Charges and Potentials at the Nerve Surface

Divalent ions and pH

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ABSTRACT The voltage dependence of the voltage clamp responses of myelinated nerve fibers depends on the concentration of divalent cations and of hydrogen ions in the bathing medium. In general, increases of the [Ca], [Ni], or [H] increase the depolarization needed to elicit a given response of the nerve. An *e*-fold increase of the [Ca] produces the following shifts of the voltage dependence of the parameters in the Hodgkin-Huxley model: m_∞, 8.7 mv; h_∞, 6.5 mv; τ_n, 0.0 mv. The same increase of the [H], if done below pH 5.5, produces the following shifts: m_∞, 13.5 mv; h_∞, 13.5 mv; τ_n, 13.5 mv; and if done above pH 5.5: m_∞, 1.3 mv; h_∞, 1.3 mv; τ_n, 4.0 mv. The voltage shifts are proportional to the logarithm of the concentration of the divalent ions and of the hydrogen ion. The observed voltage shifts are interpreted as evidence for negative fixed charges near the sodium and potassium channels. The charged groups are assumed to comprise several types, of varying affinity for divalent and hydrogen ions. The charges near the sodium channels differ from those near the potassium channels. As the pH is lowered below pH 6, the maximum sodium conductance decreases quickly and reversibly in a manner that suggests that the protonation of an acidic group with a pK_a of 5.2 blocks individual sodium channels.

INTRODUCTION

This paper presents studies of the changes of many of the voltage-dependent properties of a myelinated nerve fiber caused by changing the [Ca], the [Ni], and the [H] of the medium bathing the nerve. The results are interpreted as manifestations of the binding of these cations to negative fixed charges at the nerve surface.

Many studies show that cell membranes bear a high density of fixed negative charges. Erythrocytes and other blood cells move toward the anode in an electrophoretic measurement (Abramson, Moyer, and Gorin, 1942), and the surfaces of many cells including nerve and muscle cells are pulled toward a locally applied anodal pipette (Elul, 1967). Binding studies demonstrate cation exchange properties associated with erythrocyte membranes and with other cell membranes. Monovalent and divalent ions including hydrogen, sodium, potassium, calcium, and magnesium ions compete with each other

for a limited number of binding sites on erythrocyte membranes (Caravalho, Sanui, and Pace, 1963; Gent, Trounce, and Walser, 1964). Both the electrophoretic mobilities and the ion-binding properties of these membranes are dependent on the ionic strength and on the pH of the bathing medium.

Negative surface charges have also been invoked to explain certain experimental changes in the voltage-dependent properties of nerve membranes. Two conditions are known in which the squid axon responds as though it were hyperpolarized even though measurements of the membrane potential indicate no change. This apparent hyperpolarization can be achieved experimentally (*a*) by increasing the [Ca] of the external bathing medium or (*b*) by replacing the axoplasm with an internal solution of lower ionic strength. Voltage clamp studies have shown that the primary effect of these treatments is to displace (shift) along the voltage axis the curves that relate the activation or the inactivation of the sodium conductance to the membrane potential (Frankenhaeuser and Hodgkin, 1957; Narahashi, 1963; Moore, Narahashi, and Ulbricht, 1964; Chandler, Hodgkin, and Meves, 1965). It is as though the voltage-sensitive structures that govern the conductance changes become polarized by some local potential gradients that are not measured by the standard techniques for measuring the resting potential of nerves. These gradients may be caused by fixed negative charges at the inner and outer boundaries of the membrane whose degree of neutralization by counterions varies with the external [Ca] and with the internal ionic strength (Frankenhaeuser and Hodgkin, 1957; Chandler et al., 1965). As this paper shows, similar voltage shifts are observed with myelinated fibers.

MATERIALS AND METHODS

Single nodes of Ranvier of large fibers dissected from the sciatic nerve of *Rana pipiens* were studied at low temperatures by the voltage clamp technique of Dodge and Frankenhaeuser (1958). The procedures are discussed in Hille (1967). Basically the data are recorded with the aid of an on-line digital computer and analyzed in terms of the equations of Hodgkin and Huxley (1952).

At almost all times the nerve was clamped at a holding potential between −70 and −90 mv (all potentials are on the absolute or "E" scale of inside potential minus outside). A conditioning *prepulse* lasting 40 msec was followed immediately by the depolarizing *test pulse*. Except where otherwise noted, the prepulse was a 45 mv hyperpolarization from the holding potential.

