PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Negative Surface Charge Near Sodium Channels of Nerve: Divalent Ions, Monovalent Ions, and pH [and Discussion]

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Phil. Trans. R. Soc. Lond. B 1975 **270**, 301-318
doi: 10.1098/rstb.1975.0011

Phil. Trans. R. Soc. Lond. B. **270**, 301–318 (1975) [301]
Printed in Great Britain

Negative surface charge near sodium channels of nerve: divalent ions, monovalent ions, and pH

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Evidence is given for a high density of negative surface charge near the sodium channel of myelinated nerve fibres. The voltage dependence of peak sodium permeability is measured in a voltage clamp. The object is to measure voltage shifts in sodium activation as the following external variables are varied: divalent cation concentration and type, monovalent concentration, and pH. With equimolar substitution of divalent ions the order of effectiveness for giving a positive shift is: Ba = Sr < Mg < Ca < Co ≈ Mn < Ni < Zn. A tenfold increase of concentration of any of these ions gives a shift of +20 to +25 mV. At low pH, the shift with a tenfold increase in Ca^{2+} is much less than at normal pH, and conversely for high pH. Solutions with no added divalent ions give a shift of −18 mV relative to 2 mM Ca^{2+}. Removal of $\frac{7}{8}$ of the cations from the calcium-free solution gives a further shift of −35 mV. All shifts are explained quantitatively by assuming that changes in an external surface potential set up by fixed charges near the sodium channel produce the shifts. The model involves a diffuse double layer of counterions at the nerve surface and some binding of H^+ ions and divalent ions to the fixed charges. Three types of surface groups are postulated: (1) an acid $pK_a = 2.88$, charge density $-0.9\ nm^{-2}$; (2) an acid $pK_a = 4.58$, charge density $-0.58\ nm^{-2}$; (3) a base $pK_a = 6.28$, charge density $+0.33\ nm^{-2}$. The two acid groups also bind Ca^{2+} ions with a dissociation constant $K = 28$ M. Reasonable agreement can also be obtained with a lower net surface charge density and stronger binding of divalent ions and H^+ ions.

Introduction

Firing threshold in excitable cells depends on the concentration of divalent ions, monovalent ions, and H^+ ions in the bathing medium. All of these factors influence the voltage dependence of the Hodgkin–Huxley (1952) excitability parameters *m*, *h*, and *n* (Frankenhaeuser & Hodgkin 1957; Chandler, Hodgkin & Meves 1965; Blaustein & Goldman 1968; Hille 1968; Gilbert & Ehrenstein 1969; Mozhayeva & Naumov 1970, 1972*a*, *b*, *c*; Brismar 1973). To a first approximation, the functions relating *m*, *h*, and *n* to voltage are simply shifted along the voltage axis. Increases of external divalent, monovalent, or H^+ ion concentration increase the depolarization needed to attain a given point on these functions. For example, a tenfold increase in external $[Ca^{2+}]$ shifts the functions for sodium activation, m_∞ and τ_m, by 20–24 mV so an axon must be depolarized that much more to achieve a corresponding opening of Na channels. The depolarization needed to reach firing threshold also increases. Since other factors are changed by divalent ion concentration, the change of threshold is only roughly the same as the shifts of sodium activation.

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While individual shifts of m, h and n parameters are not identical for any given change of bathing medium, they are sufficiently similar to imply that a common underlying mechanism is responsible. The same mechanism probably underlies shifts in contraction threshold and in the Ca^{2+} spike threshold of muscle fibres as well (Hagiwara & Naka 1964; Costantin 1968; Suarez-Kurtz, Reuben, Brandt & Grundfest 1972). Two mechanisms have been suggested. The first considers that opening and closing of ionic channels is accomplished by escape or entry of Ca^{2+} into the channels (Gordon & Welsh 1948). The Ca^{2+} ion is viewed as a voltage-dependent 'plug' pulled out by depolarization or by decreased external [Ca^{2+}]. No quantitative theory of such a dynamic process has agreed with experimental observation. As is discussed by Hille (1972), the theory is particularly unsuited to explain shifts in calcium channels. A voltage-dependent block of sodium channels in elevated [Ca^{2+}] has been observed (Woodhull 1973), but with voltage dependence too weak to explain normal 'gating'. The second proposed mechanism, agreeing quantitatively with all published observations, postulates a layer of negative fixed charge on the inner and outer surfaces of the membrane and gives bathing cations a static role as counterions (A. F. Huxley, in Frankenhaeuser & Hodgkin 1957; Chandler *et al.* 1965). A negative surface potential set up by the fixed negative surface charge creates an electric field within the membrane that adds to the field from externally applied potential differences. The total field with this dual origin is sensed by whatever membrane components underlie changes in m, h and n. In this theory, shifts of excitability parameters arise through the influence of available counterions on the surface potentials. Cations 'screen' the surface charge by forming an ionic diffuse double layer at the surface and may also physically neutralize some of the surface charge by forming complexes (binding). Both effects reduce the negative surface potential; hence cations added to the outer solution increase the depolarization needed to bring the field within the membrane to a given value.

Tests of the surface potential hypothesis consist in comparing observed shifts with predictions of an appropriate form of the Gouy–Chapman–Stern theory (Grahame 1947; Davies & Rideal 1963; Barlow 1970) for potentials at planar surfaces with ionizable groups bathed by electrolyte solutions. Numerous authors have successfully applied the theory to measured voltage shifts in axons (Chandler *et al.* 1965; Gilbert & Ehrenstein 1969, 1970; Mozhayeva & Naumov 1970, 1972*a*, *b*, *c*; Brismar 1973) by choosing values for the fixed surface charge density and, in some cases, assigning acid dissociation constants (pK_a) or dissociation constants for surface complexes with various divalent ions (K_{Ca}, K_{Mg}, etc.). As originally stated (Frankenhaeuser & Hodgkin 1957), the hypothesis emphasized binding of Ca^{2+} ions. More recently in a useful clarifying paper for physiologists, McLaughlin, Szabo & Eisenman (1971) show that charged systems with screening alone do give similar surface potential changes to those with both binding and screening. Hence the observations of Frankenhaeuser & Hodgkin (1957) are readily fitted by the surface potential hypothesis under a variety of assumptions, and other types of experiments are needed to further narrow the range of acceptable charge densities, surface potentials, and dissociation constants.

This paper reports measurements of shifts of the voltage-dependent activation of sodium channels. Throughout the paper we assume that the surface charge theory is the correct explanation of shifts. Indeed our results are readily fitted by that theory. An effort is made to define the range of acceptable parameters for this fit.

When we started this study in 1969, Gilbert & Ehrenstein (1969) had just applied a form of the Gouy–Chapman–Stern theory to shifts of the activation of potassium channels for the

first time. Since increases of calcium concentration alone do not provide a complete test of the theory, we decided to test the interactions of several variables. A search for possible competition between external Ca^{2+} and H^+ ions in giving shifts was successful (Woodhull & Hille 1970), but also revealed a new difficulty in measuring shifts: The shape of the curve of peak sodium permeability P_{Na} against membrane potential E is altered by changes of pH. Thereupon Woodhull (1973) worked out a theory explaining the change in shape and permitting an unambiguous measurement of shifts. Then, inspired by the papers of Mozhayeva & Naumov (1970) on potassium channels and of McLaughlin *et al.* (1971) on artificial membranes, we looked for shifts with reductions in external monovalent salt concentration and with 'foreign' divalent ions. In particular, the results with 'foreign' divalent ions bear on the question of binding against no binding of divalent ions. Our observations were completed in 1972. Brismar (1973) has recently reported shifts of sodium and potassium channel activation with simultaneous changes of monovalent and calcium ion concentrations. Our results extend and confirm Brismar's published work, at least qualitatively.

