

Ionic Conductance Changes in Voltage Clamped Crayfish Axons at Low pH

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ABSTRACT Giant axons from the crayfish have been voltage clamped with an axial wire system. General characteristics of observed ionic currents under normal conditions are similar to those measured in other giant axons and in nodes of Ranvier. As the pH of the external bath is lowered below 7, a marked, reversible slowing of potassium currents is seen with little effect on sodium currents. The steady-state potassium conductance-voltage curve is shifted along the voltage axis in a manner consistent with the development of a hyperpolarizing surface charge. Results suggest that this potential shift accounts for part, though not all, of the observed increase in τ_n . From the behavior of the kinetics of the delayed current with external pH these alterations in potassium conductance are attributed to the titration of a histidine imidazole residue of a membrane protein. Chemical modification of histidine by carbethoxylation at pH 6 slows and strongly depresses potassium currents. The results suggest that in addition to the introduction of electrostatic forces, possibly resulting from a hyperpolarizing surface charge, protonation of a histidine group at low pH also alters the nonelectrostatic chemical interactions determining the ease with which potassium gates open and close. The evidence indicates that the modified histidine residue is closely associated with the membrane components involved in the control of potassium conductance.

INTRODUCTION

While much is known about the changes in ionic conductance responsible for excitation, relatively few details of the biochemical components involved in their control have been elucidated. Approaches that have been useful include chemical modification by enzymes (Tobias, 1955; Tasaki and Takenaka, 1964; Rojas, 1965; Abbott et al., 1972; Armstrong et al., 1973), by group-specific or cross-linking compounds (Smith, 1958; Huneus-Cox et al., 1966; Cooke et al., 1968; Shrager et al., 1969 *a, b*) and by variation of hydrogen ion and divalent cation concentrations (Frankenhaeuser and Hodgkin, 1957; Hille, 1968; Gilbert and Ehrenstein 1969; Woodhull, 1973). Several of these studies have suggested that membrane proteins may be involved in

gating ionic channels. As an example, Armstrong et al., (1973) have recently shown that Pronase, a proteolytic enzyme, applied internally in perfused squid axons, eliminates sodium inactivation. External application is without effect. Shrager et al., (1969 *a*) found that when the protein cross-linking agent tannic acid is perfused internally in crayfish axons, action potentials develop a long plateau. The membrane conductance remains high during the plateau and it is therefore likely that sodium inactivation has been inhibited. External application had no effect. Results with two rather different reagents, on different preparations, both therefore lead to identical conclusions, that the integrity of a membrane protein at the inner surface of the axon is requisite for sodium inactivation.

The present study is largely concerned with the identity of a biochemical group that is shown to be closely associated with the gating control of potassium conductance in crayfish axons. Modification of this membrane component by protonation at low pH, or by a group-specific reagent, results in a slowing of potassium current kinetics with relatively little effect on sodium currents. In addition to these results, general properties of voltage-clamped crayfish axons are described, extending the usefulness of this preparation for electrophysiological experiments. A brief preliminary report of this work has appeared (Shrager, 1974).

METHODS

The Axon

The medial giant axon from one side of the ventral nerve cord of the crayfish *Procambarus clarkii* was dissected and mounted in a Plexiglas chamber. The axon was desheathed and cleared of about 95% of adjoining fibers except in the neighborhood of a ganglion, where a small tuft of material remained adhering to the fiber. The other side of the cord was left intact to provide mechanical support. Counting the brain as ganglion 1, the axon was desheathed between ganglia 2 and 7, and was cleared between ganglia 3 and 6. Axons used in these experiments averaged about 200 μm in diameter and ranged from 160 to 250 μm .

The voltage clamp electrode was of the "piggy-back" type (Chandler and Meves, 1965) and consisted of an 18- μm diameter platinized platinum current wire (25–30 μm after platinizing) cemented to a glass capillary with Insl-X lacquer. The capillary, used to measure potential, was drawn from thin-wall hematocrit tubing. The tip was fire polished and had an OD of 40–60 μm . The capillary was filled with a solution composed of: KCl, 172 mM, K_2 citrate, 37 mM, citric acid to pH 7.5, and contained an electrically floating (unattached) platinized platinum wire to reduce the AC impedance (Fishman, 1973). The electrode was fastened to a Leitz instrument holder (E. Leitz, Inc., Rockleigh, N. J.) which, in turn, was held in a Prior micro-manipulator. This arrangement greatly facilitated aligning the electrode with the axon. The potential reference electrode was of a design similar to that of the capillary electrode, but was somewhat larger and was filled with normal Van Harreveld's

(1936) saline containing 2% agar. External current electrodes were made of platinized platinum.

A schematic diagram of the voltage clamp system is given in Fig. 1. The clamp electrode was inserted through a hole cut near ganglion 3 and was advanced until the tip of the current wire reached ganglion 6. The tip of the capillary was then about mid-way between ganglia 4 and 5. The recorded membrane potential, V_m , reached its plateau value a few millimeters before that point. The current measuring

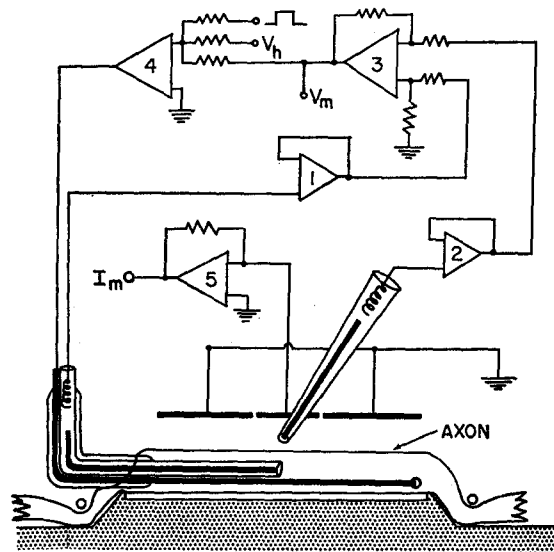


FIGURE 1. Schematic diagram of the voltage clamp system. The exposed portion of the current wire was 12 mm long. The external current electrodes were mounted on a Plexiglas backing and were positioned with a micromanipulator so that the central 2-mm current-measuring regions were centered at the tip of the potential pipette. The external electrodes were positioned within about 300 μm of the axon to minimize longitudinal current flow. Guard electrodes extended 5 mm from each side of the central portion. Control and current-measuring amplifiers (4 and 5) were Teledyne-Philbrick 1011 operational amplifiers (Teledyne-Philbrick, Dedham, Mass.). A small ball of Insul-X lacquer was applied to the tip of the current wire for protection during cannulation.

region was wholly between ganglia 4 and 5, although in early experiments it was found that the inclusion of a ganglion region (with its adhering material) in the central 2 mm did not affect the recorded currents. The electronic feedback system was of standard design and included compensation (not shown in Fig. 1) for 3–4 Ωcm^2 of the measured series resistance (4–6 Ωcm^2).

Solutions and Reagents

The normal external saline (Van Harreveld, 1936) (NVH) contained (mM); NaCl, 205; KCl, 5.4; MgCl_2 , 2.6; CaCl_2 , 13.5; NaHCO_3 , 2.3; pH adjusted to 7.5–7.6 with HCl. In all axons the external solution during dissection was NVH, and was changed to more strongly buffered solutions after the electrode was inserted and

the axon monitored for damage. Solutions used are given in the text. In general, buffers were used at a concentration of 10 mM, substituting for NaHCO₃ and/or NaCl. Diethylpyrocarbonate was obtained from Pfaltz & Bauer, Inc., Flushing, N. Y. Bis Tris (bis(2-Hydroxyethyl) imino-tris (Hydroxy-methyl)methane) was purchased from Sigma Chemical Co., St. Louis, Mo.

Temperature

Unless otherwise noted, all experiments were run at 8.5°C. A Teflon-coated aluminum block formed part of the bottom of the Plexiglas chamber, and was clamped to two thermoelectric cooling devices (Cambion #806-1067-01, Cambridge Thermionic Corp., Cambridge, Mass.). A thermistor placed close to the axon and an electronic feedback system held the temperature to within 0.6°C of the set point.