Except for the definition of the sodium conductance, g_{Na}, all the definitions of the clamp parameters are those used by Hodgkin and Huxley. In this paper the sodium conductance is defined as the product of the sodium chord conductance and a second factor:

$$g_{Na} = \frac{I_{Na}}{E - E_{Na}} \times \frac{183}{183 - E}$$

where E is the membrane potential in millivolts. The second factor in this expression varies linearly from a value of 0.75 to 1.6 in the voltage range from -75 to $+75$ mv and serves to compensate for the instantaneous rectification of the sodium system found for amphibian nerves described by Dodge and Frankenhaeuser (1958, 1959). The sodium conductance, as defined above, and the sodium permeability P_{Na}, as defined by Dodge and Frankenhaeuser, vary with voltage in the same way; i.e., the peak g_{Na} and the peak P_{Na} both approach steady limiting values as the voltage of the test pulse is increased to large values.

The legends of Figs. 3 and 4 refer to a theoretical "standard" node. See the previous paper (Hille, 1968) for references to this mathematical model.

The standard Ringer solution had the following composition (mM): NaCl 110, KCl 2.5, $CaCl_2$ 1.8 or 2.0, Tris(hydroxymethyl)aminomethane buffer (pH 7.3) 5.0. Solutions containing different amounts of divalent ions were made by substituting the stated concentration of $NiCl_2$ or $CaCl_2$ for the $CaCl_2$ of the standard Ringer solution without changing the concentration of the other salts. Solutions with different values of pH contained instead of the Tris buffer a mixture of 7 mM glycylglycine (Mann

TABLE I

VOLTAGE SHIFTS WITH E-FOLD CHANGES OF CATIONS

Test cation	Shifts					
	m_∞	τ_m	h_∞	τ_h	n_∞	τ_n
	mv	mv	mv	mv	mv	mv
Calcium (0.45–22 mM)	8.7	8.4	6.5	8.4	1.6	0.0
Hydrogen (pH 4.1–5.5)	13.5	—	13.5	—	—	13.5
Hydrogen (pH 5.5–10.1)	1.3	—	1.3	—	—	4.0

Research Labs Inc., N. Y.) and 7 mM piperazine dihydrochloride (K and K Laboratories Inc., Plainview, N. Y.) titrated with NaOH. In a few experiments a 5 mM phthalic acid buffer and a 5 mM phosphate buffer were tried.

RESULTS

In this paper the voltage dependence of many parameters in the Hodgkin-Huxley model is examined as a function of the concentration of divalent ions or of hydrogen ions. In general the changes can be described as a simple displacement or shift along the voltage axis of the relation between the measured parameter and the voltage. The results of this study are, then, primarily a list of numbers, the voltage shifts for various changes of the bathing medium. Most of these numbers are summarized in Table I as the millivolts of shift of the voltage dependence of a parameter produced by an e-fold increase of the calcium ion concentration or of the hydrogen ion concentration. The rest of the Results section gives the measurements of such shifts in detail.

Divalent Ions Shift the Voltage-Dependent Properties of Sodium Channels

Fig. 1 is a semilogarithmic plot of the measured values of the peak sodium conductance at various voltages for a node bathed in 0.45, 1.8, and 22 mM Ca. The solid line for 1.8 mM Ca (open symbols) is a smooth curve drawn to fit the measurements. This same curve is displaced 24 mv to the right for 22 mM Ca and 11 mv to the left for 0.45 mM Ca. For convenience I shall call such changes *shifts* or *voltage shifts* of +24 mv and of −11 mv. Because the voltage dependence of the peak sodium conductance in this case reflects the voltage dependence of the parameter m_∞, I shall call these shifts the shifts of m_∞. This simple identification with m_∞ is possible because, as is shown below, the other parameters that might influence the peak sodium conductance,

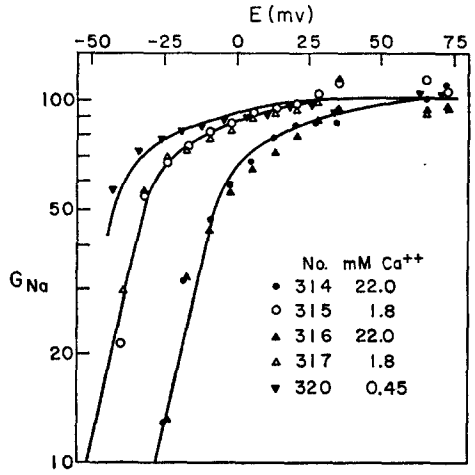

FIGURE 1. Peak sodium conductance and calcium. The peak sodium conductance as a function of voltage for a node in various [Ca]. All points are plotted on the same absolute conductance scale with 100 units corresponding approximately to 400 nmho. The value of $\bar{g}_{Na}$ for this node is then about twice as large or 800 nmho. T = 2.5°C.

τ_m, τ_h, and h_∞, are shifted by nearly the same amount as m_∞. In agreement with all previous investigations of this problem, I find that the higher the [Ca], the greater is the depolarization needed to elicit a given increase of the sodium conductance.