Materials and methods

The methods are described by Hille (1971). Briefly, nodes of Ranvier of frog myelinated nerve fibres were studied under voltage clamp conditions at 10 °C. The ionic currents were corrected for leakage and capacity currents (Armstrong & Hille 1972). Outward current is called positive. All membrane potentials are taken as inside minus outside (E scale) and are corrected for measured electrode junction potentials and 'attenuation'. Junction potentials are more significant than in our previous work, ranging up to 7 mV for solutions of the lowest ionic strength. The ends of the fibres were cut in solutions of 120 mM-KCl.

Measurement of sodium activation

The shifts studied here span a 90 mV range. Our initial observations showed that the voltage dependence of long term sodium inactivation (Adelman & Palti 1969) and of conventional sodium inactivation shift along the voltage axis with changes of monovalent, divalent, and H^+ ion concentration. To avoid the resulting variation in maximum available sodium permeability, the holding potential was adjusted after each solution change to set the resting value of h_∞ (the parameter governing conventional sodium inactivation) in the range 0.6–0.9.

Another problem was 'series resistance' on the voltage clamp (Hodgkin, Huxley & Katz 1952). This artefact distorts the peak sodium current–voltage relation in the region of negative slope, the most important region for our measure of sodium activation. With the 'northern' frogs used here effects of series resistance were not usually noticeable in experiments with positive shifts (in the same direction as in elevated calcium) where ionic currents tended to be small. For experiments with negative shifts and with solutions of low electrical conductivity, the $[Na^+]$ had to be reduced to $\frac{1}{8}$ or $\frac{1}{16}$ of the normal value to decrease the ionic current and reduce the effects of series resistance.

Currents in sodium channels were measured in the conventional manner, each test pulse being preceded by a 40 ms prepulse 45 mV more negative than the holding potential to remove ordinary resting sodium inactivation. Potassium currents were blocked by tetraethylammonium ion in all solutions. The peak sodium permeability P_{Na} was calculated from the peak sodium current and the Goldman–Hodgkin–Katz flux equation (Dodge &

Frankenhaeuser 1959). As in previous studies (Frankenhaeuser & Hodgkin 1957; Hille 1968), the curve of peak P_{Na} against potential (E) was used to measure the voltage dependence of opening of sodium channels. Shifts were measured manually by sliding a transparent standard template for the P_{Na}–E curve on the graphs of experimental points. In this way we gave weight to all points on each curve, at least down to 10 or 5 % of the maximum P_{Na}, rather than picking just a single point like the point where P_{Na} falls to 50 % of the maximum. Shift measured this way are referred to as 'shifts of sodium activation'. They reflect changes primarily in the voltage dependence of the Hodgkin–Huxley parameter m_∞. Shifts were measured relative to the standard Ringer's solution with 2 mM Ca^{2+} and 119 mM monovalent cation at pH 7.4 and are called positive when the standard P_{Na}–E template had to be slid to more positive voltages to match the observations. Control measurements in standard Ringer's solution were interposed between every one or two measurements in test solutions to give a frequent check on the condition of the fibre.

For all experiments with low pH values and for some with divalent ions, the shapes of the peak P_{Na}–E curves were obviously changed with respect to the control curve in Ringer's solution. Before shifts were measured, the curves at low pH were 'corrected' for voltage-dependent block as described by Woodhull (1973) using the average values reported in that paper for the two parameters of the blocking equation. The curves for divalent ions were similarly corrected assuming apparent dissociation constants at 0 mV of 53, 23, 10, 5, and 4 mM for block by Ca^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}. The parameter for the 'fraction of the potential drop' at the blocking site was the same as for H^+ ions. These corrections add yet another empirical procedure to the measurement of shifts, but without them the shapes of P_{Na}–E curves are too different from the standard curve to permit a shift to be measured.

TABLE 1. COMPOSITION OF EXTERNAL SOLUTIONS

	solution type		
	A	B	C
substance	mM	mM	mM
NaCl	112	104	7
KCl	0	2.3	0
chloride salt of divalent ion	0–50	0–20	0
tetraethylammonium bromide	6	12	5
tetramethylammonium chloride	0	0	$(107-x)$
sucrose	0	0	$(2x)$
MOPS buffer†	2	0	2
MCP buffer‡	0	6	0
pH	7.4	4.5–10.0	7.4

† MOPS buffer: morpholinopropane sulphonic acid, titrated to pH 7.4 with NaOH.
‡ MCP buffer: equimolar mixture of MOPS, cyclohexylaminopropane sulphonic acid, and propionic acid, titrated to final pH with NaOH or HCl.

Solutions

Three types of external solutions were used in this study: (A) solutions of constant monovalent cation concentration and pH, but variable divalent cation concentration and type; (B) solutions of constant monovalent cation concentration, but variable pH and $[Ca^{2+}]$; (C) solutions of constant pH, constant $[Na^+]$, low $[Ca^{2+}]$, and variable total monovalent cation. The compositions of the solutions are given in table 1. Buffers were chosen for their low binding of

divalent ions. Tetraethylammonium ion blocked current in potassium channels. In some solutions of type A, much of the NaCl was replaced by tetramethylammonium chloride to reduce the size of the sodium current.

Results

Divalent ions

Expectations

We start by considering the expectation of Gouy–Chapman–Stern theory for a negatively charged surface of high charge density in a medium containing a constant concentration of monovalent cations and a variable concentration of divalent cations M^{2+}. At sufficiently low $[M^{2+}]$ there is a large negative surface potential ψ_0 set by the monovalent cation concentration. As $[M^{2+}]$ is increased from a low to high value there is a gradual transition from a high negative value of ψ_0 to a zero value. The $\psi_0 - \lg [M^{2+}]$ curve is sigmoid with a middle region approximating to a straight line of maximum possible slope of 28.1 mV per tenfold increase in $[M^{2+}]$ at 10 °C. Even in systems with high surface charge density this maximum slope may not be realized if there also are dissociating acid or base groups acting to 'buffer' the changes of ψ_0. The $\psi_0 - \lg [M^{2+}]$ curve is similar for divalent ions that bind and ions that do not, except that binding reduces the $[M^{2+}]$ needed to reach a corresponding value of ψ_0. In the following we avoid using the expression 'ionic strength', for as McLaughlin *et al.* (1971) show, the formula for ionic strength weights ions of different valuence in the wrong way for 'screening' at planar surfaces.

Frankenhaeuser & Hodgkin (1957) reported that changing external $[Ca^{2+}]$ in the concentration range 4.4 to 112 mM shifts the voltage dependence of sodium activation 21.5 mV per tenfold increase in squid giant axons at 6 °C. Shifts with Ni^{2+} ion replacing Ca^{2+} ion are similar, but less Ni^{2+} than Ca^{2+} is needed to reach any point on the relation between shift and lg $[M^{2+}]$ (Dodge 1961; Blaustein & Goldman 1968; Hille 1968). Hence in the Gouy–Chapman–Stern theory, Ca^{2+} binds less strongly than Ni^{2+}. Our first goal in these experiments was to explore the available divalent ions systematically to see in a similar way if any ions bind less than Ca^{2+}. If so, this would be proof, within the context of the theory, that Ca^{2+} ions do bind to a degree. Preliminary experiments indicated that Mg^{2+} may be an example of an ion which is bound less than Ca^{2+} (Frankenhaeuser & Hodgkin 1957). Our second goal was to look for a flattening out of the shift against lg $[M^{2+}]$ relation at low $[M^{2+}]$. The concentration range where this occurs sets important bounds on the possible surface charge densities and M^{2+} dissociation constants of the theory.