Analysis and Notation

Potassium conductances are calculated using the formulation of Hodgkin and Huxley (1952), but with an exponent of n equal to 5 rather than 4, as this was found to provide better agreement with the data (see Results). Eq. 6 of Hodgkin and Huxley (1952) then becomes:

$$g_K = \bar{g}_K n^5, \quad (1)$$

where the potassium conductance, g_K , is calculated from the voltage clamp data as

$$g_K = I_K / (V_m - V_K) \quad (2)$$

and where the variation of n with time and V_m is given by

$$\frac{dn}{dt} = \alpha_n (1 - n) - \beta_n n, \quad (3)$$

with the solution

$$n = n_\infty - (n_\infty - n_0) \exp(-t/\tau_n), \quad (4)$$

where

$$n_\infty = \alpha_n / (\alpha_n + \beta_n) \quad (5)$$

and

$$\tau_n = (\alpha_n + \beta_n)^{-1}. \quad (6)$$

g_K may then be expressed as

$$g_K = \{(g_{K\infty})^{1/5} - [(g_{K\infty})^{1/5} - (g_{K0})^{1/5}] \exp(-t/\tau_n)\}^5. \quad (7)$$

RESULTS

Voltage Clamped Crayfish Axons: General Properties

The resting potential of axons cannulated with the combination electrode varied only slightly among different preparations. Measured at 8.5°C in axons

bathed in normal Van Harreveld's (1936) solution (NVH), V_m was -76 ± 0.2 mV ($n = 24$) (uncorrected for liquid junction potentials) with a range of -74 to -78 mV. Voltage clamp currents recorded from an axon in NVH are illustrated in Fig. 2 *a*. Fig. 2 *b* shows $I-V$ curves for peak early current and steady-state current, measured on another nerve in NVH. Results appear similar to those recorded from other axons (Hodgkin et al. (1952); Dodge and Frankenhaeuser, 1958; Binstock and Goldman, 1969).

Tetrodotoxin (TTX) specifically inhibits the transient increase in sodium conductance in axon membranes, while the delayed outward current remains

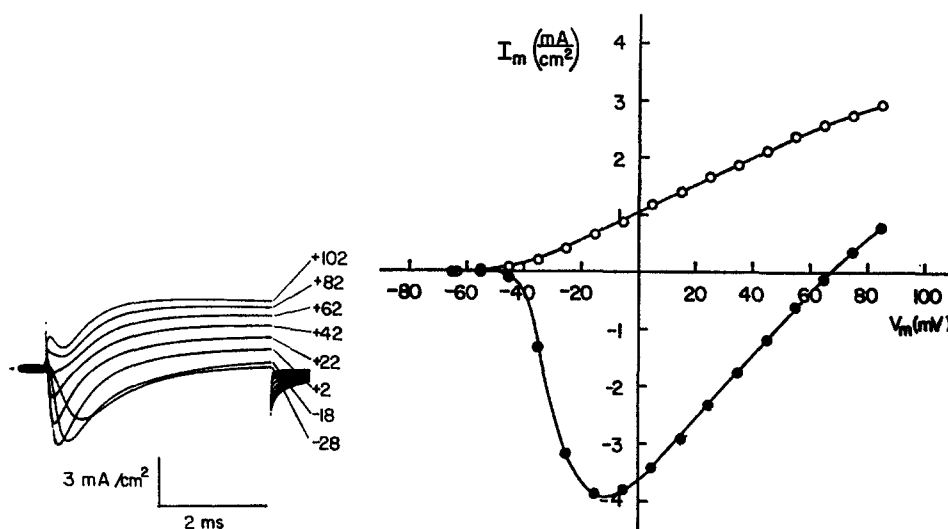


FIGURE 2. Voltage clamp currents from crayfish axons. (Left) Family of currents recorded under voltage clamp during depolarizing steps from the holding potential (-78 mV) to membrane potentials indicated. External solution: NVH. (Right) $I-V$ curves for peak early current (filled circles) and steady-state current (open circles) from another axon in NVH. Holding potential (V_h), -75 mV.

unchanged (Narahashi et al., 1964). The action of TTX on voltage clamp currents in crayfish axons (Fig. 3) is similar to that observed in other preparations. Tetraethylammonium ion (TEA) is known to block potassium currents in squid axons when applied at the internal surface of the membrane (Armstrong and Binstock, 1965). Impedance measurements on internally perfused crayfish axons have shown that TEA added to the perfusate blocks the delayed increase in conductance during the action potential (Shrager et al., 1969 *a*). Wallin (1966) has measured intracellular concentrations of K^+ , Na^+ , and Cl^- , and in axons bathed in NVH, equilibrium potentials calculated at $8.5^\circ C$ are $V_K = -92$ mV, $V_{Na} = +60$ mV, and $V_{Cl} = -63$ mV, subject to possible errors due to unequal activity coefficients between axoplasm and external bath. For the axon of Fig. 2 *b*, the reversal potential for

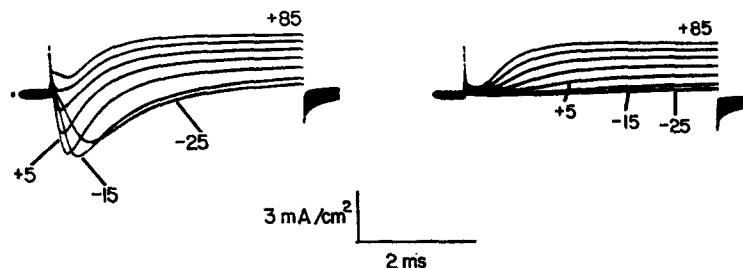


FIGURE 3. Families of voltage clamp currents from an axon before (left) and after (right) addition of 100 nM TTX to the external saline (NVH). Depolarizations from the holding potential (-75 mV) to -25 , -15 , $+5$, $+25$, $+45$, $+65$, $+85$ mV.

the early current was $+67$ mV. The reversal potential for tail currents measured after a short depolarizing prepulse in NVH + 100 nM TTX was -80 mV. Further, the kinetics of the delayed currents measured in solutions containing TTX (see below) were similar to those of other axons (Hodgkin and Huxley, 1952; Hille, 1967). It is therefore likely that in the response of a crayfish axon to a depolarizing pulse, the early transient current is carried predominantly by sodium ions and the delayed outward current largely by potassium ions. Strickholm and Wallin (1967) have demonstrated an increased permeability to Cl^- in axons depolarized by high external $[\text{K}^+]$ and it is therefore possible that some fraction of the steady-state current is carried by Cl^- ions. This point has not as yet been investigated in detail.

Variation of External pH

Voltage clamp currents from an axon at external pH values of 7.5 and 5.8 are compared in Fig. 4. At the lower pH the amplitudes of peak sodium currents and steady-state currents are reduced by approximately 20% and the time to peak sodium current is increased by about 50%. The most marked change is observed in the kinetics of the potassium current. Measured at $+65$ mV (close to V_{Na}) the time required for I_{K} to reach half its final value increased from 0.7 ms at pH 7.5 to 2.6 ms at pH 5.8. The currents recorded at low pH are somewhat similar to voltage clamp records obtained when an instability is present in the feedback loop (Cole and Moore, 1960 *a*). The "notch" however, is more apparent at high depolarizations rather than at potentials in the negative conductance region of the I - V curve. Of greater importance, the apparent shape of the delayed current as a function of time is reproduced after external application of TTX. This is illustrated in Fig. 5, which shows data at pH 5.4. The recorded currents are therefore not the result of clamp instability. Fig. 5 also shows clearly the complete separation in time of sodium and potassium currents that may be achieved at low pH in crayfish axons. The remainder of this paper will be concerned mainly with