In the experiment of Fig. 1 the points for high and low [Ca] are fit by purely horizontal displacements of the curve, and, therefore, the maximum sodium conductance, $\bar{g}_{Na}$, is constant despite the changes in the [Ca]. However, in one node of five tested with high calcium, $\bar{g}_{Na}$ was reduced by 35% in high [Ca] and in another node $\bar{g}_{Na}$ was reduced by 30% in 20 mM Ni. Before the voltage shift can be measured in these cases, the vertical scale for each set of measurements must first be normalized so that the final amplitude at the highest depolarizations is the same in all solutions. Then the voltage shift can be measured as before.

Although the successive changes of the solutions follow at 5 min intervals in these experiments, the measurements are found to reflect the full effect of the applied concentration of divalent ions. Indeed, the effect is nearly fully established within 5 sec of the application of the new solution and does not change appreciably if monitored for an additional 30 min. Thus the reproducibility of duplicate measurements in Fig. 1 is good.

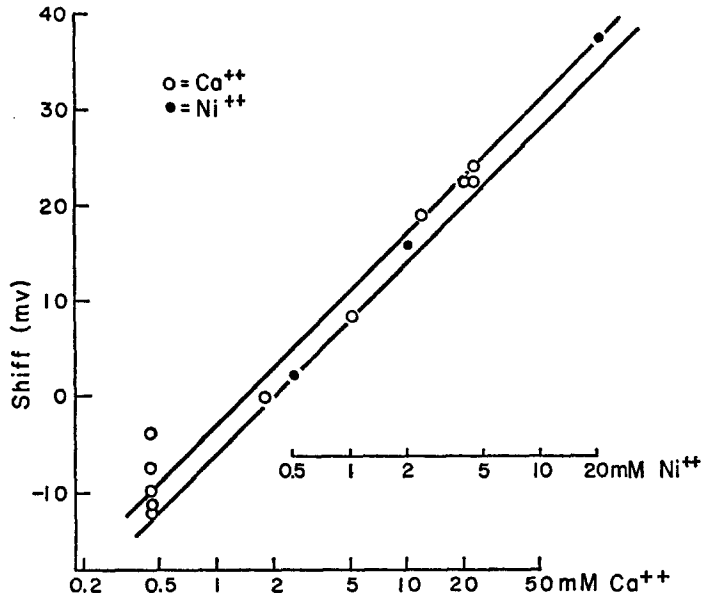

FIGURE 2. Voltage shifts and divalent ion concentration. The shifts of the peak sodium conductance relation along the voltage axis in solutions with various [Ca] (open circles) and with various [Ni] (filled circles). Notice that the logarithmic scale of [Ni] has been displaced relative to the calcium scale by a factor of five in concentration. The zero point of the voltage shift axis has been chosen to correspond with the [Ca] in the standard Ringer solution. The two straight lines have a slope of 8.7 mv per e-fold change of divalent ion concentration and a separation of 3 mv, the estimated uncertainty. The data are taken from seven different nodes. T = 2.5° to 11.5°C.

The collected measurements of the voltage shifts of m_∞ in various [Ca] and [Ni] are plotted against the logarithm of the divalent ion concentration in Fig. 2. The shifts are always referred to measurements in 1.8 or 2 mM Ca. Notice that the logarithmic scale of [Ni] has been displaced relative to the scale of [Ca], so that 1 mM Ni is equivalent to 5 mM Ca. The two lines have a slope of 20 mv per 10-fold change in divalent ion concentration or 8.7 mv per e-fold change and a separation of 3 mv, the estimated uncertainty of the measurement. The points seem to satisfy a semilogarithmic relationship over a 50 mv range of voltage shift. The possible meaning of such a relationship

will be discussed later. The measurements in Fig. 2 are taken over a 9°C range of temperatures. In my experience variations of temperature from 1° to 19°C seem to have little influence on the voltage dependence of m_∞, of h_∞, and of n_∞ in normal Ringer's solution.

The concentration of divalent ions also influences the voltage dependence of the parameter, h_∞. The steady-state sodium inactivation is easily measured in experiments in which the voltage of the prepulse is varied and the voltage of the test pulse is held constant. The amplitude of the transient sodium conductance increase elicited by the test pulse is proportional to the value of h_∞ at the voltage of the prepulse. Again divalent ions produce a shift which can be measured as a displacement of the curve of h_∞ along the voltage axis relative to a control in the standard Ringer solution. The few measured shifts of h_∞ were 11.2 and 14.5 mv in 20 mM Ca, 12.0 mv in 2 mM Ni, and 26.3 and 27.0 mv in 20 mM Ni. All these shifts are positive; i.e., in the direction of greater depolarization. When these points are plotted on a semilogarithmic plot like that of Fig. 2 with the scale of [Ni] displaced as before, they fall near a line of slope 15 mv per 10-fold change or 6.5 mv per *e*-fold change of divalent ion concentration. Thus the shifts of h_∞ are about 25% smaller than the shifts of m_∞ in the same solutions. More points should be measured before this can be considered to be a complete description, however.