Observations

Figure 1 gives the measured shifts of sodium activation as a function of the concentration of eight different divalent ions. The symbols are averages of several measurements and the smooth curves are from a form of the surface potential theory to be discussed later. Solutions were of type A in table 1 and the only divalent ion in each solution is that indicated by the symbol. For an equimolar substitution, the order of effectiveness for shifting is

$$Zn^{2+} > Ni^{2+} > Co^{2+} = Mn^{2+} > Ca^{2+} > Mg^{2+} > Ba^{2+} = Sr^{2+}.$$

The total scatter of the observations averaged for each point is typically ± 1.5 mV around the mean so there is no known uncertainty regarding this sequence. The observations are

interpreted to mean that Ba^{2+} and Sr^{2+} are the least bound, Mg^{2+} is more bound and Ca^{2+}, still more. For each divalent ion, sodium activation is shifted 20–23 mV per tenfold concentration increase in the steepest part of the observed relation.

While between 2 and 22 nM Ca^{2+} the observed shifts fall roughly on a straight line, below 2 nM they definitely do not. The shift at 0.1 mM-Ca^{2+} is -14.0 mV rather than the -27 mV expected by linear extrapolation, and several experiments with '0 mM' Ca (no added Ca^{2+} ions) applied for up to 30 min gave shifts of only -18.5 mV. There are three possible

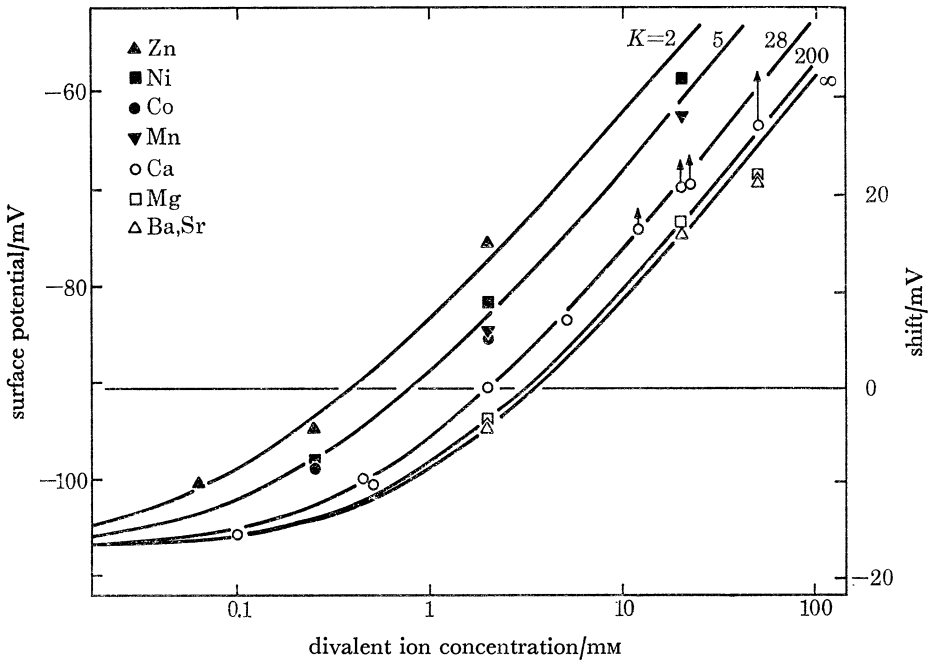

FIGURE 1. Voltage shifts with different divalent ions. Shifts (right scale) are plotted against the concentration of added divalent ion with the horizontal line marking the control value for 2 mM Ca^{2+}. External solutions of type A. Each point is the mean of about four observations. Arrows on the points for 12, 20, 22, and 50 mM Ca^{2+} show a correction attributable to the effect of raised osmolality of those solutions as explained in the text. The curves are calculated from 'model I' (table 2) using various values for the dissociation constant K for divalent ions. The curve with $K = \infty$ corresponds to 'no binding'. The scale at the left gives the calculated surface potential.

explanations. First the result is free from artefact and correctly reveals the theoretically expected limit on shifts with constant monovalent cation concentration. Second the correct relation is linear below 2 mM-Ca^{2+}, but the solutions are contaminated with divalent ion equivalent to 0.5 mM-Ca^{2+}, so the intended low [Ca^{2+}] was never reached. Third the solutions are adequate, but there is poor exchange in the chamber or a diffusion barrier at the node preventing the surface [Ca^{2+}] from falling below 0.5 mM. Several points argue against the last two interpretations. The water used to mix solutions was twice distilled, the second time in a glass still, and collected and stored in polyethylene plastic bottles. Analysis by atomic absorption spectrophotometry revealed less than 10 μM-Ca^{2+} in any of the concentrated stock solutions of monovalent salts and buffers. Exchange in the chamber and at the node was good enough for the properties of a node to return to normal within less than 10 s of the replacement of a 20 mM Ca^{2+} Ringer's with the standard 2 mM Ca^{2+} Ringer's. Changes back and fourth between '0.1 mM Ca^{2+}' and '0.5 mM Ca^{2+}' also gave rapid and reproducible 5 mV

shifts. Finally, Ulbricht (1964) studied changes in the action potential of nodes of Ranvier using steps of [Ca^{2+}] in a fast flow device. In his work the changes take less than 200 ms to go to completion.

In two experiments, 1 mM ethylendiaminetetraacetic acid was included in a 0.5 mM Ca^{2+} solution (retitrated to pH 7.4) to give an estimated free [Ca^{2+}] of 10^{-8} M. The solution was applied to a node under voltage clamp conditions at -90 mV. Within 10 s a large inward current developed at the holding potential of -90 mV, exceeding by several fold the maximum inward sodium current of a normal node. The conductance of the node rose to a high value and no voltage-dependent responses were evident. A Ringer's solution without the chelating agent was applied after 30 s and within another several minutes the node seemed to recover its normal properties. As other authors have found, such extremely low divalent ion concentrations outside a cell may disturb normal membrane structure in general. The loss of responses of the ionic channels may be secondary.