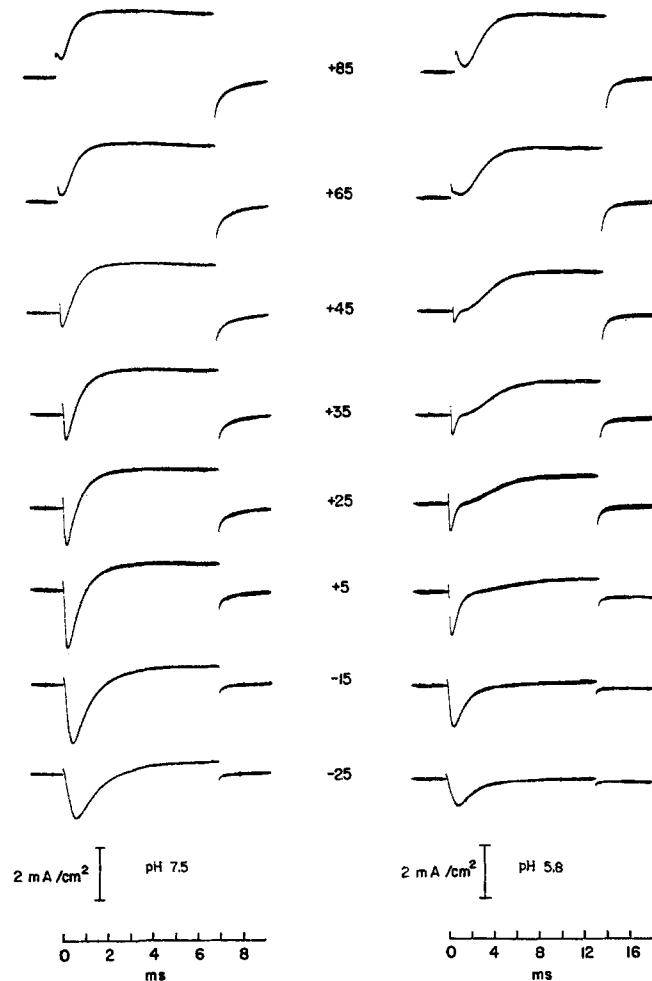


FIGURE 4. Variation of the external pH. (Left) Currents recorded at pH 7.5 during depolarizations from $V_h = -75$ to potentials shown. (Right) Same, pH 5.8. Note difference in time scales. The external solution contained (mM): NaCl, 197.3; KCl, 5.4; $MgCl_2$, 2.6; $CaCl_2$, 13.5; bis-Tris, 10. pH adjusted with HCl.

the alteration of potassium conductance caused by pH changes and by chemical modification.

Changes in potassium currents induced at low pH are almost completely reversible, as shown in Fig. 6. Effects were also independent of the buffer system used. Buffers employed in these studies (generally present at a concentration of 10 mM) included: Tris-HCl, Tris-maleate, maleic acid-Na maleate, bis-Tris-HCl, and phosphate. Results were also similar whether or not HCO_3^- was present in the external solution.

One possible way in which protonation of a membrane chemical group

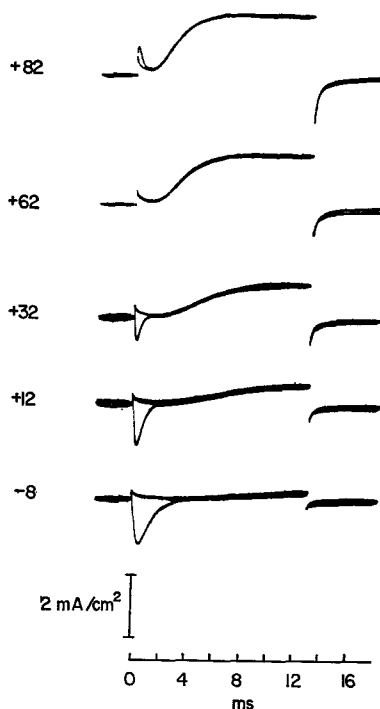


FIGURE 5. Current records before and after addition of 100 nM TTX to the external solution, superimposed photographically. $V_h = -78$ mV. Depolarizations to the levels given. The external solution contained (mM): NaCl, 197.3; KCl, 5.4; MgCl₂, 2.6; CaCl₂, 13.5; NaH₂PO₄-Na₂HPO₄, 10; pH 5.4.

may affect ionic conductances is by alteration of fixed surface charge. Hydrogen ions bound to the external surface of the membrane at low pH might exert electrostatic forces on other membrane charges similar to those that would result from a hyperpolarization of the membrane. This charge due to bound protons, which will be referred to in this paper as a hyperpolarizing surface charge, would therefore alter the effective electric field at more interior positions in the membrane, but would not result in a change in the measured potential difference between cytoplasm and external bath, since the potential due to surface charge decays rapidly with distance at normal ionic strength. This condition would then be similar to that postulated for the action of calcium ions on squid axons by Huxley (Frankenhaeuser and Hodgkin, 1957). Further, this surface charge might induce or alter rectification in ionic channels (Adrian, 1969). Instantaneous $I-V$ curves for potassium at pH 7.5 and 5.8 are given in Fig. 7. Results are shown after both short and long (I_K at its steady-state value) depolarizing prepulses. Capacitative transients were subtracted by applying paired hyper- and depolarizing pulses (Binstock and Goldman, 1971). These curves show rectification, devia-

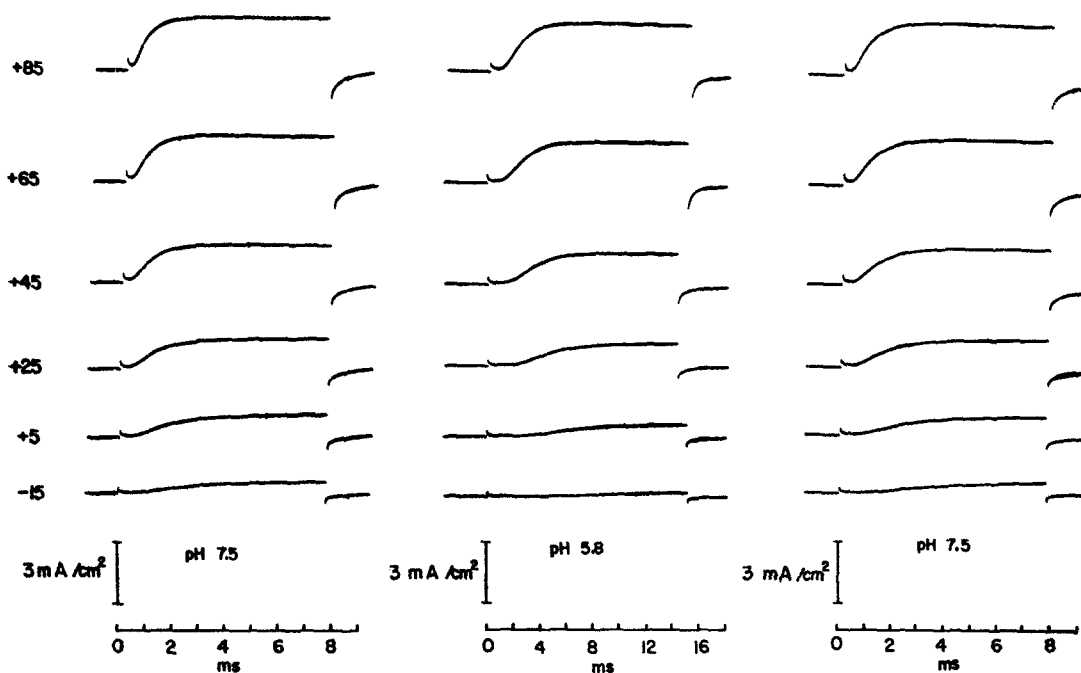


FIGURE 6. Reversibility of effects of low pH on potassium currents. External pH was 7.5 before (left) and after (right) a shift to 5.8 (center). $V_h = -75$ mV. Depolarizations to levels given. External solutions contained: NaCl, 197.3 mM; KCl, 5.4 mM; $MgCl_2$, 2.6 mM; $CaCl_2$, 13.5 mM; bis Tris, 10 mM; TTX, 100 nM; pH adjusted with HCl. Note different time scales.

tions from a straight line appearing mainly at large, negative membrane potentials. The degree of curvature is not very different after lowering the external pH to 5.8, although the additional rectification below -75 mV, visible in Fig. 7 *b*, may reflect a change in membrane fixed charge on protonation. In Fig. 7 *b* (long prepulse) the reversal potential is considerably more positive than the expected V_K of about -90 mV, due most probably to accumulation of potassium in the Schwann cell space (Frankenhaeuser and Hodgkin, 1956). A difference in this accumulation might be responsible for the small difference in reversal potentials between the data at pH 7.5 and 5.8 since the integral of current over time was greater at the lower pH. In the data for a short prepulse (Fig. 7 *a*) where potassium accumulation would be considerably reduced, the reversal potential is found to be approximately -80 mV at both pH 7.5 and pH 5.8. Invariance of the reversal potential with pH would suggest that the selectivity of the channel has not been altered. However, accumulation in the Schwann cell space and difficulties in measuring instantaneous currents after short depolarizations introduce uncertainties and since, in general, small differences were measured, as in Fig. 7 *b*, this point should be investigated further, perhaps at elevated external potassium concentrations.