The time course of the sodium current at a given voltage becomes slower as the [Ca] is raised. I have not analyzed these changes in detail. It is clear from a few experiments, however, that the prolongation arises from voltage shifts of the parameters, τ_m and τ_h, in parallel with the shifts of m_∞ and h_∞. The left side of Fig. 3 shows an example of a 20 mv shift of τ_h for an 11-fold increase in the [Ca]. The dashed curve is the same as the solid curve but displaced to the right by 20 mv. The shift of τ_m in this case (not illustrated) was also 20 mv.

Nickel and calcium have different effects on the rate constants τ_m and τ_h. Whereas the voltage dependence of m_∞ is only slightly shifted when 0.5 mM Ni replaces 2 mM Ca, the time course of the sodium current is much slower in the nickel solution. I concur with Dodge's (1961) conclusion that in addition to shifting the voltage dependence, nickel lengthens the time constants, τ_m and τ_h, by a factor of approximately two, as though the node had been cooled by 5° to 10°C. Thus these effects of 0.5 mM Ni cannot be imitated by a suitable choice of [Ca] unless the temperature is also lowered. These phenomena are not examined in this paper.

Potassium Parameters Are Shifted Less Than Sodium Parameters by Divalent Ion Changes

The steady-state value of n can be studied in experiments with prepulses of various voltages followed by a test pulse at a voltage near E_{Na} (Dodge, 1963).

The kinetics of the potassium current during the test pulse are a convenient index of the value of n achieved during the prepulse. The right side of Fig. 4 shows the potassium currents seen in such an experiment. As the voltage of the test pulse is a few millivolts above E_{Na} in this case, there are brief (lasting 1 msec) outward sodium currents in a few of the records. These small sodium currents do not interfere with the calculation of n_∞. The actual value of n_∞ can be computed readily by a comparison of the observations with a set of theoretical curves that are calculated for different values of n at the beginning of the test pulse. The center of Fig. 4 shows the results with calcium. There

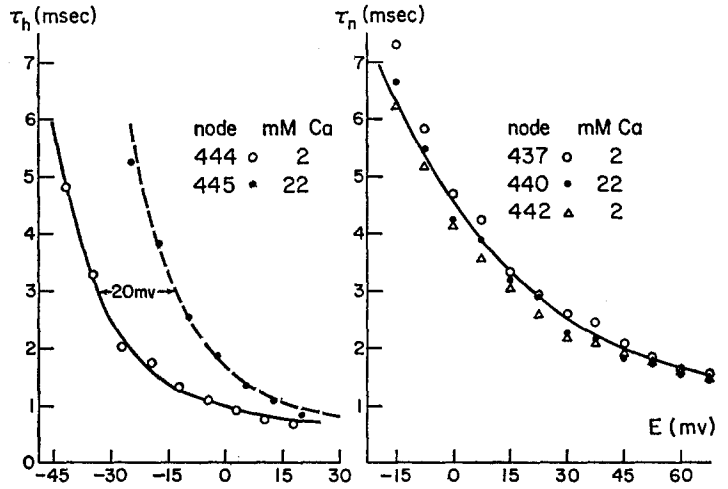

FIGURE 3. Calcium and the time constants. The time constants τ_h (left) and τ_n (right) of a node in normal and in high [Ca] solutions. The dashed line drawn for τ_h of record 445 is the same as the solid line for record 444 but displaced 20 mv along the voltage axis. The solid lines in both graphs are taken directly from the theoretical standard node assuming a threefold increase in the time constants at 11°C compared with the standard 22°C. The solutions bathing the node in records 444 and 445 contain 6 mM tetraethylammonium chloride. Experiments with this node are shown in Figs. 4 and 7 of Hille (1967). T = 11°C.

is only a small (3.5 mv) shift of n_∞ in 20 mM Ca. Similarly, high [Ca] has no effect on the voltage dependence of τ_n (Fig. 3, right) although, in the same node, it does affect τ_h considerably (Fig. 3, left). I have found no changes in τ_n on going from 2–20 or 22 mM Ca and back in five different observations with three different nodes, and no changes on going from 2–0.45 mM Ca in five observations with four nodes. Thus the potassium channel is insensitive to the [Ca] over a 50-fold range of concentration.

Substitution of nickel for calcium *does* affect the potassium channels. The left side of Fig. 4 shows a clear 20 mv voltage shift (in the positive direction) of n_∞ in 20 mM Ni. The time constants τ_n (not illustrated) are also shifted by

about 13 mv in the same experiment. I have not measured the shift of n_∞ in other nickel concentrations. The shift of τ_n in 2 mM Ni is about 8 mv.