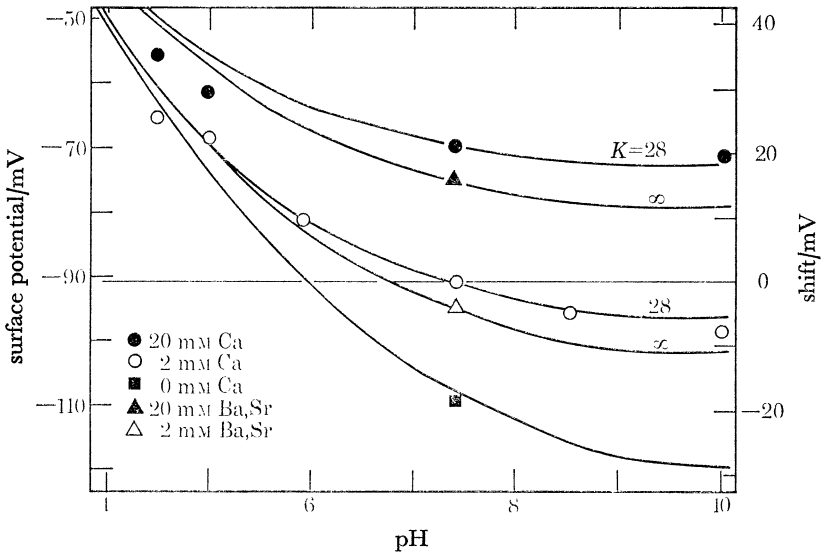

FIGURE 2. Shifts (right scale) with pH changes at various divalent ion concentrations. External solutions of type B. The horizontal line marks the control value in standard Ringer's solution. Each point is the mean of about four observations. Curves are calculated from ('model I') (table 2) for no binding ($K = \infty$) and $K = 28$ mM at 0, 2, and 20 mM divalent concentration.

pH

In the Gouy–Chapman–Stern theory applied to surfaces with ionizable acid groups, surface potentials become less negative as the pH of the bathing solution is lowered and fewer acid groups remain dissociated. Lowering the pH around a node of Ranvier also gives a positive shift of sodium activation (Hille 1968; Drouin & The 1969; Woodhull 1973). Figure 2 shows a remeasurement of the shifts with pH in the range from pH 4.5–10.0 for a variety of divalent ion concentrations. Solutions are of type B. With 2 mM-Ca^{2+} (open circles) the points agree with earlier measurements (Hille 1968) except at pH 5.0 and 4.5 where the shifts are not as large. Actually, the observed peak P_{Na}–E relations agree even at low pH, but in the new work we have corrected peak P_{Na} for voltage-dependent block by H^+ ions (Woodhull 1973) before measuring shifts. The small currents at low pH and the necessity for this correction make these points less reliable than those at pH 5.9 and above.

Woodhull & Hille (1970) showed that the shift caused by increasing [Ca^{2+}] tenfold is largest at high pH and smallest at low pH. That result is confirmed in figure 2. Changing from 2 mM Ca^{2+} to 20 mM shifts +27.9 mV at pH 10.0, +21.0 mV at pH 7.4, and +9.8 mV at pH 4.5, as if there were some kind of competition between the effects of H^+ ions and of Ca^{2+} ions.

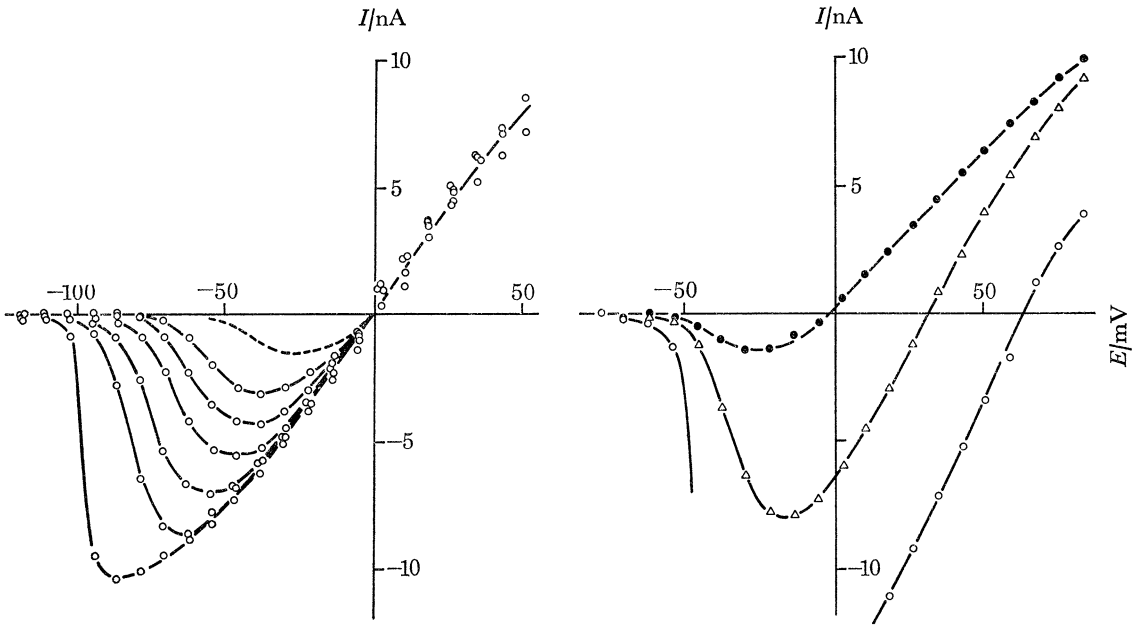

FIGURE 3. Peak sodium current–voltage relations in low sodium solutions of different total monovalent salt concentration. External solutions of type C. Right: normal total monovalent cation concentration (C^+ = 120 mM) and 2 mM Ca^{2+} with [Na^+] reduced from (○) 112 mM to (△) 28 and (●) 7 mM. Left: total monovalent cation concentration decreased in steps from 120–15.3 mM with a constant 7 mM Na^+ and 0.0 mM Ca^{2+}. The largest inward currents are from solutions with the lowest total C^+. Dashed curve is the same as the curve through filled circles on the right with 2 mM-Ca^{2+}. Curves have no theoretical significance.

Monovalent cation concentration

According to the Gouy–Chapman theory, 'screening' of surface charges by monovalent ions can be significant, particularly when divalent ion concentration is low. Maximum slopes of 56.2 mV per tenfold concentration change can be obtained with high surface charge densities. Experiments were designed to look for shifts of sodium activation as monovalent salts are replaced by sucrose (solutions of type C). Figure 3 shows the sequence of measurements as peak current–voltage relations in sodium channels. First with a normal [Ca^{2+}] of 2 mM (right side of figure), [Na^+] is lowered from 112 to 28 mM and then to 7 mM by replacing NaCl by tetramethylammonium chloride. This decreases the maximum inward sodium current to 1.5 nA and decreases detectable error due to series resistance. Pulses to −50 mV activate a just barely detectable increase in P_{Na}. Finally solutions with no added Ca^{2+} and a constant [Na^+] of 7 mM are applied (left side of figure). The degree of activation of P_{Na} with pulses to −50 mV increases because of a −18.5 mV shift from the elimination of divalent ions. When the monovalent cation concentration, symbolized by C^+, is still the normal 120 nM, the maximum inward current is roughly 3 nA and half maximal activation of P_{Na} occurs near −50 mV. As C^+ is reduced progressively to 15.3 mM, the maximum inward current eventually becomes larger than 10 nA and there is significant activation of P_{Na} even at −100 mV, a −50 mV

shift relative to normal Ringer's. With the lowest value of C^+, the solution conductivity is too low and the sodium currents again too high for good control of the sodium currents. In all solutions except the most dilute one, the steep part of the relation between lg (P_{Na}) and E has a slope of an e-fold increase in P_{Na} per 6–8 mV of depolarization as in normal fibres.

The many current–voltage relations in figure 3 with a constant 7 mM external Na^+ have nearly the same maximum P_{Na} and differ from each other primarily by shifts of sodium activation. Some of the shifts in this and four other experiments with solutions of lowered C^+ are summarized in figure 4. With no added Ca^{2+}, the shifts are about 42 mV per tenfold increase of C^+. In the presence of 0.5 mM or 2 mM-Ca^{2+}, change of C^+ has a much smaller effect.