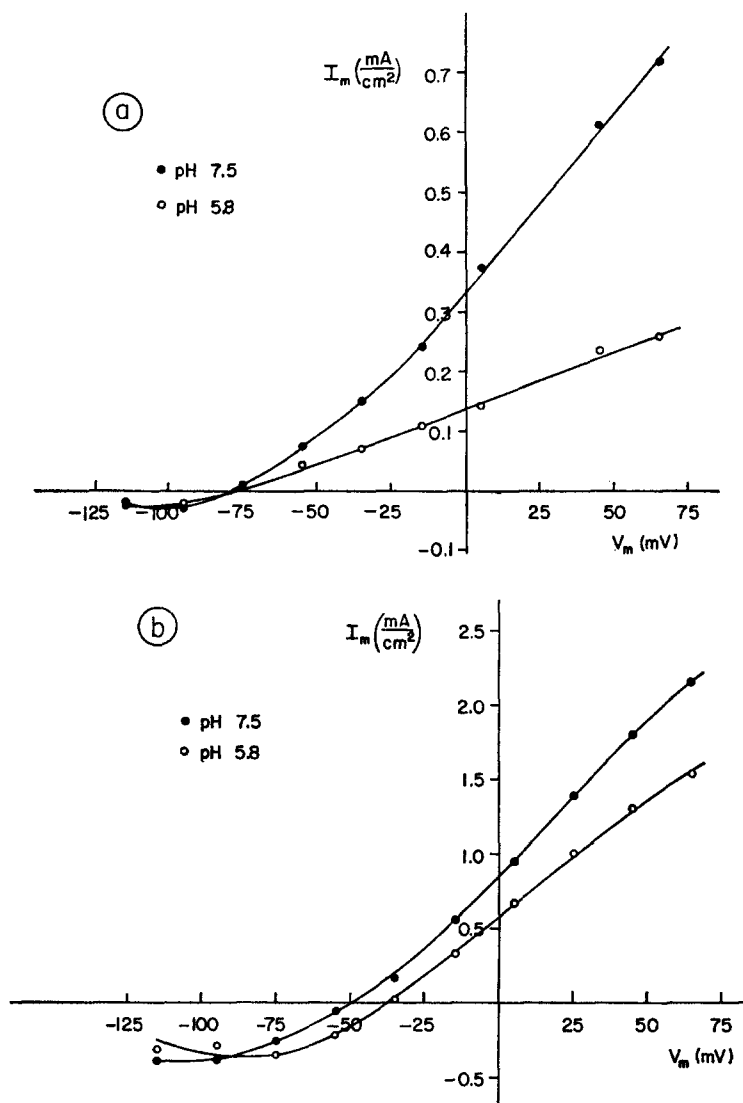


FIGURE 7. Instantaneous I - V relationship in external solutions of Fig. 6. Filled circles, pH 7.5; open circles, pH 5.8. $V_h = -75$ mV. Prepulse to +25 mV. Currents measured 75 μ s after test pulse to potentials plotted on the abscissa. (a) Short prepulse (current reached about 25% of its steady-state value before test pulse). (b) Long prepulse (current at steady-state level before test pulse).

The dependence of steady-state potassium conductance ($g_{K\infty}$) on membrane potential as a function of external pH is illustrated in Fig. 8. $g_{K\infty}$ has been calculated from Eq. 2 as the conductance corresponding to the steady-state value of I_K . Depolarizing pulses up to 40 ms in duration were used to allow I_K to reach its maximum value. The conductance-voltage curve is shifted approximately 25 mV to the right by the reduction in pH from 7.5

to 5.8. The voltage shift in $g_{K\infty}$ is reasonably constant over the range of potentials studied, varying only between 23 and 28 mV. In this analysis V_K has been taken as -90 mV. Accumulation of K^+ in the Schwann cell space during a strong depolarization reduces V_K in crayfish axons as discussed earlier. The slower rise of g_K at the lower pH resulted in a larger total outward K flux to the time of measurement. For shifts of V_K similar to those in Fig. 7 *b*, a correction for accumulation increases all conductances in Fig. 8, but the voltage shift in $g_{K\infty}$ is essentially unchanged, decreasing by a few millivolts. The common asymptote for $g_{K\infty}$ at pH 7.5 and 5.8 suggests that g_K is

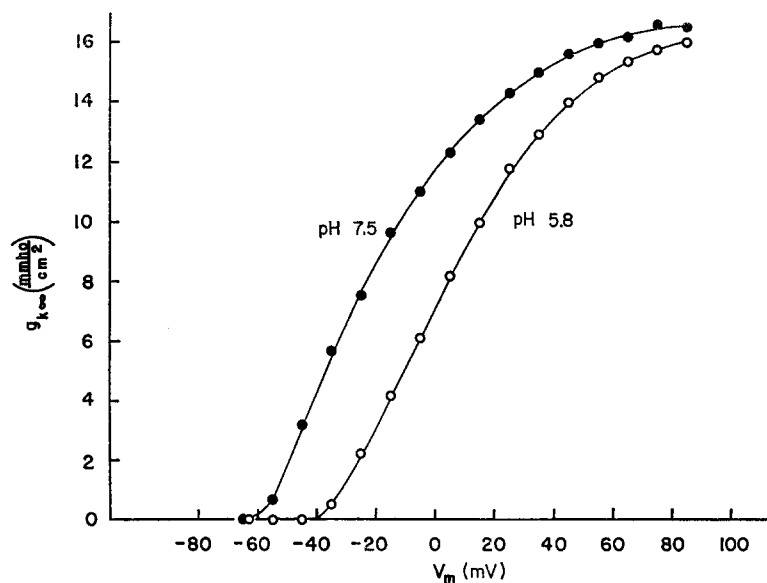


FIGURE 8. Steady-state potassium conductance-voltage curves at pH 7.5 (filled circles) and pH 5.8 (open circles). Lines drawn by eye. External solutions as in Fig. 6. $V_h = -75$ mV.

not altered by this reduction in pH. The voltage shift in $g_{K\infty}$ is therefore applicable to n_∞ as well. The observed constant voltage shift would be consistent with an alteration of the electric field within the membrane resulting from a hyperpolarizing surface charge due to hydrogen ion binding at the external axon surface. Such a field could slow potassium conductance changes in two ways. In the Hodgkin-Huxley (1952) formulation the voltage shift in $g_{K\infty}$ above would result from shifts in the rate constants α_n and β_n . Secondly, hyperpolarizing prepulses induce delays in the rise of g_K during a depolarization in squid axons (Frankenhaeuser and Hodgkin, 1957; Cole and Moore, 1960 *b*) and in *Myxicola* axons (Goldman and Schauf, 1973). This latter effect is found also in crayfish axons (Fig. 9). The currents shown are traced from the oscilloscope records for an axon depolarized to $+25$ mV from prepulse

potentials of -75 to -215 mV. As in *Myxicola* axons (Goldman and Schauf, 1973) the effect is essentially saturated for hyperpolarizing prepulses to potentials more negative than 125 – 135 mV.