All the measurements on the potassium currents indicate that the potassium channels are less sensitive to the divalent ion concentration than the sodium channels. The shifts of all the voltage-dependent parameters in the Hodgkin-Huxley model are summarized in Table I.

Changes in the pH Have a Dual Effect on the Node

The two major effects of changes in pH can be seen in Fig. 5. The sodium currents are greatly attenuated at low values of pH (record 716), and all

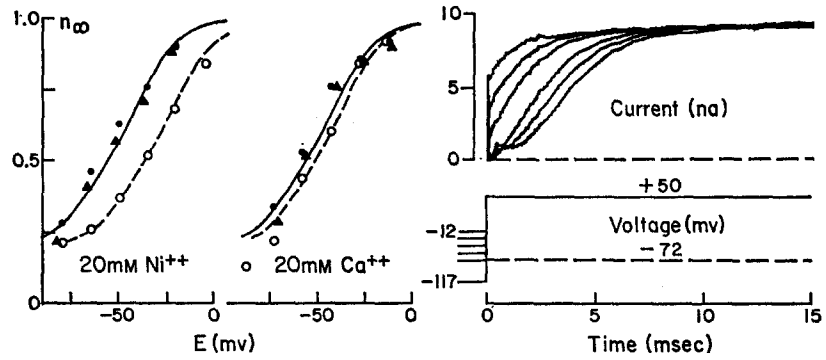

FIGURE 4. Divalent ions and n_∞. The voltage dependence of n_∞ in two nodes treated with Ca and Ni. The open circles are measurements in 20 mM Ca and 20 mM Ni, as labeled, and the filled circles and triangles are measurements in the control Ringer solution (2 mM Ca) from before and after the exposure to the test solution. The inset to the right shows the currents, after subtraction of the capacity current and the leak, that were used to obtain the filled triangles of the second graph. The solid curves on the graphs are taken from the theoretical standard node while the dashed curves are the same lines shifted by 20 mv in the case of 20 mM Ni and by 3.5 mv for 20 mM Ca. Node records 740–742 and 747–751. T = 11.5°C.

voltage-dependent parameters of the node are shifted to more positive voltages by any decrease in pH. Thus, for example, the lower the pH the slower is the rise of the potassium currents and the greater is the depolarization needed to elicit a given increase in the sodium conductance. The effects are independent of whether the buffer solution is made from the mixture of glycylglycine and piperazine or from phosphate or from phthalic acid. Over a range of 6 pH units from 4.1–10.1 there are no irreversible changes in the voltage clamp properties. Indeed the node responds more rapidly to pH changes than to the application and removal of any other agent I have used, perhaps because protonations and deprotonations are very fast reactions and because the hydrogen ion concentration in each solution is strongly buffered.

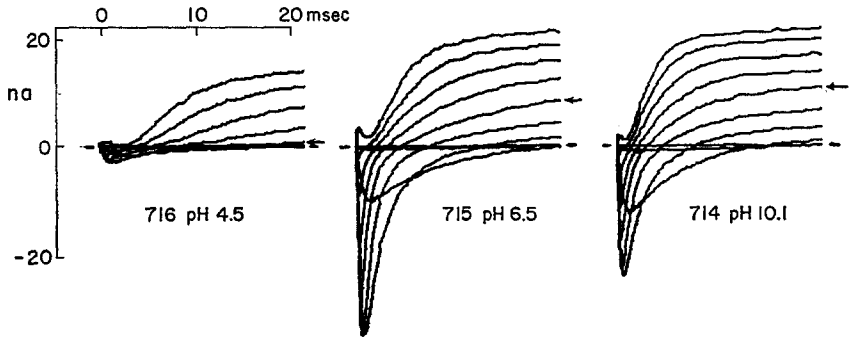

FIGURE 5. The excitability mechanisms and pH. The voltage clamp currents of a node, minus capacity current and leak, in solutions buffered to different values of pH. The voltages for the 10 curves in each family are spaced at 15 mv intervals from −65 to +70 mv. The arrow to the right of each family of curves indicates the current record for −5 mv. T = 11°C.

