydroxylamine (OHNH_3^+), hydrazine (NH_2NH_3^+), and methylamine (CH_3NH_3^+) cations are very similar molecules. Nevertheless the sodium channel is less than 1% as permeable to methylamine as to hydroxylamine and hydrazine. Methylamine is very much smaller than formidine, guanidine, and aminoguanidine which have one, two, and three more nitrogens; there is some striking differ-

ence between $-\text{CH}_3$ groups and $-\text{OH}$ and $-\text{NH}_2$ groups which the selectivity filter can sense.

HYDROGEN BOND ENERGY One way to distinguish $-\text{CH}_3$ from $-\text{OH}$ and $-\text{NH}_2$ is through the ability to form hydrogen bonds. Methyl groups cannot form hydrogen bonds, whereas hydroxyl and amino groups are good donors whose hydrogens can bond to any oxygen-containing group to form $\text{O}-\text{H}\cdots\text{O}$ and $\text{N}-\text{H}\cdots\text{O}$ linkages. Selectivity might be based on the formation of a hydrogen bond with an energy on the order of 3 kcal/mole which could contribute a Boltzmann factor of 100 to binding of the ion to the channel.

Arguments can be given against using the *energy* of hydrogen bonding to explain the profound permeability differences between methylated and unmethylated cations. It would be difficult for the channel to provide as adaptable and as concentrated a hydrogen bonding environment as water provides. Thus, as the various $-\text{NH}_2$ and $-\text{OH}$ groups enter the channel, they may not hydrogen bond as completely to the groups in the channel as they did to solvent molecules. Looked at in this way, the ability to form hydrogen bonds might tend to keep ions in the solvent and out of the pore. On the other hand, it is probable that within the pore the effective dielectric constant is lower and hydrogen bonds are stronger than in the bathing solution. Another more compelling argument against the importance of the energy of the hydrogen bond is that methylhydroxylamine, methylhydrazine, acetamidine, and methylguanidine can make many hydrogen bonds yet their one methyl group makes them impermeant. The channel seems to select against methyl groups and not for hydrogen bonds.

HYDROGEN BOND LENGTH Strong selection against methyl groups could be achieved by a pore narrower than 3.8 Å. Such a pore would also be narrower than the van der Waals thickness of many permeant cations. However, there is a special circumstance which nevertheless could make the permeant cations exhibit a smaller effective size than their van der Waals size, and this will be the basis of a molecular model of the pore. In the formation of a hydrogen bond, the donor ($-\text{OH}$ or $-\text{NH}$) and the acceptor ($-\text{O}$) may approach up to 0.9 Å closer than van der Waals contact. For example, in ice the hydrogen of one water overlaps the oxygen of the next by 0.84 Å (Pauling, 1960). In effect, the donor and acceptor approach as if there were no hydrogen on the donor. Thus hydrogen bond donors have two effective sizes: the compound appears small if probed by an oxygen atom, and it has its usual van der Waals size if probed by any other atom. With respect to an oxygen probe, amino and hydroxyl groups would be 3.0 Å in diameter while methyl groups would be 3.8 Å. Using this fact, it is possible to design a pore lined with oxygen atoms which can pass hydroxyl and amino groups but not methyl groups.

A Molecular Model of the Pore

Fig. 9 is a diagram of a pore with the appropriate properties. The circles represent (to scale) eight oxygen atoms outlining an area about 3.1×5.1 Å which is the opening of the pore. This pore is the absolute minimum size which might still fit the observations.

The model pore excludes the five impermeant cations listed in Table I because their methyl groups, 3.8 Å across, are too wide. The first seven impermeant cations of Table II have more than one methyl or methylene group

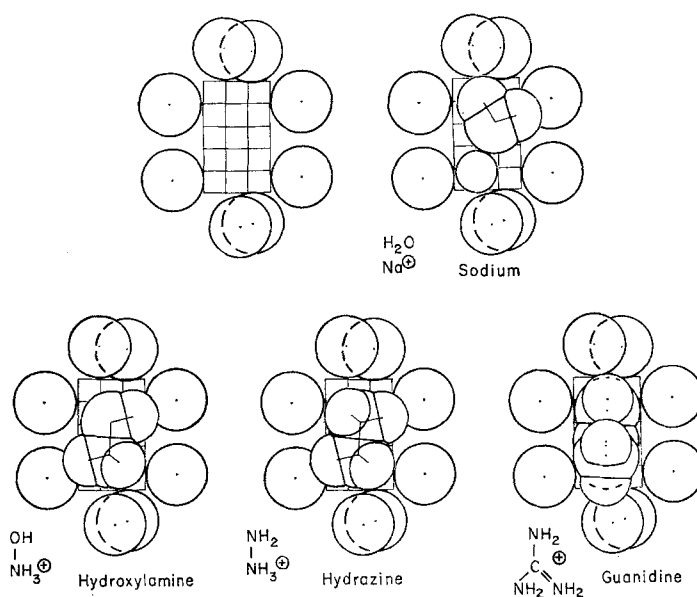


FIGURE 9. A model of the sodium channel consisting of an oxygen-lined pore. The grid inside is 3 Å by 5 Å. The successive frames show a sodium ion and a water molecule, hydroxylamine, hydrazine, and guanidine in the pore. Hydrogen bonds are formed wherever the hydrogens on the permeating cation overlap with oxygen atoms forming the pore. Methylamine would look like hydrazine in such a drawing, but overlap between methyl hydrogens and the oxygens of the pore would not be permitted.

and are in several ways too large to fit in the pore. Biguanide $((\text{NH}_2)_2\text{CNH}:\text{C}(\text{NH}_2)_2^+)$ is nonplanar and again does not fit the 3.1 Å dimension of the pore. Although they are planar, triaminoguanidine $(\text{NH}_2\text{NH}_2:\text{C}(\text{NH}_2\text{NH}_2)_2^+)$ and imidazole are excluded because they exceed the 5.1 Å dimension even after taking into account possible hydrogen bonds.