If the changes in potassium conductance at low pH are due to a hyperpolarizing surface charge, the dual effects of this perturbation of the electric field should be simulated by equal hyperpolarizing shifts in both prepulse and test pulse levels at normal pH. The prepulse hyperpolarization would induce the Cole-Moore (1960 *b*) effect (Fig. 9) and the test pulse shift would allow for the less depolarized electric field acting on potassium gates after protonation of the external membrane surface. The conductance therefore might respond similarly to shifts in field effected by voltage clamp currents as it does to shifts due to surface charge. Experiments on two axons are illustrated in Fig. 10. The symbols represent experimental points recorded during the test pulse, V_2 (inset), with conductances calculated from Eq. 2. Filled

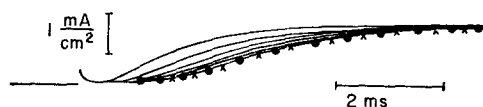


FIGURE 9. The Cole-Moore (1960 *b*) effect in crayfish axons. $V_h = -75$. 50-ms hyperpolarizing prepulses preceded the test pulse to $+25$ mV. Current records were superimposed by tracing. The most rapidly rising curve was the result with no prepulse. Successive curves were recorded with prepulses to (in order) -95 mV, -115 mV, -135 mV, -155 mV, -175 mV, -195 mV (filled circles), -215 mV (X's). External solution identical to that of Fig. 6, pH 7.5.

symbols and X's were recorded at pH 7.5; open circles at pH 5.8. In Fig. 10 *a* filled and open circles represent data at $V_1 = -75$ mV, $V_2 = +65$ mV (no prepulse). Other points represent successive hyperpolarizing shifts in V_1 and V_2 in 20-mV steps, at pH 7.5. The points at pH 5.8 cannot be matched to data recorded at pH 7.5 with a constant hyperpolarizing shift in V_1 and V_2 . It may be seen that while $g_{K\infty}$ in this axon was shifted about 20 mV at pH 5.8, the potassium conductance rose at a markedly slower rate than that recorded at pH 7.5 with a 20-mV shift in potentials. The calculated curves, fitted by eye using Eq. 7, match the data reasonably well at pH 7.5, and somewhat less so at pH 5.8. Voltage shifts in τ_n and in $g_{K\infty}$ should be equal if lowering the external pH results solely in a simple change in surface charge. Between pH 7.5 and 5.8 the shift in τ_n calculated from these curves was 48 mV, while the shift in $g_{K\infty}$ was 20 mV. Possible explanations for this difference are explored below. Fig. 10 *b* illustrates results from another axon at the same pH values, but different potentials. In this case V_2 was stepped down from $+25$ mV, and V_1 from -125 mV. Since V_1 was in all cases 50 mV or more hyperpolarized from the holding potential of -75 mV, the delay in g_K on hyperpolarization was essentially saturated (Fig. 9). Results are nonethe-

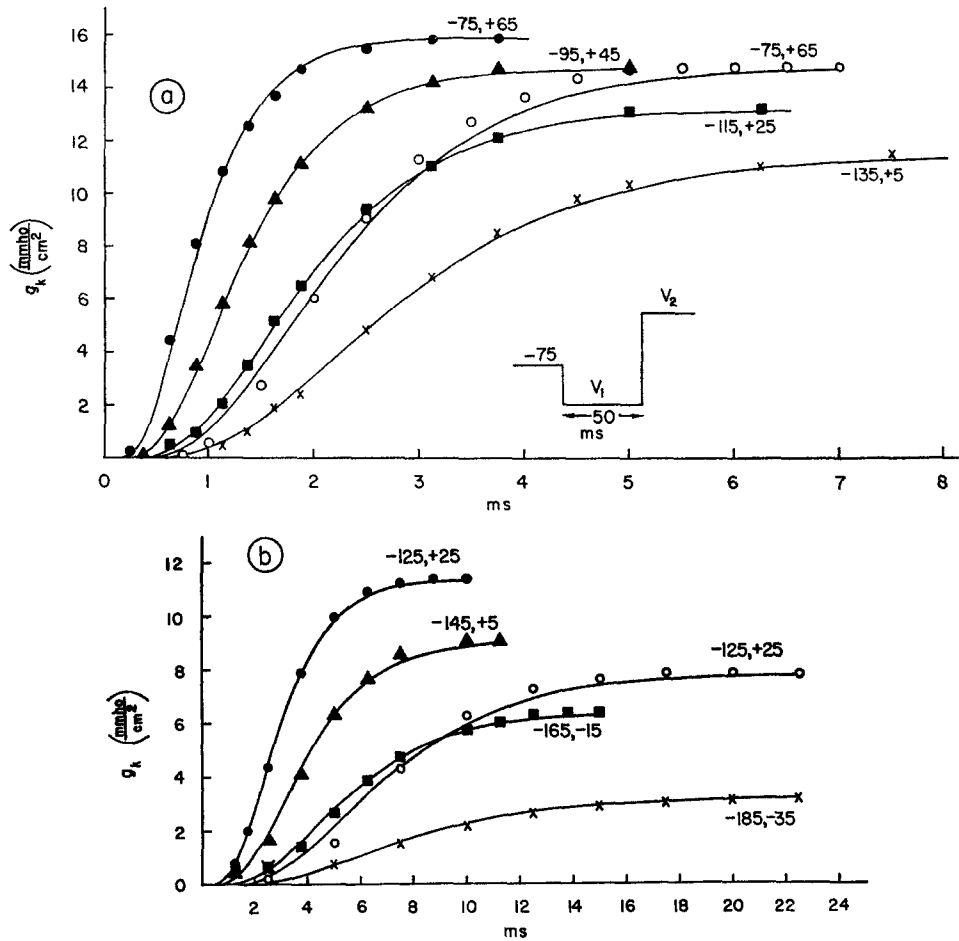


FIGURE 10. Comparison of the time-course of g_K at pH 5.8 with that at pH 7.5 with hyperpolarizing potential shifts in prepulse (V_1) and test pulse (V_2) levels. $V_h = -75$ mV. Points represent experimental data, calculated using Eq. 2, and $V_K = -90$ mV. Filled symbols and x's represent data at pH 7.5; open circles were recorded at pH 5.8. Capacitative transients were measured by applying hyperpolarizing steps, and were subtracted from clamp currents. Leakage currents were subtracted assuming a time-invariant, linear leak. Both of these corrections were small, were similar at both pH levels, and did not affect the basic results or the conclusions drawn from them. Curves are drawn using Eq. 7 with $g_{K_0} = 0$, and are fitted by eye. Numbers denote V_1 , V_2 for each set of points and associated curve. External solutions as in Fig. 6.

less similar to those of Fig. 10 a. The voltage shift in $g_{K\infty}$ was in this case 30 mV and that in τ_n was 56 mV.

Identity of Titratable Group

In order to extract information about the identity of the protonated group from the recorded voltage clamp data, a scheme is used that parallels in

many respects a model for the pH dependence of the rate of an enzymatically catalyzed reaction developed by Johnson et al. (1954). As a possible physical model for conductance changes Hodgkin and Huxley (1952) noted that gating particles in potassium channels might occupy either of two positions, with the relative probability of occupancy determined by voltage-sensitive rate constants. The distribution of gating components then depends on electrostatic forces, and may in addition be influenced by a variety of other intra- and intermolecular chemical interactions. The results illustrated in Fig. 10 suggest that in addition to inducing changes in the electric field across the membrane, lowering the external pH may alter these latter interactions and thereby contribute to the slowing of g_K kinetics. If we use, as in the notation of Hodgkin and Huxley (1952), α_n to denote the rate constant for gating components to move from a closed to an open state, and β_n to denote the rate constant for the reverse transition, then a hyperpolarizing surface charge has the effect of decreasing α_n and increasing β_n , while an increase in a nonelectrostatic energy barrier between closed and open positions would decrease both α_n and β_n . The two effects are therefore additive with respect to α_n . At large depolarizations (e.g., to V_{Na}) $g_{K\infty}$ is close to g_K at normal and at low pH. Then, for this situation, n_∞ is close to unity, and τ_n is approximately equal to α_n^{-1} ($\beta_n \ll \alpha_n$). Measuring τ_n as a function of pH, at a large depolarization, could then provide information on the correlation of potassium kinetics with degree of protonation.

In Fig. 11 the rate of rise of g_K at different pH values relative to that at pH 7.5, measured as the ratio of the reciprocals of the corresponding times to reach one-half the final steady-state value, is plotted as a function of the external pH. Results for axons in several different buffer systems are shown (symbols represent experimental points) and all points were taken at $V_m = +65$ mV. In order to correlate this data with theory, it is assumed that the ratio of half-times equals the corresponding ratio of τ_n values. This will be true if $g_{K0} = 0$. The holding potential in all cases was -75 to -78 mV and although g_{K0} was therefore small, it could not be measured with precision in these experiments. As the pH is lowered, protons are considered to bind to a membrane component, A , according to the reaction



where the charge on A is unspecified. Then, at a given pH the fraction of A that is unprotonated is given by:

$$\frac{[A]}{[A] + [A(H^+)_m]} = [1 + 10^{(pK_a - mpH)}]^{-1}, \quad (9)$$

where $K_a = 1/K'$ and $pK_a = -\log K_a$. Or, in different terms, since rates of protonation and deprotonation are very fast compared to α_n and β_n , the

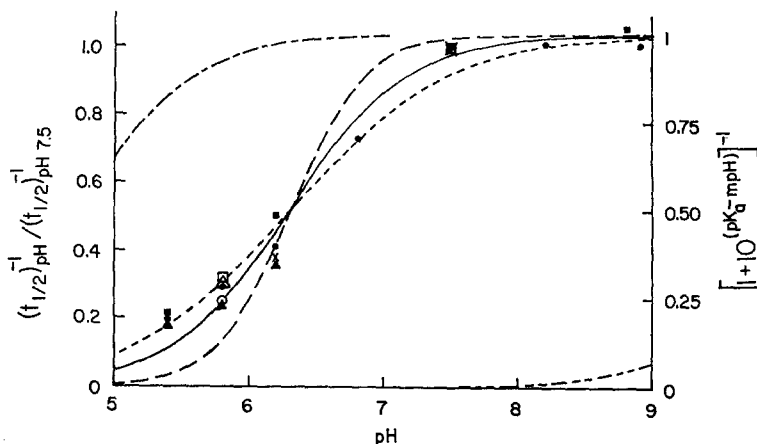


FIGURE 11. pH dependence of potassium kinetics. Points represent experimental data, measured as the ratio of the reciprocal of the time required for I_K to reach one-half its steady-state level at the pH value on the abscissa, relative to that at pH 7.5. Depolarizations were from the holding potential (-75 to -78 mV) to $+65$ mV. External solutions: filled triangles, phosphate-buffered solution of Fig. 5, but without TTX; filled squares, (mM) NaCl 197.3, KCl 5.4, $MgCl_2$ 2.6, $CaCl_2$ 13.5, Tris-maleate, 15; filled circles, identical for solution with filled squares, but with 100 nM TTX; open circles, bis-Tris solution as in Fig. 6, but no TTX; open squares, and open triangles, as in Fig. 6 (including TTX); X's, (mM) NaCl 197.3, KCl 5.4, $MgCl_2$ 2.6, $CaCl_2$ 13.5, Na maleate-maleic acid 10. Curves calculated from function on right ordinate. —, $pK_a = 6.3$, $m = 1$; ---, $pK_a = 4.7$, $m = 1$; - · - · -, $pK_a = 10.1$, $m = 1$; ····, $pK_a = 4.7$, $m = 0.75$; — — —, $pK_a = 10.1$, $m = 1.6$.

right-hand side of Eq. 9 represents the fraction of time that any particular residue A is unprotonated. We consider the possibility that α_n is proportional to this "unprotonated time," i.e., that during the time that component A is protonated, in the course of normal fluctuations, the probability is effectively zero that a gating particle associated with that component will move from "closed" to "open." Curves calculated from Eq. 9 are given in Fig. 11 for several different cases. Typical values for the pK_a of several amino acid side chains in proteins given by Tanford (1961) are: side chain COO^- , 4.7; histidine (imidazole) 6.5; $\alpha-NH_2$, 7.8; phenolic OH, 9.95; $\epsilon-NH_2$, 10.2; guanidyl, >12 . The solid curve in Fig. 11 is drawn for $m = 1$ and $pK_a = 6.3$, i.e., a first order reaction of hydrogen ions with histidine. The experimental points are fitted reasonably well except at very low pH, where the curve predicts a steeper decrease in rate than was recorded. First order curves for carboxyl and ϵ -amino groups clearly do not fit the data. In theory, it is possible to calculate m independently as the slope of $\log(r - 1)$ vs. pH, where $r = (t_{1/2})_{pH} / (t_{1/2})_{pH 7.5}$ (Johnson et al., 1954). However, excessive scatter of the data precludes an accurate determination here, and values of m less than 1 are not ruled out. In Fig. 11 an attempt is made to fit curves for carboxyl

groups ($pK_a = 4.7$) and ϵ -amino groups ($pK_a = 10.1$) to the experimental points. Choosing m to match the data at a 50% decrease in rate, we have for carboxyl groups, $m = 0.75$, and for ϵ -amino groups, $m = 1.6$. The curve for carboxyl groups fits the data reasonably well at low pH but less so near pH 7. The calculated values for ϵ -amino groups do not seem to fit the experimental values, even for $m \neq 1$. If α_n is reduced, but is not equal to zero during the time A is protonated, then the theoretical curves would be drawn with the lower asymptote above zero. The experimental points might be better fitted by this formulation, but in view of the approximations used in this analysis the additional complexity is probably not justified.

The above scheme is not necessarily unique in its applicability to these data, and other mechanisms (see e.g., Bass and Moore, 1973) might also be fitted. The data of Fig. 11 have all been taken at $V_m = +65$ mV. Measured on two axons in the presence of TTX, the titration curve was found to be identical at all potentials between +5 and +85 mV, although the approximations used in the above analysis are valid only at the higher potentials. Measurements of individual rate constants over the entire potential range will be useful in further tests of the applicability of this scheme.

Chemical Modification of Histidine

While the simplest reaction consistent with the experimental results is therefore a first order titration of histidine residues, other possibilities are not ruled out. In an attempt at a further resolution of these possibilities experiments aimed at chemical modification of histidine side chains were performed. Muhlrád et al., (1967, 1969) and Ovádi et al. (1967) have reported that diethylpyrocarbonate ($C_2H_5OOCOCOC_2H_5$) specifically carbethoxylates histidyl residues in proteins at pH 6 (Fig. 12). Diethylpyrocarbonate hydrolyzes rapidly (half-life 1.15 h at 20°C) to CO_2 and ethanol (Fedorcsák and Ehrenberg, 1966). The reagent was dissolved by vigorous stirring imme-

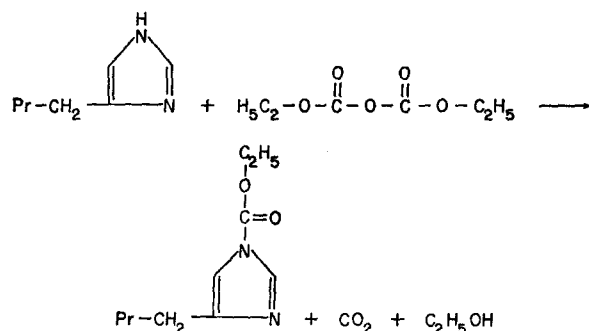


FIGURE 12. Reaction of diethylpyrocarbonate with histidine imidazole groups in protein (Pr) (from data of Muhlrád et al., 1967; see also Hill and Branch, 1940).

diately before use. The external solution contained (mM) NaCl 185, KCl 5.4, MgCl₂ 2.6, CaCl₂ 13.5, NaHCO₃ 2.3, Na maleate-maleic acid buffer (pH 6) 20. Buffering was sufficiently strong so that over the time of application (generally about ½ h) the change in pH due to CO₂ production was less than 0.1 pH. The small amount of ethanol liberated by diethylpyrocarbonate is unlikely to have any effect since results were the same if 40 mM ethanol was present in solutions before and during the reaction.

Fig. 13 illustrates changes in action potentials after carbethoxylation. Fig. 13 *a* shows an action potential in normal Van Harreveld's (1936) solution.

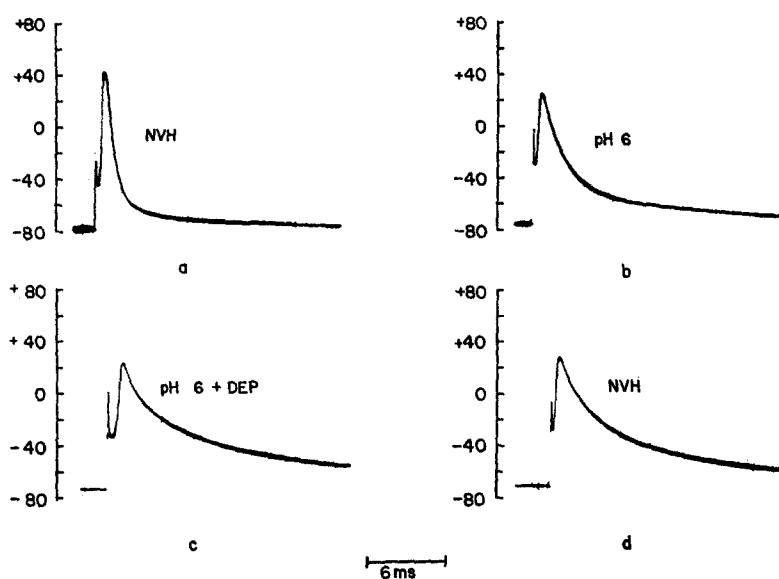


FIGURE 13. Effects of low pH and diethylpyrocarbonate on action potentials. The axon was stimulated by applying a brief pulse of current through the current wire. (*a*) Normal saline (NVH) (pH 7.5). (*b*) External solution (mM): NaCl, 185; KCl, 5.4; MgCl₂ 2.6; CaCl₂, 13.5; NaHCO₃, 2.3; Na maleate-maleic acid, 20; pH 6.0. (*c*) After 25-min exposure to 0.75 mM diethylpyrocarbonate (DEP) added to the solution of (*b*). (*d*) After return to NVH.

An action potential recorded in the above external solution at pH 6 is shown in Fig. 13 *b*. The reduction in overshoot and slowing of the falling phase of the action potential are consistent with voltage clamp results at low pH. Fig. 13 *c* shows a record after a 25-min exposure to 0.75 mM diethylpyrocarbonate, and Fig. 13 *d* the result after a return to normal Van Harreveld's solution. The rising phase and overshoot of the spike are little affected by the reagent, while the falling phase is slowed markedly. This slowing is not reversed on removal of diethylpyrocarbonate and return to pH 7.5.

Voltage clamp records before and after carbethoxylation at pH 6 are illustrated in Fig. 14. Sodium currents are unchanged by the reaction, while

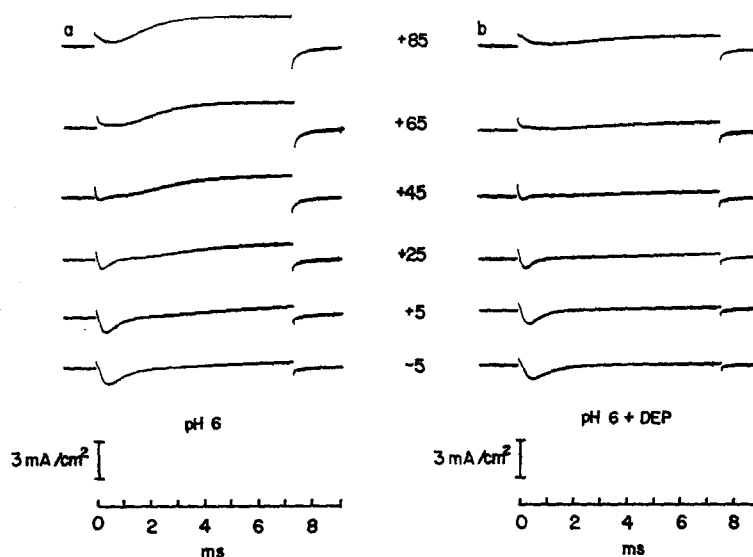


FIGURE 14. Voltage clamp results in diethylpyrocarbonate. (a) Records in maleate-buffered saline, pH 6.0 (see Fig. 13 b). Depolarizations from $V_h = -75$ mV to the levels indicated. (b) 20 min after addition of 0.75 mM diethylpyrocarbonate (DEP) to the solution of (a). Potentials as in (a).

steady-state currents are strongly depressed. These results were not reversed on removal of reagent at pH 6. Application of TTX after treatment with diethylpyrocarbonate eliminated the early transient current, but had no effect on the delayed current, suggesting that diethylpyrocarbonate acted specifically on the potassium conductance. At lower concentrations of diethylpyrocarbonate, e.g., 0.25 mM, it was possible to observe voltage clamp currents after partial inhibition. Potassium currents were slowed as the reaction proceeded and inhibition progressed.

DISCUSSION

The results of the experiments reported here suggest that at low external pH protonation of a membrane component introduces charge that lowers the electrostatic energy for potassium gates in closed positions relative to that for open positions. This perturbation was detectable as a voltage shift in n_∞ on the order of 25 mV for a pH change from 7.5 to 5.8. The experiments illustrated in Fig. 10 were designed as one test of the possibility that this potential shift could also account for the slower rise of g_K during a depolarization at low pH. The data indicated that the observed voltage shift in n_∞ was of the order of one-half of that required for agreement with the observed increase in τ_n . An important limitation on these data is the influence of potassium accumulation in the Schwann cell space on V_K . In Fig. 10, the early portions of the conductance curves would be negligibly affected by this accu-

mulation, and the inference from the experimental points that the delay in the early rise of g_K is shifted by about 50 mV at pH 5.8 therefore seems justified. The situation at long times is less clear. Assuming a constant V_K , the voltage shift in steady-state properties was on the order of 20–30 mV. A correction for the shift in V_K due to accumulation tends to increase steady-state conductances and reduce differences among values at high depolarizations (Adelman et al., 1973). If the data of Fig. 10 *a* and *b* are adjusted by calculating the shift in V_K from the integral of I_K over time, the voltage shift in steady-state conductance is less certain due to the above compression, but remains close to 30 mV. Further, the independence principle predicts that potassium currents at high, positive membrane potentials are relatively invariant with $[K^+]_o$. The results therefore suggest that a hyperpolarizing surface charge is introduced at low pH, but that the resulting voltage shift is only partially responsible for the slowing of potassium kinetics. However, a more accurate analysis will require more detailed data on potassium accumulation in the Schwann cell space.

Several reports of changes in ionic conductances with alteration of membrane surface charge have appeared. Huxley has suggested that a depolarizing shift in the steady-state $g_K - V$ curve and slower rise of g_K of squid axons with increases in $[Ca^{++}]_o$ seen by Frankenhaeuser and Hodgkin (1957), could be due to the adsorption of Ca^{++} at the external surface of the membrane, creating an internal hyperpolarizing field. This work was later confirmed and extended by Gilbert and Ehrenstein (1969) and by Ehrenstein and Gilbert (1973). Working on frog node of Ranvier, Hille (1968) found voltage shifts in τ_n of the order of 30–40 mV for a decrease in pH to 4.8–4.1. However, shifts in n_∞ were not measured in these experiments. In later work Hille (1973) reported a depression in potassium currents at pH levels almost 2 pH units below those effective on crayfish axons, and concluded that groups with pK_a 's between 5 and 10 were absent from potassium channels in the node. These results were consistent with those of Drouin and The (1969) who found that potassium current depression in the node at low pH could be fitted by the titration of a group with a pK_a of 4.6. No data on kinetics were given. Mozhayeva and Naumov (1970) found that the pH dependence of the steady-state potassium conductance in the node required the presence of more than one titratable species, possibly including carboxyl and amino groups. Again, changes in kinetics were not treated. Woodhull (1973) has described a voltage dependent block of sodium channels at low pH, and has interpreted her results on the node in terms of protons both binding to sites within channels and also altering the effective surface charge. Block of open channels is considered to be essentially instantaneous as compared to gating time constants, and therefore would not account for the changes in potassium kinetics reported here. Further, the shift in the $g_{K\infty} - V$ curve with low pH

(Fig. 8) is fairly constant with potential, in contrast to the behavior of P_{Na} in the node. Among other studies relevant to the present work are those by Chandler et al. (1965) and by Begenisich and Lynch (1974).

Hodgkin and Huxley (1952) have suggested that control of ionic conductances might depend on the distribution of specialized membrane components with a net charge or dipole moment. In one scheme fitting the voltage clamp data these gating components exist in a time- and voltage-dependent distribution between open and closed states. In the case of potassium channels, the power of n signifies the number of components required to exist simultaneously in the open configuration for the channel to conduct potassium ions. At a constant membrane potential, the gating components approach an equilibrium distribution determining the steady-state conductance at that potential. Visualized as a first order reaction, the energy levels for an individual gating component may be diagrammed as in Fig. 15. The solid line represents the hypothetical situation at pH 7.5. The dotted lines illustrate a possible perturbation of these levels for the case of a hyperpolarizing surface charge. This shift has the effect of decreasing α_n and increasing β_n . The data discussed above suggest that this perturbation may not completely account for the observed changes in potassium conductance at low pH. The dashed line in Fig. 15 represents a nonelectrostatic modification at low pH affecting the ease with which potassium gating components are able to move from one state to the other. This shift would effect a reduction in both α_n and β_n , and could therefore contribute to the slowing of the rise of g_K during a depolarization at low pH. One prediction of this model is that β_n should not increase as much at low pH as it would if the only changes were electrostatic (it might even decrease). This possibility has not yet been examined in detail, but in one axon in which measurements were made, β_n was found to be similar at pH 5.8 to its value at pH 7.5, while α_n decreased by a factor of about 3.5 at

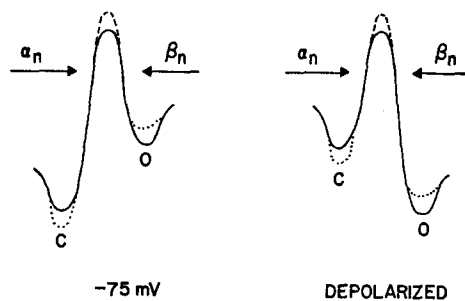


FIGURE 15. Hypothetical energy level diagram for individual potassium gating components showing energy minima for closed (C) and open (O) states. Dotted lines illustrate a possible electrostatic perturbation caused by proton binding at or near the gate component. Dashed lines show the result of a chemical alteration that hinders a transition from one state to another. (Left) $V_m = -75$ mV; (right) depolarized.

pH 5.8. Further work will be required to substantiate this point. It should also be noted that the experimental points in Fig. 10 were not as closely fitted by Eq. 7 at pH 5.8 as at pH 7.5. It is possible, for example, that not all gating components at a channel were affected equally.

Results of experiments with diethylpyrocarbonate contribute additional information on these phenomena. Carbethoxylation of histidine at pH 6 decreases the pK_a of the imidazole group from 6.3 to 4.4 (Muhlrad et al., 1967) and therefore *reduces* the positive charge introduced by protonation of this amino acid side chain, yet diethylpyrocarbonate inhibited the opening of potassium channels. Introduction of a covalently bound carbethoxy group at a membrane protein residue could provide steric hindrance to a conformational change in the neighborhood of, or involving, that amino acid. The residues affected are therefore likely to reside close to, or comprise part of, potassium gating components. In the experiments at low pH the results suggested that electrostatic effects alone were inadequate to account completely for the observed changes in potassium kinetics, although, as discussed, some aspects of the analysis were inconclusive. Future work, particularly with instantaneous currents, will hopefully resolve some of these difficulties. The modification experiments, on the other hand, suggest that the voltage shifts observed at low pH do not arise from proton binding at random sites on the membrane surface. In this regard, it is evident from Fig. 4 that there is little if any voltage shift in peak early current at pH 5.8 suggesting that sodium activation gates are little affected by the charge introduced at potassium channels, and results are similar with respect to steady-state sodium inactivation. Therefore, in both pH and reagent modification experiments evidence supports the idea that the membrane groups affected are closely associated with potassium gates, and this is clearly a required condition if proton binding affects nonelectrostatic interactions.

To what extent can we identify the reactive membrane group involved? The simplest scheme fitting the titration data of Fig. 11 is a first order titration of histidine imidazole groups. Involvement of other residues could not be ruled out but require more complex systems. In the case of carboxyl groups, for example, the data would be fitted about equally well if the membrane component contained four COO^- groups, with the protonation of any three sufficient to decrease α_n . Another potential source of error in estimating pK_a is the possible concentration or dilution of hydrogen ions at the external membrane surface due to the presence of fixed negative or positive charge. Further data on voltage shifts as a function of pH, or experiments including variation of the external ionic strength might allow an assessment of the correction required (Gilbert and Ehrenstein, 1970; D. Goldstein, personal communication). Muhlrad et al., (1967, 1969) and Ovádi et al. (1967) have shown that both for free amino acids and in proteins, reaction with

diethylpyrocarbonate is specific for histidine at pH 6. At more alkaline pH, or at much higher concentrations of reagent, other residues, including sulfhydryl, guanidyl, phenolic, indole, and amino groups are also carbethoxylated (Muhlrad et al., 1967; Rosen et al., 1970; Wolf et al., 1970). Other compounds known to react with histidine groups (as well as other residues) have also been found to suppress potassium conductance changes. Among these are 1-fluoro-2,4-dinitrobenzene (Cooke et al., 1968), glutaraldehyde, and rose bengal (Shrager, unpublished observations). Finally, Clark and Strickholm (1971) have reported sharp transitions in membrane impedance of potassium-depolarized crayfish axons at pH 6–6.5. Taken together, results of titration and modification experiments are therefore reasonably suggestive that a histidine residue of a membrane protein constitutes part of, or is closely associated with the mechanism responsible for the control of potassium conductance.

Since it has been postulated that on protonation chemical interactions, as well as the effective field, are perturbed, it is perhaps worthwhile to consider a possible mechanism fulfilling both requirements. Several protein side chains and phospholipids contain groups that can participate in hydrogen bonds. If either the proton donor or acceptor, or both, in a hydrogen bond are ionizable, then the bond will be stable only in the pH range in which the donor is protonated and the acceptor is not (Scheraga, 1963). Thus, a histidyl-carboxylate bond will exist only in the pH range 4.5–6.5, and a tyrosyl-histidyl bond is stable between pH 6.5 and 10.5. Lowering the pH below 6.5 therefore creates or stabilizes hydrogen bonds in which histidine is the donor, and breaks those bonds in which histidine is the acceptor. The present data do not allow a distinction between these two possibilities, nor is it possible to distinguish from these experiments whether the protonated group is in the neighborhood of the gate component, or is actually part of it. Both electrostatic and chemical interactions could result equally well from either situation.

Finally, this work demonstrates that the crayfish axon is a useful preparation for voltage clamp experiments. It is readily available at all seasons and, additionally, it may be internally perfused (Shrager et al., 1969 *a*). The temporal separation of sodium and potassium currents with moderate decreases in pH, not seen in other preparations, might be of value in the analysis of gating currents recently measured in squid axons (Armstrong and Bezanilla, 1973; Keynes and Rojas, 1973).

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