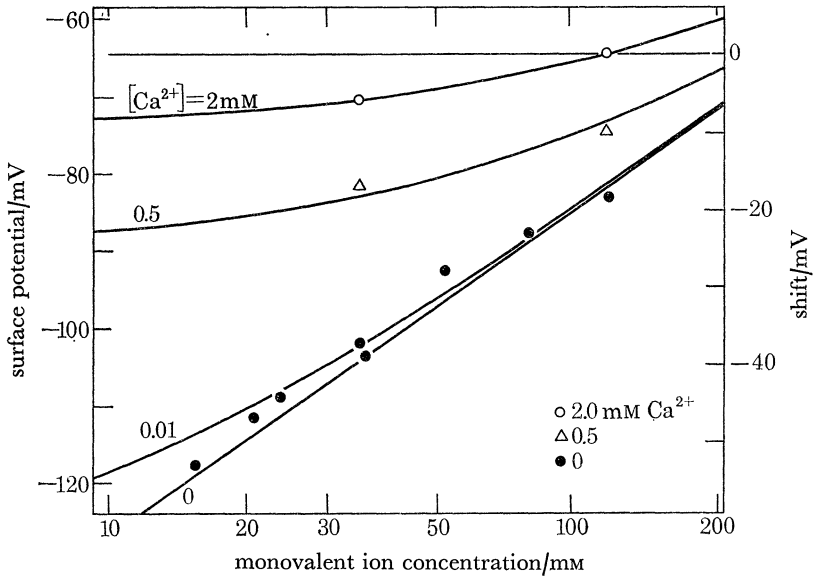

FIGURE 4. Shifts (right scale) with reduction of total monovalent ion concentration at three Ca^{2+} concentrations. Experiments like that of figure 3 with external solutions of type C. Each point is the mean of one to three observations. The curves are calculated from ('model I') (table 2) with K = 28 mM and divalent concentrations 0, 0.01, 0.5, and 2.0 mM.

Other observations

Shifts of sodium activation were also measured in single experiments with UO_2^{2+}, La^{3+} and Tb^{3+} ions. In 1 μM UO_2^{2+} there was no shift (± 2 mV) but a 50 % depression of sodium currents. In 100 μM UO_2^{2+} sodium currents were reduced to less than 1 % of their former value. The effects of 1 μM or 100 μM UO_2^{2+} were not reversed by rinsing with standard Ringer's but were quickly and completely reversed by a rinse with a Ringer's solution containing 50 mM tris-(hydroxymethyl)aminomethane. Shifts of +10 and +16 mV were recorded in 40–80 μM La^{3+} and 50 μM Tb^{3+}, respectively, the concentration of the La^{3+} solution being uncertain because of the quantity of water imbibed by the salt used. Sodium currents were also depressed to 75 % of the control value at 0 mV.

In a few experiments (Woodhull 1972) shifts of sodium inactivation were measured for comparison with shifts of sodium activation. The measured shifts (with shifts of activation given in parentheses) were: +16.2 mV (+21.0) in 20 mM Ca^{2+} pH 7.4, +22.3 mV (+21.6) in 2 mM Ca^{2+} pH 5, and −8.2 mV (−15.9) in 2 mM Ca^{2+} pH 10.

In addition to shifts and blocking of sodium conductance, some solutions had other effects

on the node. For example, the leakage conductance was reduced by 8–9 % in 100 μM UO_2^{2+}, 2 mM Ni^{2+}, 2 mM Zn^{2+}, and 20 mM Mn^{2+}. The trivalent ions, and UO_2^{2+}, Mn^{2+} and Ni^{2+} also slowed the kinetic changes of sodium current beyond the amount expected from shifts of time constants (cf. Dodge 1961). Both activation and inactivation could be slowed more than threefold.

Discussion

A possible correction to the results

Before discussing how well the observations can be explained by surface potentials on the outer surface of the membrane, we consider a possible unintended source of shifts. This is the effect of internal ionic concentrations on surface potentials of the inner surface of the membrane. When in other experiments a Ringer's solution is made deliberately hypertonic by adding tetramethylammonium salts, the reversal potential for current in sodium channels falls as if axoplasmic salts become more concentrated by shrinkage of the cell. Therefore there probably also is shrinkage in the solutions of type A which contained added divalent salts. The concentrating of internal salts would increase screening of internal negative surface charge, giving a negative shift, partly off-setting the positive shift from elevated external divalent ion. We estimated the magnitude of this effect assuming that axoplasm is a 1–1 salt solution that remains iso-osmotic with the external medium and that the inner membrane surface of nodes of Ranvier bears the same negative charge density as Chandler *et al.* (1965) found for squid giant axons. The 'corrections' are indicated as arrows on points for 10, 20, 22, and 50 mM Ca^{2+} in figure 1. Similar arrows could be applied to measurements with high concentrations of other divalent ions. At 20 mM M^{2+} the correction adds 2.4 mV to the shift. We advance no specific evidence for or against the presence of internal negative charge in the node of Ranvier, but believe that some should be assumed to be there until proven otherwise.

Theoretical interpretation of results

We now examine the hypothesis that all shifts are accounted for by changes of the surface potential of the outer surface of the membrane. The Gouy–Chapman theory of surface potentials at the interface of a charged solid with an electrolyte solution is well known (Grahame 1947; Davies & Rideal 1963; Barlow 1970). It is not discussed here except to note that at several points the theory neglects consequences of discreteness and molecularity in the solvent, ions, and fixed charges. The assumption of a perfect impenetrable plane surface with uniformly smeared charge density ignores the structural specificity and heterogeneity of cell membranes. Hence, even if all observed shifts are a consequence of changes of surface potential, the theory might still not apply perfectly. For the experiments described here, potential changes (shifts) extend over a range of 90 mV and the solutions contain ions of various valences, so a full theoretical expression for ψ_0 with mixed electrolytes and without small-potential approximations is needed. The relation at $T = 10$ °C between free surface charge density σ_f (elementary charges per square nanometre) and surface potential ψ_0 (millivolts) becomes (Grahame 1947)

$$\sigma_f = \frac{1}{2.7}\left[\sum_i c_i \left(\exp\frac{-z_i\psi_0}{24.4} - 1\right)\right]^{\frac{1}{2}}, \qquad (1)$$

where c_i is the bulk concentration (molar) of the ith ion with charge z_i.