The seven permeant organic ions fit into the pore by making several hydrogen bonds to the surrounding oxygen atoms. Some of the ways for permeant organic ions to fit into the pore are given schematically in Fig. 9. Hydrogen bonds are indicated in the diagram by the overlap of hydrogen atoms, belonging to the cations, with the oxygen atoms of the pore. As drawn,

the lengths and angles of the hydrogen bonds are well within the typical range (Pauling, 1960; Chidambaram et al., 1970). Hydrazine (NH_2NH_3^+) and hydroxylamine (OHNH_3^+) could pass through in many possible orientations making hydrogen bonds with oxygens in several directions. The drawing of hydroxylamine, for example, shows two hydrogen bonds to the pore, one from the hydroxyl group above, and one from the ammonium group below. All the compounds related to guanidine ($\text{NH}_2:\text{C}(\text{NH}_2)_2^+$) are more restricted. They just fit the 3.1 Å dimension of the pore, making no hydrogen bonds laterally, and, provided they are oriented in one of only a few possible ways, they also just fit the 5.1 Å dimension, making several hydrogen bonds. As shown in Fig. 9, there is little room to spare when one of these cations is in the pore. The lower permeability of sodium channels to guanidine compounds relative to hydrazine and hydroxylamine might arise from the lower probability that guanidine compounds strike the channel in one of the few acceptable orientations. Because several hydrogen bonds would have to be broken simultaneously, the organic cations may leave the pore more slowly than sodium ions do. Slow leaving could explain the reduction of currents below the size expected from the independence principle with most of these cations, for the cation might "tie up" the channel so long that other ions are prevented from entering.

One frame of Fig. 9 shows a sodium ion (diameter 1.90 Å, Pauling, 1960) in the pore. The ion is accompanied by a water molecule hydrogen bonded to the pore (Hille, 1971 *a*). Analysis with a three-dimensional model of the pore shows that there is more than enough room for two more water molecules touching the sodium ion, one from "behind" and one from the "front" of the ion in the pore. Thus, the sodium ion can keep at least three waters of hydration and come into close contact with three more oxygen atoms of the pore. The pore is also wide enough to accept lithium, potassium, rubidium, and calcium ions. A later paper will report measurements of the permeability of the sodium channel to these and other ions and will argue that an important factor governing selectivity among these ions of small radius is how easily waters of hydration can be adjusted to give a good fit between the partly hydrated ion and the pore. The ammonium ion is also so small that waters of hydration would be needed to "adapt" the ion to the pore. Further discussion of ammonium and inorganic cations, including sodium, is best deferred to that paper where the importance of water molecules is more fully described.

The pore is large enough to accommodate some nonelectrolyte molecules. Several water molecules would fit into the empty pore. Hydrogen peroxide, formaldehyde, formamide, and urea also fit. Methyl and sulfur-containing groups are too large so that methane, methanol, and thiourea would not pass through. Benzene rings are also too large.

Experiments with changing the pH of the medium bathing the node of Ranvier have revealed an acid group associated with sodium channels (Hille, 1968; Drouin and The, 1969). At low pH the sodium conductance of the node is reversibly reduced as though a single acid group with a pK_a of 5.2 is being titrated. Apparently the normal functioning of the sodium channel requires that a certain group bear a negative charge. The pK_a indicates that the group is probably an oxygen acid such as a carboxylic acid or a phosphate. This observation can be incorporated into the model pore by supposing that two of the oxygen atoms of the pore are the oxygens of the essential acid group. Then the pore would normally bear a single negative charge, helping to attract cations. This charge would be lost at low pH by protonation of the acid. Fig. 9 was drawn with the idea that the overlapping pair of oxygens forming the lower margin of the pore is an ionized carboxylic acid. The penetrating ions are shown with their cationic ends oriented towards the negative charge.

REMARKS ON THE PORE This proposal is intended to be a realistic model to account for ionic *selectivity* in the sodium channel. The model is not intended to account for the voltage-dependent opening and closing of the sodium channel. Additional structural components will have to be added to explain this important *gating* function. The most important feature of the model is an oxygen-lined cavity roughly 3×5 Å. If the model pore were not formed by hydrogen bond-accepting oxygen atoms, the geometrical explanation for selection against methyl groups would have to be abandoned and a new explanation sought. The pore is much narrower than the 7.3 Å diameter pore postulated in a previous realistic model of the sodium channel (Mullins, 1959 *a, b*, 1961). Again, the impermeability to methylamine is the major argument for making the pore so very narrow. Other arguments are given in a later paper. The exact number and position of oxygen atoms is certainly not known, nor is the chemical nature of the oxygen-bearing groups, but there are reasons to suggest that one pair of oxygens is an ionized carboxylic acid. Some structure containing many other atoms is needed to support the important oxygen atoms, as for example in the cation-permeable, oxygen-lined pore formed by the polypeptide gramicidin A (Urry, 1971; Urry et al., 1971; Hladky and Haydon, 1970). The framework of supporting atoms would have some flexibility so that the static view in Fig. 9 should be supplemented by some idea of motion and a blurring of the perimeter of the pore.

Arguments will be given in a later paper that the constricted part of the sodium channel is only a few Ångstrom units long. Indeed it might be no longer than the groupings of oxygens in Fig. 9, with a much wider passage reaching the rest of the way through the membrane. A later paper will also

show that the structure in Fig. 9 is a good receptor for binding of tetrodotoxin and saxitoxin in a blocking position.

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