The collected measurements of the pH dependence of $\bar{g}_{Na}$ (always referred to the value at pH 7.3) are indicated by open circles in Fig. 6. The smooth curve drawn through these points has the shape of the theoretical dissociation curve of a weak acid with a pK_a of 5.2. These observations suggest that the permeability of individual sodium channels is blocked when an acidic group

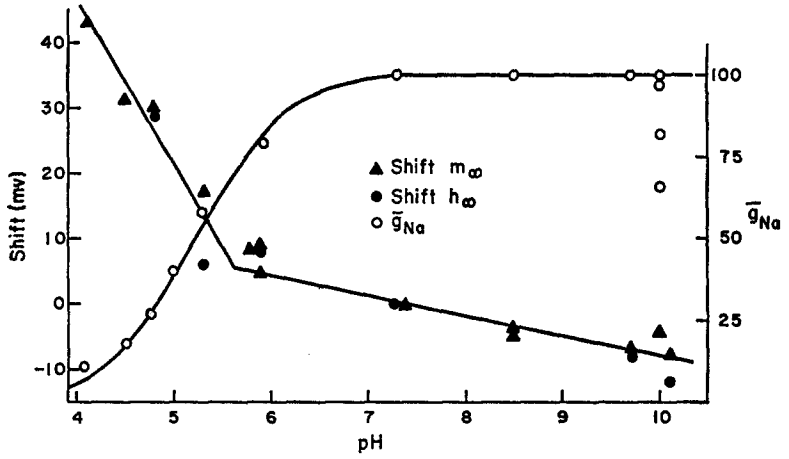

FIGURE 6. The changes of the sodium channels with changing pH. The shifts of the voltage dependence of the parameters, m_∞ and h_∞, and the relative amplitudes of the sodium conductance, $\bar{g}_{Na}$, are plotted as a function of the pH of the medium. All measurements are with the glycylglycine-piperazine buffer. The smooth curve drawn for $\bar{g}_{Na}$ has the shape of the dissociation curve of a weak acid with a pK_a of 5.2. The solid line drawn for the voltage shifts have slopes of 2.6 mv per e-fold change of hydrogen ion concentration above pH 5.5 and of 13.5 mv below pH 5.5. The data are taken from six different nodes. T = 6.5° to 9°C.

associated with them becomes protonated. Another reason for thinking that a component of the sodium channels is specifically affected by low pH is that the sodium conductance is usually enhanced after a treatment at low pH. Frequently $\bar{g}_{Na}$ stays at 120% of its original value for many minutes following an exposure to pH 4.5. This effect is like the restorative action of low concentrations of saxitoxin reported in the preceding paper (Hille, 1968). Possibly a macromolecular component of some of the sodium channels "denatures" in the course of normal experimental manipulations thus reducing the sodium conductance. The presence of an acid solution or the binding of saxitoxin may favor "renaturation" of this component.

Fig. 6 also shows the measurements of the shifts of m_∞ (filled triangles) and of the shifts of h_∞ (filled circles). Two features apparent from the graph are the nearly equal shifts of the two parameters and the marked increase of sensitivity of both parameters to pH changes below pH 5.5. Above pH 5.5 the solid line has a slope of 3.0 mv per 10-fold change of hydrogen ion concentration and below pH 5.5, a slope of 31 mv per 10-fold change. These slopes correspond to 1.3 and 13.5 mv per e-fold change. The shifts produced by increasing the concentration of the hydrogen ion and those produced by increasing the concentration of divalent ions are in the same direction.

pH Changes Shift the Potassium Parameters More Than the Sodium Parameters

Fig. 5 clearly shows the shortening at high pH of the time constant, τ_n, associated with the activation of the potassium channels. The changes at pH 10.1 correspond to shifts of τ_n of -24 mv, considerably larger than the -4 and -12 mv shifts of the parameters, m_∞ and h_∞, of the sodium channels. In other nodes the shifts of τ_n ranged between -22 and -26 mv at pH 10.1.

In acidic solutions the shifts of τ_n and the shifts of m_∞ and of h_∞ are nearly equal. In one node studied at pH 4.8, 4.5, and 4.1, the shifts of τ_n were 27, 30, and 41.5 mv and the shifts of m_∞ were 30, 31.5, and 42 mv. The results with several other nodes were very similar.

Unfortunately in none of the experiments was the parameter, n_∞, studied by the direct method used with divalent ions, nor was the test pulse made long enough to obtain the steady-state values of the potassium conductance at the smaller depolarizations. The current-voltage relations derived from my observations are consistent with the supposition that the shifts of n_∞ are similar to the shifts of τ_n, but direct evidence is lacking. The shifts of some of the voltage-dependent parameters in the Hodgkin-Huxley model are summarized in Table I.

The Hydrogen Ion Does Not Carry Significant Membrane Current

In the range of pH studied there was no evidence of a shift of the equilibrium potentials, E_{Na} and E_K, such as would occur if the sodium or potassium

channels were very permeable to the hydrogen ion. Of course, even in Ringer's solution at pH 4, the hydrogen ion concentration is only 0.1% of the sodium ion concentration, so the hydrogen ion permeability, P_H, of the nodal membrane might still be equal to the sodium ion permeability, P_{Na}. However, it seems certain that at pH 7 the contribution of the hydrogen ion to the early or late currents is insignificant.