Stern's extension of Gouy–Chapman theory takes into account the finite size of ions and specific binding of ions to the surface. Following the practice of Gilbert & Ehrenstein (1969), Mozhayeva & Naumov (1970, 1972*a*) and McLaughlin *et al.* (1971), we consider only specific binding and ignore the finite-size corrections in Stern's theory. Preliminary trials showed that a minimum of three different acid dissociation constants are needed to explain the broad pH dependence of the observed shifts. The following assumptions are made. The charge on the membrane surface comprises three dissociable chemical groups: two monobasic acid groups of surface density σ_1 and σ_2 with intrinsic acid dissociation constants K_{H1} and K_{H2} and a basic group of density σ_3 with intrinsic acid dissociation constant K_{H3}. Divalent ions bind to groups of type 1 and 2 with intrinsic dissociation constants K_{Ca}, K_{Mn}, etc. Two groups of the same type (type 1 or 2) are neutralized when one divalent ion binds. At very low pH the acid groups are neutral and the basic group is positively charged, while at high pH the basic group is neutral and the acid groups negatively charged. From conventional kinetics of equilibrium binding, all of these assumptions are summarized in the following equation for free surface charge (with Ca^{2+} as the divalent ion)

$$\sigma_f = \frac{\sigma_1}{1+K_{H1}/[H^+]_0+K_{Ca}/[Ca^{2+}]_0}+\frac{\sigma_2}{1+K_{H2}/[H^+]_0+K_{Ca}/[Ca^{2+}]_0}+\frac{\sigma_3}{1+[H^+]_0/K_{H3}}, \quad (2)$$

where σ_1 and σ_2 are negative numbers and σ_3 is positive and $[H^+]_0$ and $[Ca^{2+}]_0$ stand for volume concentrations at the membrane surface where the dissociable groups are. Concentrations at the surface are related to bulk concentrations by Boltzmann factors expressing the influence of potential on ion distribution. At 10 °C the relations are:

$$[H^+]_0 = [H^+] \exp(-\psi_0/24.4), \quad (3)$$

$$[Ca^{2+}]_0 = [Ca^{2+}] \exp(-\psi_0/12.2). \quad (4)$$

Again ψ_0 is in millivolts. Equations (1) through (4) are solved by substituting (3) and (4) into (2) and then setting (2) and (1) equal to each other, giving a single implicit function for surface potential. The surface potential is then obtained on a computer by an iterative numerical procedure, the Newton–Raphson method.

Our primary goal was to see if all shifts could be accounted for on the basis of surface-potential theory, and the secondary goal was to determine the significance of 'binding' relative to simple 'screening'. Some trials showed that the seven parameter-theory outlined could be fitted to the observations with a range of acceptable values for the parameters. An example of one satisfactory fit is given as smooth curves in figures 1, 2 and 4. Varying any one of the seven parameters significantly disturbs the fit. The values used for figures 1, 2 and 4 are given in table 2. under the columns labelled 'model I'. If one parameter is changed, all others must also be changed to restore the fit. Satisfactory fits may be defined in the seven dimensional 'parameter space' by a single line segment proceeding from moderate charge densities, binding, and surface potentials to high charge densities, binding, and surface potentials. Model I is near the high surface potential end of this line. A second satisfactory choice of parameters, now from near the low surface potential end of the line, is designated 'model II' in table 2 and is used to draw smooth curves in figures 5, 6 and 7.

Adjustments of parameters for fit was done by a crude trial and error method and certainly could have been refined further to improve the fit. Once a value for ψ_0 and hence σ_f in standard

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Ringer's solution was selected, suitable values for K_{Ca}, K_{Mn}, etc., were determined primarily from plots like figures 1 or 5. The allocation of total charge among the three types of charged groups and suitable values of K_{H1}, K_{H2}, and K_{H3} were determined from plots like figures 2 or 6. Several reiterations of the fitting process were needed. Points at pH 4.5 and all experiments with low monovalent ion concentration were ignored in choosing parameters. Nevertheless the measurements in low monovalent salt are well fitted and serve to confirm the validity of the surface potential theory.

The upper and lower limits on acceptable charge densities are determined by different factors. In a system with no binding of divalent ions, the steep dependence of ψ_0 on $[M^{2+}]$ extends to lower and lower $[M^{2+}]$ as the surface charge density is increased, and hence the predicted shift from lowering $[M^{2+}]$ from 2 to 0 mM increases. For example, compare lines

TABLE 2. SURFACE PARAMETERS OF THE TWO MODELS

(*a*) Charge densities and dissociation constants

	model I				model II			
type	σ / nm^{-2}	pK_a	K_{Ca} / M	K_{Ba} / M	σ / nm^{-2}	pK_a	K_{Ca} / M	K_{Ba} / M
1 acid	−0.90	2.88	28	∞	−0.66	3.76	1.2	3.0
2 acid	−0.59	4.58	28	∞	−0.30	5.66	1.2	3.0
3 base	0.33	6.28	∞	∞	0.21	7.16	∞	∞

(*b*) Surface conditions in control Ringer's at pH 7.4

	model I	model II
ψ_0	−90.6 mV	−64.3 mV
pH_0	5.79	6.26
$[Ca^{2+}]_0$	3.35 M	0.34 M
σ_f	1.04 nm^{-2}	−0.50 nm^{-2}
bound Ca^{2+}	0.077 nm^{-2}	0.087 nm^{-2}

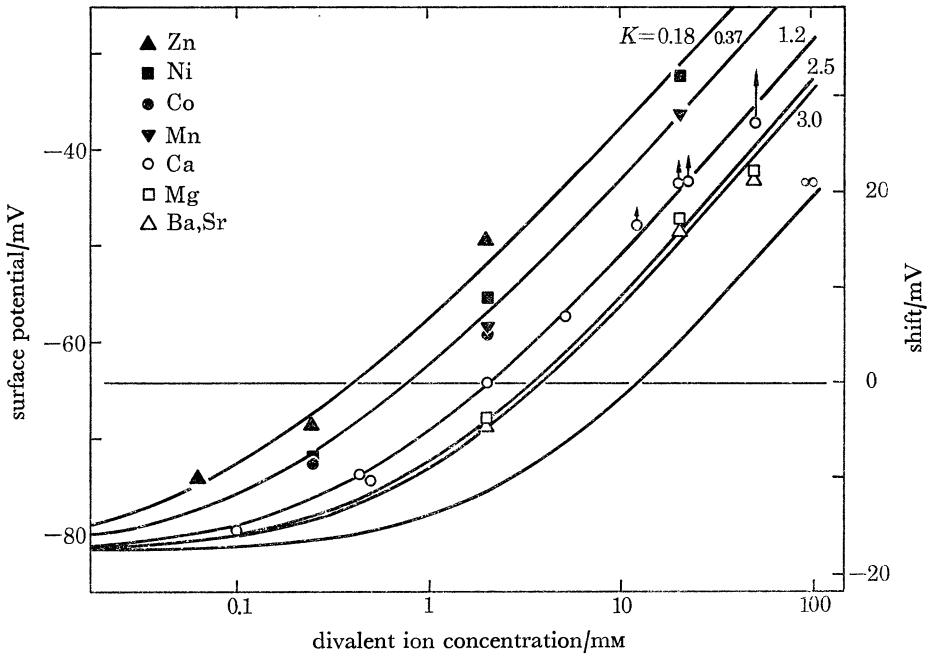

FIGURE 5. The observations of figure 1 are replotted with theoretical curves from 'model II' (table 2).

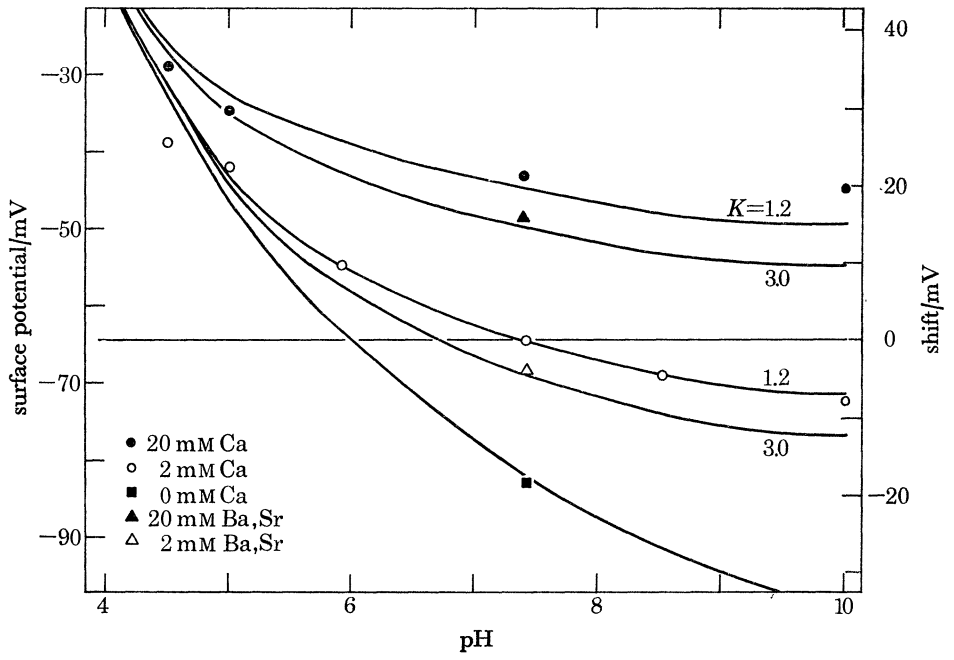

FIGURE 6. The observations of figure 2 are replotted with theoretical curves from 'model II' (table 2).

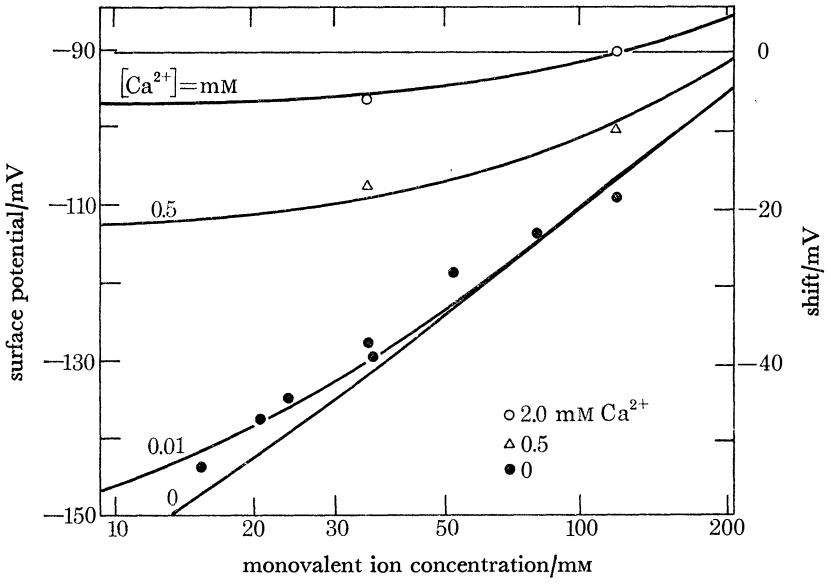

FIGURE 7. The observations of figure 4 are replotted with theoretical curves from 'model II' (table 2).

labelled '$K = \infty$' in figures 1 and 5. Experimentally, there is only a -14.6 mV shift when $[Ba^{2+}]$ or $[Sr^{2+}]$ is lowered from 2 to 0 mM. Model I is at the high charge density limit because systems with more charge give more than the -14.6 mV shift on removing divalents.

The lower charge density limit is harder to define. As charge density is lowered, the maximum slope of the ψ_0 against lg $[M^{2+}]$ relation gradually decreases. At the physiological $[Ca^{2+}]$ there is an upward curvature of this relation, and the region of high slope must be sought at abnormality high $[Ca^{2+}]$. The reliability of such measurements can be questioned since 'corrections' are applied for a small voltage-dependent block of sodium channels by Ca^{2+}

ions, for junction potentials, and for the effects of cell shrinkage. Without the correction for shrinkage, the experimental shift on changing from 2 to 20 mM-Ca^{2+} is 21.0 mV, while with correction it becomes 23.4 mV. For the same change of [Ca^{2+}], model I predicts 21.5 mV, model II 20.1 mV, and another model with 5 mV less surface potential than model II predicts 19.5 mV. Believing that the experimental value is somewhere between 21.0 and 23.4 mV, we suggest that model II is at or below the low limit of acceptable surface charge.

Comparison with other work on sodium activation

There are numerous published observations of threshold changes with changes of external divalent ion concentration, but few direct studies on shifts of activation of sodium channels with a voltage clamp. Our 'model I' gives a value of -70.3 mV for ψ_0 in 'seawater' with 11 mM Ca^{2+} and no Mg^{2+}. The potential falls by 19.8 mV when the [Ca^{2+}] is elevated tenfold. By comparison Frankenhaeuser & Hodgkin (1957) found shifts of 21.4 mV per tenfold change in squid giant axon and Blaustein & Goldman (1968), 17.1 mV in lobster. Blaustein & Goldman (1968) ranked the polyvalent ions in the following sequence for shifts with equimolar replacement:

$$La^{2+} > Ni^{2+} > Zn^{2+} \approx Co^{2+} > Cd^{2+} \approx Ba^{2+} > Ca^{2+} < Mg^{2+}.$$

The sequence is very close to ours except for the position of Ba^{2+}. Vogel (1973) measured shifts in myelinated nerve of $+10$ mV in 100 μM La^{3+} and $+31$ mV in 500 μM La^{3+}. We found $+10$ mV in 40–80 μM. Our model I predicts shifts of -2.4, $+1.0$ and $+11.6$ mV for 50, 100 and 500 μM of a trivalent ion that does not bind.

Shifts with lowered external monovalent ion concentration or pH have not been studied in marine axons. The only other study with low external monovalent ion concentration is Brismar's (1973) on the node of Ranvier. His results are qualitatively like ours but in the lowest monovalent ion concentrations, his shifts are 7–10 mV more negative than our models predict. The discrepancy is probably explainable since Brismar's reference solution in this experiment contained 90 mM K^+, 27.5 mM Na^+, and 2 mM Ca^{2+}, while ours contained 107 mM quaternary ammonium ion, 7 mM Na^+, and 2 mM Ca^{2+}. Isotonic K^+ solutions give a positive shift relative to a standard Ringer's solution reference and a $+8$ mV shift relative to solutions consisting largely of quaternary ammonium ions (Hille 1972). Such effects point to the difficulty of identifying 'neutral' monovalent cations for diluting Na^+ ions in these experiments, a problem we do not claim to have resolved. Other experiments have shown appreciable positive shifts on replacing Na^+ ions with Li^+, Tl^+, guanidinium and others (Hille 1971, 1972). Replacing Cl^- ions with I^-, Br^-, NO_3^-, and a long list of other anions leads to negative shifts.