Similarly the leakage current is not carried by hydrogen ions (or by hydroxyl ions). If the leakage conductance at pH 7.3 is called 100, the mean conductance in five measurements at pH 4.1–4.8 is 95 ± 7 (mean ± SD), and in five measurements at pH 9.7–10.1 it is 106 ± 7.

DISCUSSION

This paper has concentrated on the voltage shifts that occur with changes in the [Ca] or with changes in the pH. The measurements show that divalent ions and hydrogen ions control the voltage dependence of the sodium and potassium channels to different degrees. The sodium channels are sensitive to the [Ca] and the potassium channels are not. Above pH 5.5 the sodium channels are less sensitive than potassium channels to changes in the pH, whereas below pH 5.5 the shifts of the sodium and potassium parameters are equal.

The order of magnitude and the sign of the shifts that I have measured are similar to those found by others with other axons, although the relative sensitivities of the sodium and potassium channels to changes of the [Ca] seem to differ. Using the squid giant axon, Frankenhaeuser and Hodgkin (1957) have reported the following shifts for an *e*-fold increase of the [Ca]: m_∞, 9.4 mv; h_∞, 4.0 mv; and n_∞, 8.9 mv. The difference between these numbers and those in Table I for the myelinated nerve fiber is probably an example of the small differences among axons of different origin. With the lobster giant axon shifts of m_∞ of 6.3 mv and of 8.0 mv per *e*-fold change of the [Ca] have been reported (Julian, Moore, and Goldman, 1962; Blaustein and Goldman, 1966). There are no published voltage clamp studies on the shifts produced by pH changes, although a similarity between the effects of low pH and of high [Ca] has been noted before (see Shanes, 1958).

Surface Charges on Cell Membranes

As Huxley proposed (cited by Frankenhaeuser and Hodgkin, 1957), voltage shifts would be obtained if the outer boundary of the axon membrane bore negative fixed charges that could bind divalent cations to an extent that varied with the concentration of the cation. These fixed charges would produce a surface potential. Then even when the transmembrane potential is held constant, the potential profile within the membrane would reflect the divalent ion concentration, because of the changes of the surface potential.

As more divalent ions bind to the outer surface, the surface potential would become more positive and the electric field within the membrane would change in the same direction as it would when the membrane is hyperpolarized. The following paragraphs consider some of the evidence for and various consequences of this hypothesis.

If there are negatively charged groups on the nerve surface, they must be ionized, acidic groups that can be neutralized as the pH is lowered. The neutralization of the acids, like the binding of the divalent ions, would change the surface potential so that the nerve would respond as if it were hyperpolarized. A few earlier studies (see Shanes, 1958) and my observations show that low pH does cause a positive shift of the voltage-dependent responses of the nerve, as expected in the theory.

As noted in the Introduction, fixed negative charges are found on some cell membranes. Abramson et al. (1942) estimated from electrophoretic studies that in a physiological solution the net surface charge density of the erythrocyte of man is 7×10^{12} cm^{-2} elementary negative charges. The surface charge reverses sign at pH 1.7. A calculation from the binding studies of Gent et al. (1964) on erythrocytes suggests that there are a maximum of 57×10^{12} cm^{-2} binding sites for calcium ions at zero ionic strength and that in physiological conditions there are about 2×10^{12} cm^{-2} sites. In making this calculation I used 1.5×10^{12} g as the dry weight of an erythrocyte ghost (Dodge, Mitchell, and Hanahan, 1963).

Chandler et al. (1965) proposed that the shifts produced by lowering the ionic strength of the internal medium of the squid giant axon could be explained by a negative surface charge. In this case the charge would be at the inner boundary of the membrane, and the shifts would be due to the variation of the surface potential with the ionic strength. According to formula 8.0 of Chandler et al. the observations on the shifts of the sodium parameters in the squid axon could be explained by assuming a surface charge density of 14×10^{12} cm^{-2}. Because the shifts of the potassium parameters were much smaller than those of the sodium parameters in their experiments, Chandler et al. suggested that the density of charge near the sodium channel is greater than that near the potassium channel.

The Node of Ranvier

Can a fixed charge theory account for my observations with the node of Ranvier? Four main facts must be explained: (*a*) shifts that span a range of at least 65 mv (from −24 mv to +41 mv in the case of pH and the potassium parameters), (*b*) a marked difference between the sensitivities of the sodium and potassium channels to [Ca] changes, (*c*) a greater sensitivity of both channels to pH changes below pH 5.5, and (*d*) a semilogarithmic relationship between the [Ca] or the [H] and the magnitude of the shift (see Fig. 2).

Formula 8.0 of Chandler et al. (1965) shows that a negative surface charge density of 35×10^{12} cm^{-2} would produce a -70 mv surface potential in a medium with the ionic strength of Ringer's solution. If all these charges are neutralized, the surface potential would fall to zero. This change of surface potential is large enough to explain all the shifts that I have observed. The hypothetical charge density is also of the same order of magnitude as those that have been suggested for other cell membranes.

It can readily be shown that the charges need not be distributed at this density over the entire membrane surface. If they were, there would be 10^7 charges on a node of 30 μ^2 area. This is about 2000 times as great as the estimated number of sodium channels on a node (Hille, 1968). As the estimated distance between individual sodium channels is 800 A, only a small fraction of the possible 2000 charges could be near enough to the channel to contribute to the electrostatic forces at the channel. Therefore much fewer than 2000 charged groups located in the vicinity of the sodium and potassium channels could account for the observations.

As suggested by Chandler et al. for the charges on the inner boundary of the squid axon, the charged groups near the sodium and potassium channels may be quite different. The differing chemical properties of these groups might account for the difference in the relative sensitivities of the sodium and potassium channels to pH changes and to divalent ion concentration changes. Divalent ions would be bound most firmly by multiply ionized acidic groups or by paired carboxylic acid groups. They would bind only weakly to monoacidic groups and not at all to basic groups. The differences between the effects of various divalent ions (e.g., Ca and Ni) may reflect the differences in their binding. Basic groups (e.g., amino groups) protonate above pH 7. Weakly acidic groups protonate at lower values of pH, and stronger acidic groups do not protonate at all in the range of pH studied. It is apparent that combinations of these ionizing groups can be chosen that would account for the observed pattern of shifts. The differences between the squid axon and the frog node in this pattern may simply be due to a difference in the number and nature of the charged groups in the vicinity of the ionic channels in the two cases.

The picture of the surface potentials developed here involves both the ions bound specifically to fixed surface charges and a diffuse double layer of ions in the transition region between the surface and the bulk solution. The theory of the surface potentials in such a situation, developed by Stern, actually predicts an approximately semilogarithmic relationship between the concentration of the ion that binds specifically and the surface potential (see formula 2.45 in Davies and Rideal, 1963). The Stern theory might be an adequate beginning for the theory of the shifts produced by changes in the pH and in the divalent ion concentration.

If, indeed, Huxley's suggestion of negative surface charges that bind cations is to be accepted, further experiments should be done. The effects of changes of the ionic strength of the external solution should be studied, and the results should be similar to those with changes in the internal ionic strength. Chemical modifications of the charged groups on the membrane should be attempted. This kind of experiment with neuraminidase has led to a clear identification of sialic acid as the major contributor to the negative charges on the erythrocyte membrane (Seaman and Uhlenbruck, 1963). Voltage clamp investigations of the interactions between the effects of pH changes and [Ca] on nerves could also be useful.

The Reduction of $\bar{g}_{Na}$ at Low pH

Low pH values lead to two changes in the responses of the nerve: a positive shift of all parameters and a reduction of the sodium conductance. There is not enough evidence to decide whether both changes have the same cause, or whether, instead, they have independent causes. The evidence for a single cause is that the changes become apparent at about the same value of pH (between pH 6 and 5) and that the reduction of $\bar{g}_{Na}$ could be attributed directly to the reduction of the surface potential by the following type of theory. A negative surface potential would tend to dilute the anions and to concentrate the cations in the region of the diffuse double layer. Thus if the surface potential is reduced, the [Na] near the sodium channel, and hence $\bar{g}_{Na}$, might also be reduced. This electrostatic argument clearly is not consistent with the lack of effect of the [Ca] on $\bar{g}_{Na}$ and therefore may also not apply to the effects of pH. With frog skeletal muscle, Hutter and Warner (1967) have shown that the anion conductance of frog skeletal muscle is reversibly abolished by low values of pH. They noted that this change was in the opposite direction from that expected with the above electrostatic argument, for protonation of the muscle surface should increase the local anion concentration and thus the anion conductance. For these reasons, I suggest that the shifts in low pH may be produced by the general decrease of negative charge on the nerve surface, whereas the reduction of $\bar{g}_{Na}$ is caused more specifically by the protonation of some acidic group that must be ionized for the sodium channel to function normally. Conceivably this acidic group is part of the receptor(s) for tetrodotoxin, for saxitoxin, and for local anesthetics, anesthetic substances which seem to block sodium channels in their cationic forms (see Camougis, Takman, and Tasse, 1967; Ritchie and Greengard, 1966; and Hille, 1968).

In the preceding paper (Hille, 1968) and in a paper by Camougis et al. (1967) it was observed that tetrodotoxin and saxitoxin are much less active anesthetics at high pH than at neutral pH, and it was suggested that the activity was lost at high pH because a basic group on the toxin molecules

became neutralized. This paper shows that raising the pH has almost no effect on the parameters of the sodium channels (although lowering the pH does) and strengthens the conclusion that it is the toxins that change significantly at high pH and not the channels.

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