Other voltage-dependent membrane properties

Shifts of sodium inactivation with external monovalent, H^+, and divalent ion concentration have not been studied much. As in our work, Frankenhaeuser & Hodgkin (1957) found up to 30 % smaller shifts of inactivation than of activation as [Ca^{2+}] is varied. Vogel (1973) found the same for effects of added La^{3+}. On the other hand Chandler *et al.* (1965) found 30 % larger shifts of inactivation than activation as *internal* monovalent ion concentration is varied. These differences suggest that the field-detecting mechanisms giving voltage dependence to activation and inactivation of sodium channels 'see' similar, but not identical, local electric fields from neighbouring charged groups.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Shifts of potassium activation have been studied extensively and fitted to a variety of surface potential models. Divalent, monovalent, and H^+ ion concentration all play a significant role as with sodium activation, although shifts of potassium activation are usually somewhat smaller. The sequence of effectiveness of various divalent ions includes

$$Ni^{2+} > Co^{2+} > Ca^{2+} > Mg^{2+},$$

as for sodium activation (Blaustein & Goldman 1968; Khodorov & Peganov 1969; Mozhayeva & Naumov 1970). On the basis of fairly scattered measurements on K^+-depolarized squid giant axons, Gilbert & Ehrenstein (1969) suggested a -53 mV surface potential in standard seawater with very weak Ca^{2+} binding. From experiments with an extremely slowly (seconds) varying 'ramp clamp' on K^+-depolarized myelinated nerve, Mozhayeva & Naumov (1970, 1972*c*) proposed a -30 mV surface potential in standard Ringer's with very strong Ca^{2+} binding. The same axons with a fast step clamp led Brismar (1973) to a -55 mV standard surface potential with no Ca^{2+} binding. These various measurements differ in recording method (possibility of attenuation artefact) and in the significance of low potassium inactivation. The smaller size of these surface potentials relative to our models I and II reflects the generally smaller shifts of potassium activation. Evidently local fields near potassium channels are fundamentally similar to those near sodium channels, but smaller.

Remarks on the Ca '*plug*' *theory*

The successful quantitative fit of surface potential equations to shifts over a wide range of conditions weakens the case for the voltage-dependent 'plug' theory of Ca^{2+} action (Gordon & Welsh 1948). The most difficult observations to square with that theory are the shifts obtained with changing monovalent ion concentration and the preservation of a very steep relation between lg (P_{Na}) and E in the absence of added divalent ions. Armstrong, Bezanilla & Horowicz (1972) showed that frog muscle fibres continue to conduct action potentials for up to 20 min in solutions with 1 mM EGTA and an estimated free [Ca^{2+}] below 10^{-8} M. Other arguments against 'plug' theories for normal sodium activation or potassium activation are given elsewhere (Hille 1972). We believe that the 'field sensors' of ionic channels and the 'gates' they control are part of the membrane structure rather than diffusible components of the medium.

Remarks on surface potential theory

The reasonable fits obtained with quite different choices of surface parameters in this paper demonstrate the difficulty of drawing exact conclusions from measurements of surface potential. The problem is to find a procedure to identify the shift corresponding to zero surface potential using physiologically acceptable solutions. Without this we can simply say that the postulated surface charge densities are somewhat less than those for pure phospholipid membranes (McLaughlin *et al.* 1971) and the acid and divalent ion dissociation constants are reasonable for side chains and carboxy-terminal ends of proteins, for phosphate, ethanolamine, and serine groups of phospholipids, and for carboxy groups of sialic acid. Chemical modifications of the membrane might be used to attack this identification more directly.

Figure 8 shows hypothetical potential profiles across the membrane including the proposed surface potentials. The potential and distance scales are intended to be realistic. Three nm has been allotted to the insulating portion of the membrane. Undoubtedly there are many hydrophilic membrane components extending beyond this narrow region. A free negative

charge density of 0.14 nm^{-2} or 1 per 7.25 nm^2 has been assumed for the inner surface of the membrane (Chandler *et al.* 1965). This is about 15 and 30 % of the external free charge densities in models I and II. When the membrane potential observed between electrodes in the bulk solution is −75 mV, the potential drop within the membrane itself is only −9 to −35 mV. The diagram neglects contributions of dipoles which can be very large (Haydon & Myers 1973) and of fixed charge groups attached to the membrane but located at some distance from the membrane as in a ('fuzzy coat' extending 10 nm from the surface. Such groups would make little contribution to the surface potential if they lie beyond the *ca.* 0.8 nm thickness (Debye length) of the surface double layer. Taken at face value these results imply that the membrane potential range from −60 to −40 mV where the most dramatic changes of *m*, *n* and *h* parameters occur, corresponds to a change of the electric field within the membrane from an inward to an outward direction.

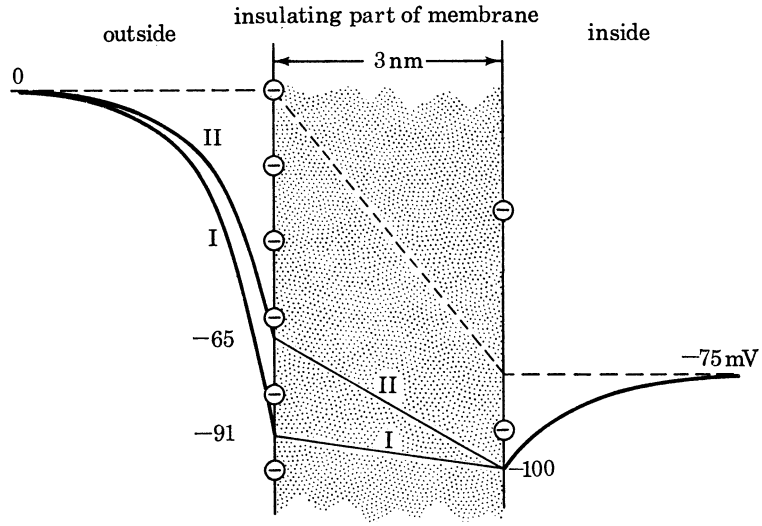

FIGURE 8. Potential profile near and within the membrane according to the surface potential hypothesis. The diagram is intended to be to scale with an insulating membrane 3 nm thick, bearing external charges every 1 nm (like the free charge in model I) and internal charges every 2.7 nm (Chandler *et al.* 1965). Debye lengths for the fall-off of potential are 0.9 nm in the internal solution and 0.8 nm in the external solution. The lines labelled I and II correspond to potential profiles in models I and II. The internal field is assumed to be constant and contributions of membrane dipoles are omitted.

McLaughlin & Harary (1974) have suggested that the internal potential drop is small and the surface potentials asymmetrical as drawn because the electrical driving force on charged phospholipids tends to redistribute them between inner and outer membrane leaflets to reduce the internal field. The 'flip-flop' redistribution is thought to be very slow with a time scale of hours or days. Conceivably the opening and closing or long term inactivation of ionic channels involves a similar 'flip-flop' of charged components that move more easily than the average phospholipid in a bilayer.

Quite aside from affecting gating, surface potentials should also influence the effective permeability of open channels by modifying local concentrations of permeant ions and by changing the electric driving forces within the membrane (Frankenhaeuser 1960). However, virtually no permeability changes of this type were seen with the shifts caused by variations of ions not thought to bind, e.g. variations of monovalent concentration or of Sr^{2+} concentration. We suggest therefore that the conducting mouth of the pore is significantly further

from the region of surface potentials than the voltage sensing structures or that the ionic double layer at the mouth is strongly disturbed as soon as current flows through the pore. Finally we repeat that the quantitative deductions given in this paper are derived from a theory that is itself only approximate, so the values chosen in our models are more guidelines than precise conclusions.

We are indebted to Susan A. Morton for invaluable secretarial help and to Kaaren Nichols for help in data analysis. This research was supported by U.S. Public Health Service grants NS08174, NS05082 and RR00374.

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Discussion

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When you reduce the external sodium, do you assume that the internal sodium stays constant?

B. Hille

Whether internal sodium stays constant is not believed to have any effect on shifts measured the way we do. With isotonic solutions and healthy fibres, the resting exchange of sodium at the node is probably extremely slow relative to diffusion down the internode from the cut ends of the fibre.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES