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Specific and Unspecific Charges at the Sodium Channels of the Nerve Membrane*

H. Drouin and B. Neumcke

I. Physiologisches Institut der Universität des Saarlandes
Homburg (Saar), Germany

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Summary. Voltage clamp experiments were performed on myelinated nerve fibres of Rana esculenta. The combined effects of increasing extracellular H^+ concentration and lowering the Na^+ concentration on sodium currents were studied. The presence of negative charges of two types at the external membrane surface could explain the observed shifts in the I_{Na}-V curves and the reduction of I_{Na} induced by H^+. Accordingly, from the data of shifts of the conductance voltage curves we calculate a density of $-1\ e_0/(20\ Å)^2$ for unsepecific charges with an intrinsic $pK_a = 4.3$. From the reduction of I_{Na} induced by H^+ we estimate a density of $-1\ e_0$/Na channel for specific charges with an intrinsic $pK_a = 4.5$. Na^+ ions would bind to these specific charges when passing through the sodium channels and Na^+ transport would be blocked if these groups are protonated.

Key words: Ranvier Node — Sodium Channels — Surface Charges.

Several reports of the effects of pH of the external solution bathing a nerve fibre indicate that pH reduction has basically a dual effect on the ionic currents. The curves relating ionic conductances and membrane potentials shift along the voltage axis towards more positive membrane potentials, and the sodium and potassium conductances are reduced (Hille, 1968, 1973; Drouin and The, 1969). These two effects were described in an empirical form (Drouin and The, 1969; Drouin, 1971). However, physical interpretations were presented for one of these effects only.

Lowering the internal ionic strength in perfused giant nerve fibres shifts the curve relating sodium conductance and potential. Chandler, Hodgkin and Meves (1965) explained this effect as being due to changes of the electrical surface potential at the inner nerve membrane. In subsequent publications (Rojas and Atwater, 1968; Gilbert and Ehrenstein, 1969, 1970; Gilbert, 1971; Ehrenstein and Gilbert, 1973; Mozhayeva and Naumov, 1970, 1972a–d; D'Arrigo, 1973; Brismar, 1973; Vogel, 1973;

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Hartz and Ulbricht, 1973; Hille, Woodhull, and Shapiro, 1974) this concept has been applied with great success to describe the effects of various ionic compositions of extra- and intracellular solutions on sodium and potassium currents of squid giant axons and myelinated nerve fibres. Experiments with artifical lipid bilayer membranes (McLaughlin, Szabo, and Eisenman, 1971; Muller and Finkelstein, 1972; Haydon and Myers, 1973; Hladky and Haydon, 1973) confirm the importance of membrane surface potentials for the description of conductance properties.

The second effect of low pH solutions, the decrease of the ionic currents, was investigated by Woodhull (1972, 1973) for the sodium system. Woodhull postulates an ion-binding site in the sodium channel and assumes that the sodium channel is blocked if H^+ is bound at this site. In this investigation we adopt this idea with two modifications. We assume that the ion-binding site is located at the external membrane surface and take into account a pH dependent negative surface potential at this interface. There is evidence for the existence of negative surface charges at the outside of nerve membranes near the sodium channels (Hille, Woodhull, and Shapiro, 1974). It seems logical, therefore, to infer that the Na^+ concentration at the entrance of sodium channels might be higher than in the bulk of the extracellular solution. At low pH, the negative surface charges are protonated and the Na^+ concentration at the membrane surface will decrease. Thus the rate constants for the movement of Na^+ between the external solution and the entrance of the sodium channel become pH dependent, and this feature will be considered in our analysis.

Our work differs from previous investigations in the following respects. We studied the effects of pH at various sodium concentrations and we introduce corrections to take into account the effects of the resistance in series with the membrane in the nodal region. In all calculations of surface potentials and binding constants the measured ion activities in the test solutions were used and not ion concentrations. In the concentration range of our test solutions, the effective charge of ions is decreased both by screening and association with counterions (Davies, 1962). Therefore, the use of ion activities will give a more accurate picture of the actual charge distribution. The transport of Na^+ is formulated in terms of chord conductance and not in terms of the usual permeability. Finally, both the shifting and blocking effects of protons will be described by a physical model.

Methods

Single myelinated nerve fibres were dissected from the sciatic nerve of Rana esculenta (Stämpfli, 1952, 1969). Motor and sensory fibres were investigated. However, for the experiments described here no marked difference in the results obtained with these two types of fibres could be found.

Single nodes of Ranvier were studied at 15°C under voltage clamp conditions using the method described by Nonner (1969).

Fibres were mounted in an acrylic chamber with three movable troughs fitted into a base. Thus, four saline pools were obtained. The fibre was fixed by layers of silicone grease and vaseline seals. An additional air gap between the guard and the input electrode of the amplifier abolished the attenuation artefact (Dodge and Frankenhaeuser, 1958). The internodes were not cut. The nodes in the neighbourhood of the node under investigation (test node) were kept in isotonic KCl solution. A constant flow of Ringer's solution across the test node was applied. Rapid solution changes were possible by means of a multichannel stopcock (improved model of Kilb and Stämpfli, 1955).

Recording and Analysis

At the beginning of the experiment the holding potential of the node was adjusted in such a way that only 30% of the sodium system was inactivated.

A digital computer (Honeywell DDP-516) generated the stimulating pulses and digitalized the membrane currents at intervals of 20 μsec. To reduce noise, these currents were transmitted through a 40 kHz, second order, Bessel filter. In addition, for each record stored on magnetic tape, two records taken at intervals of 2 sec were averaged. For each solution the following records were taken:

a) Capacity and Leakage Currents. During a hyperpolarizing pulse (−50 mV, 50 msec), 100 current values were measured within the first 2 msec and 64 measurements were averaged during the last 1.28 msec of the pulse.

b) Sodium Currents. A hyperpolarizing prepulse (−40 mV, 50 msec) was followed by a depolarizing test pulse (50 msec). The amplitude of the test pulse was varied between 12 and 140 mV in steps of 4 or 8 mV. During the first 2 msec of the test pulse 50 current values were taken each being the average of 2 consecutive measurements. For the late currents 5 similar current values from 10 measurements between 4 and 4.2 msec were taken. During the last 1.28 msec of the test pulse, 64 measurements were averaged as in (1).

The analysis of the voltage clamp data was performed by off line computer programs. From the first group of records the leakage current was taken as the steady state current. This current was subtracted from the remaining values to give the capacity current transients. These were approximated by a single exponential function. From the second group of records, capacity and leakage currents were subtracted assuming an ideal membrane capacity and an ohmic leakage conductance. The remaining ionic currents, I, were separated into sodium currents and potassium currents, I_K. We used the procedure described by Hille (1967), in which I_K is determined by fitting the function

$$I_K = I_{Kss}[1 - \exp(-t/\tau_n)]^4 \quad (1)$$

to the late currents. I_{Kss} is the steady state potassium current and τ_n the time constant for the activation of the potassium system. This fit was used when 'I' exceeded a prescribed lower or upper limit; in this case the peak sodium current, I_{Na}, was taken as the minimum of $I-I_K$ for small values of the test pulse V and as the maximum of $I-I_K$ if V exceeded the sodium equilibrium potential V_{Na}. The already mentioned averaging and filtering procedures reduced noise to a great extent, thus no appreciable errors were introduced into the determination of I_{Na}.

This procedure of separating the sodium and potassium currents was checked experimentally by applying this method to records taken in Ringer's solution with

and without 5 mM TEA (tetraethylammonium chloride). In TEA-Ringer the potassium channels are partially blocked (Hille, 1967). However, for both solutions almost the same peak sodium currents were obtained. The blocking effect of TEA is pH dependent (Mozhayeva and Naumov, 1972a). Since the pH value is one of the parameters which was varied in our experiments, we had to avoid the addition of TEA to our test solutions. Therefore, the above described mathematical separation of sodium and potassium currents was used.

Solutions

The reference Ringer's solution had the following composition: 110 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 5.0 mM Tris (hydroxymethyl) aminomethane buffer, pH = 7.3 at 15° C. (For the use of Tris buffer see Durst and Staples, 1972.)

Three types of test solutions were used for the experiments:

a) Solutions with reduced pH (solutions No. 1, 2, 3, 4 in Table 1). They were buffered with 5 mM sodiumhydrogenphthalate and adjusted to the requested pH values with NaOH or HCl. The concentration of sodium ions was kept at 110 mM.

b) Solutions with reduced sodium ion concentration (solutions No. 5, 6, 7 in Table 1). For these solutions NaCl was replaced by choline · Cl or by Tris · Cl. But in most experiments Tris was used as sodium substitute. The solutions used in this series contained 2.5 mM KCl and 1.8 mM $CaCl_2$, in addition 55 mM NaCl and 60 mM Tris (No. 5 in Table 1), 27.5 mM NaCl and 87.5 mM Tris (No. 6 in Table 1), 13.75 mM NaCl and 101.25 mM Tris (No. 7 in Table 1), pH = 7.3 of these solutions was adjusted with HCl.

c) Solutions with reduced pH and sodium ion concentration (solutions No. 8, 9, 10 in Table 1). All solutions contained 2.5 mM KCl and 1.8 mM $CaCl_2$, in addition 65 mM NaCl and 60 mM Tris, pH = 5.5 (No. 8 in Table 1), 55 mM NaCl and 60 mM Tris, pH = 5.0 (No. 9 in Table 1), 27.5 mM NaCl and 87.5 mM Tris, pH = 5.4 (No. 10 in Table 1). The pH values were adjusted by adding HCl. Solutions of series c) were unbuffered.

For all solutions we measured pH (at 15° C), activity of sodium ions, a_{Na}, osmolality and conductivity. The results are compiled in Table 1 (see p. 211).

The pH of the solutions was checked electrometrically (Knick pH Meter, Type 34, and Ingold Electrode, Type HA-401-S-NS). The activities listed in Table 1 were measured using cation selective electrodes and a digital pH/mV meter (Orion Sodium Specific Electrode, Model 94-11; and Orion Calcium Activity Electrode, Model 92-20; Orion Research, Model 801).

Liquid junction potentials were measured with reference to an isotonic KCl solution at 15° C. Agar bridges containing isotonic KCl were applied. Osmotic pressure of the various solutions was evaluated measuring the decrease in the freezing point (Knauer Elektronisches Halbmikro-Osmometer, Type M). The conductivity was measured with an ohmmeter (Radiometer Conductivity Meter, Type CDM2, and Radiometer Electrode, Type CDC 104).

Corrections

In the analysis of our data, changes in membrane surface potential are deduced from shifts in conductance voltage curves. The accuracy of our measurements depends on an accurate measurement of V across the membrane. V is in turn affected by possible changes in the resistance in series with the membrane.

Table 1. Parameters of solutions

Solution	a_{Na} [mM]	pH (15°C)	Osmolality [m moles/kg]	Conductivity [Ω^{-1} cm^{-1}]	Liquid junction potential[a] [mV]
Ringer	85	7.3	219	0.011	3.4
1	80	6.4	209	0.010	2.7
2	80	5.3	205	0.010	2.7
3	80	4.9	213	0.010	2.8
4	80	4.3	214	0.011	2.9
5	44	7.3	215	0.010	4.7
6	23	7.3	212	0.009	5.7
7	12	7.3	211	0.008	6.0
8	51	5.5	234	0.010	5.2
9	44	5.0	214	0.009	5.5
10	25	5.4	225	0.009	6.7

For Ringer's solution and test solutions No. 1, 2, 3, and 4 the activity a_{Ca} of calcium ions varied between 0.64 and 0.75 mM.

[a] With reference to isotonic KCl.

Voltage Drop at Membrane Series Resistance

Fig. 1 shows the relevant part of the equivalent circuit in our experiments. All the parameters needed for the discussion of the effects of the series resistance are indicated (for a drawing of the voltage clamp arrangement see Fig. 1 b of Nonner, 1969). The voltage $u_3 - u_0$ between the electrode A, electrically connected to the stimulator, and the axoplasm at the node is the sum of the voltage across the resistance R_m of the nodal membrane, $u_1 - u_0$, the voltage across the resistance R_g in the nodal gap, $u_2 - u_1$, and the voltage across the resistance R_A of the electrolyte solutions between the fibre and electrode A, $u_3 - u_2$. Under voltage clamp conditions a current I_m is driven through the resistance R_m and R_g, in series with the internodal resistance R_{ax}, to keep the potential difference $u_3 - u_0$ at a fixed value. However, the current flowing through R_A is greater than I_m because some current leaks through the vaseline seal between the test node and the node on the left of the diagram. So $u_3 - u_1$ is equal to

$$u_3 - u_1 = R_g I_m + R_A (I_m + I_S) . \tag{2}$$

From Kirchhoff's second law we know that

$$R_S I_S = (R_{ax} + R_m + R_g) I_m . \tag{3}$$

If Eqs. (2) and (3) are combined, $u_3 - u_1$ can be expressed in terms of I_m as

$$u_3 - u_1 = \left[R_g + R_A + R_A \left\{ \frac{R_{ax} + R_m + R_g}{R_S} \right\} \right] \cdot I_m . \tag{4}$$

The electrolyte resistance in the nodal gap, R_g, is greater than the resistance in the bulk solution, R_A, and the internodal resistance R_{ax} will be much greater than R_m or R_g, whence

$$u_3 - u_1 = \left[R_g + \left\{ \frac{R_A R_{ax}}{R_S} \right\} \right] \cdot I_m . \tag{5}$$

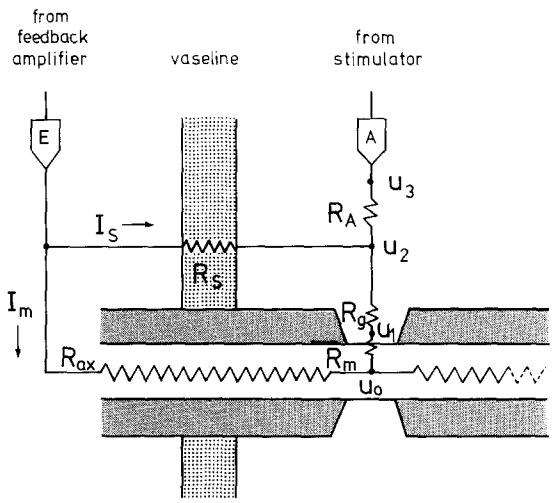

Fig. 1. Schematical illustration of voltage drop $u_3 - u_1$ at membrane series resistances

As the ends of the fibre were not cut in our experiments, we have to add to R_{ax} the membrane resistance of the node on the left side which was kept in isotonic KCl. The resistance of this node is estimated as 10 MΩ and R_{ax} as 25 MΩ (Stämpfli, 1952).

The ratio R_A/R_S was determined as follows. A microelectrode (internal resistance 50 kΩ) was placed in the extracellular solution near the test node. The voltage between this electrode and the corresponding chamber electrode A, $u_3 - u_2$, was measured and compared with the voltage between the electrodes E and A. When the electrode A was grounded and a potential step of 300 mV was applied to electrode E, $u_3 - u_2 = 0.5$ mV was measured. Under these conditions almost all current flows through R_S because the internodal resistance R_{ax} is much higher than the resistance R_S of the vaseline seal ($R_S \cong 3$ MΩ, see Nonner, 1969). Therefore, the ratio of measured and applied voltages must be equal to the ratio $R_A/R_S = 0.5$ mV/300 mV = 1/600. Thus, taken R_{ax} as 10 MΩ + 25MΩ we get

$$R_A \cdot R_{ax}/R_S \cong 5.8 \cdot 10^4\ \Omega\ .$$

The electrolyte resistance in the nodal gap, R_g, cannot be measured directly. However, a rough estimate can be obtained if we consider the nodal gap as a cylindrical slit between the insulating myelin sheets, whence

$$R_g = \frac{1}{2\pi d \varkappa} \ln \frac{r_1}{r_2} \tag{6}$$

where r_1, r_2 are the outer and inner radius of the cylinder respectively, d is the cylinder width, and $\varkappa$ the specific conductivity of the electrolyte solution between the radii r_1 and r_2. Taking $r_1 = 7$ µm, $r_2 = 3.5$ µm, $d = 0.5$ µm and $\varkappa = 10^{-2}\ \Omega^{-1}\ \mathrm{cm}^{-1}$ we obtain $R_g \cong 2.2 \cdot 10^5\ \Omega$.

For the sum $R_g + R_A R_{ax}/R_S$ we, therefore, use the value $2.8 \cdot 10^5\ \Omega$. Due to infoldings of the nodal membrane, its area A_m will be larger than the geometrical

value $2\pi r_1 d = 11\ \mu m^2$ and we, therefore, assume $A_m = 30\ \mu m^2$. Then the voltage across the series resistance is $u_3 - u_1 = 0.084\ i_m$, where $u_3 - u_1$ is in mV if i_m, the current density through the test node, is in mA/cm². If, for example, the current density i_m drops from 100 mA/cm² to 20 mA/cm², $u_3 - u_1$ will change from 8.4 mV to 1.7 mV and the observed voltage shift has to be corrected by 8.4 − 1.7 mV = 6.7 mV.

To test the magnitude of this correction, we measured sodium current voltage relations in Ringer's solution using various conditioning prepulses. In all experiments, the current voltage relation which gave the smallest peak sodium currents was displaced towards more positive potentials. As an example −94 mA/cm² peak inward sodium current was located at $V = 46$ mV (for a 40 mV conditioning hyperpolarizing prepulse) whereas the peak I_{Na} of −26 mA/cm² was located at $V = 52$ mV for a 5 mV depolarizing prepulse. The calculated voltages, $u_3 - u_1$, across the series resistances are 7.9 mV and 2.2 mV. Therefore, the observed shift of 6 mV can be predicted using Eq. (5).

Eq. (5), relating the potential drop $u_3 - u_1$ across the series resistances with the current I_m flowing through the nodal membrane, contains the electrolyte resistance. At a fixed membrane current, I_m, test solutions with different electrolyte resistance, therefore, will give different values of $u_3 - u_1$. From conductivity measurements shown in Table 1 it follows that the conductivity did not change much and the corresponding corrections of $u_3 - u_1$ calculated with Eq. (5) are less than 1 mV. Whence, changes in potential drop across the series resistance due to changes in electrolyte resistance can be neglected.

Another source of error will be introduced by changes in liquid junction potentials between Ringer and test solutions. Junction potentials between Ringer and test solutions were measured and are presented in Table 1. If the test solution is positive with respect to Ringer's solution (solutions 5—10), this potential difference has to be subtracted from measured voltage shifts. If the test solutions are negative with respect to Ringer's solution (solutions 1—4), this amount must be added.

Nomenclature and Current Calibration

Absolute membrane potentials, E, are taken as the potential of the axoplasm minus the potential of the outside solution. Consequently, outward membrane currents are positive. Displacements of the membrane potential E from its resting value E_r are denoted by V, $V = E - E_r$.

The current density i_m through the nodal membrane can be calculated from the measured voltage V_E across the resistance R_{ax} as $i_m = V_E/A_m R_{ax}$. According to Dodge and Frankenhaeuser (1958) we assume $A_m R_{ax} = 10\ \Omega\,cm^2$. This calibration factor is in agreement with the values $R_{ax} = 35\ M\Omega$ and $A_m = 30\,\mu m^2$, which we used in the calculation of the voltage drop across the series resistances. In principle, absolute values of I_m are not relevant for our calculations because in our analysis we use current ratios. Only the corrections discussed in the previous section depend on the absolute value of i_m.

From Eq. (5)

$$\Delta\,(u_3 - u_1) = \left[R_g + \left\{\frac{R_A \cdot R_{ax}}{R_S}\right\}\right] A_m \cdot \Delta i_m\,. \tag{7}$$

If Δi_m is expressed by the measured voltage $\Delta V_E = A_m R_{ax} \Delta i_m$ we obtain

$$\Delta\,(u_3 - u_1) = \left\{\frac{R_g}{R_{ax}} + \frac{R_A}{R_S}\right\} \Delta\, V_E\,. \tag{8}$$

In most of our experiments, $\Delta\ (u_3 - u_1)$ is less than 10 mV. Therefore, if our estimate of $R_g/R_{ax} + R_A/R_S$ does not deviate by more than 10% from the exact value, the uncertainty in our estimation of $\Delta\ (u_3 - u_1)$ is below 1 mV and can be neglected.

Results

A. Effects of Reduced pH and Sodium Ion Concentration on I_{Na}-V Curves

Figs. 2, 3 and 4 illustrate a few examples of reduced external sodium and low pH:

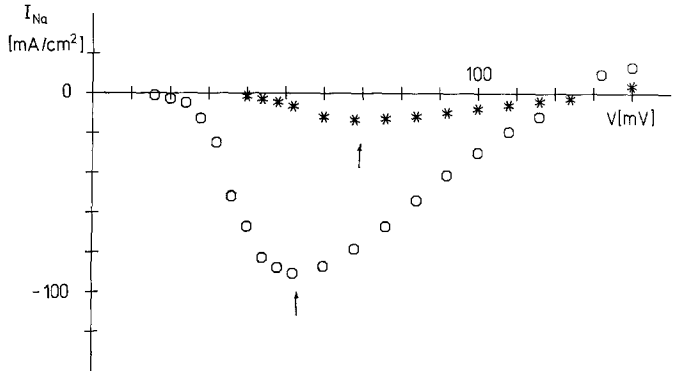

Fig. 2. Effect of low pH. Peak sodium current density I_{Na} as function of depolarization V. o Reference Ringer's solution (a_{Na} = 85 mM, pH = 7.3), * Test solution No. 3 (a_{Na} = 85 mM, pH = 4.9). Experiment 20. 7. 73 GNR = 1, 2, motor fibre. Minima of the current voltage curves are marked by arrows in this figure and in Figs. 3 and 4

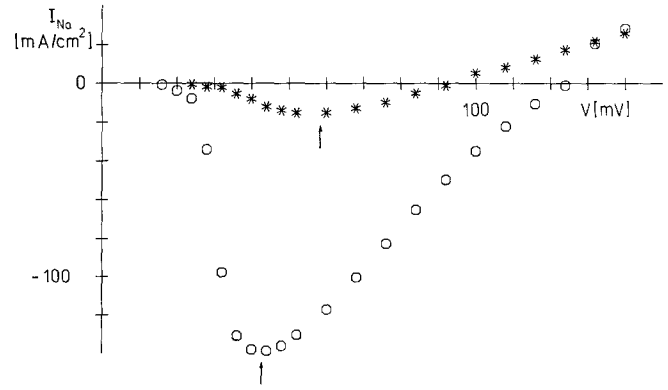

Fig. 3. Effect of low a_{Na}. Peak sodium current density I_{Na} as function of depolarization V. o Reference Ringer's solution (a_{Na} = 85 mM, pH = 7.3), * Test solution No. 6 (a_{Na} = 23 mM, pH = 7.3). Experiment 9. 8. 73 GNR = 3, 4, 5, motor fibre

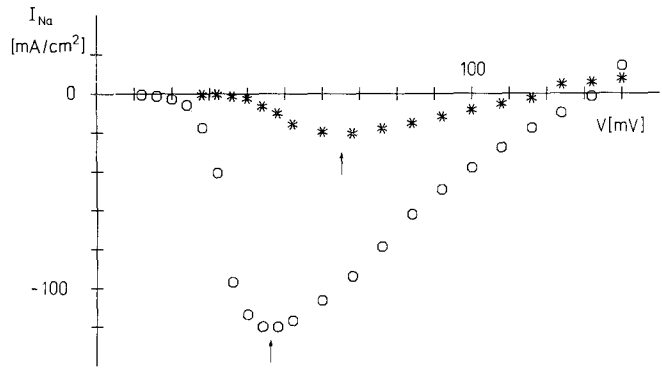

Fig. 4. Combined effects of low pH and low a_{Na}. Peak sodium current density I_{Na} as function of depolarization V. o Reference Ringer's solution (a_{Na} = 85 mM, pH = 7.3), * Test solution No. 8 (a_{Na} = 51 mM, pH = 5.5). Experiment 24. 10. 73 GNR = 20, 21, sensory fibre

a) Effects on Reversal Potentials V_{Na}. First let us consider the effects on reversal potentials which are not affected by the voltage error due to the series resistance. Reversal potentials were determined as the intercept of the I_{Na}-V curve with the V axis. When these values were corrected for liquid junction potentials, they followed the Nernst relationship between equilibrium potential and ion activity.

b) Effects on Position of Peak I_{Na}-V. All other parameters characterizing the I_{Na}-V curve were affected by the voltage error due to the series resistance. Thus, if we examine the position of the peak I_{Na} value in the I_{Na}-V curve shown in Fig. 3, although the pH value of the test solution is the same as in Ringer, nevertheless, a shift from V = 43 mV, in Ringer's solution, to V = 58 mV, for test solution No. 6, is observed. We estimate that for the decrease of maximal current density from -139 mA/cm² in Ringer to -16 mA/cm² in the test solution gives a 10.3 mV (123 · 0.084) change in the voltage drop across the series resistance. Additional 2.3 mV arise from the liquid junction potential between Ringer and test solution No. 6 (compare Table 1). Thus 12.6 mV of the observed shift of 15 mV can be accounted for by the changes in the voltage drop across the series resistances and changes in liquid junction potentials.

Usually after a run in a test solution of low pH, the sodium currents recorded in Ringer's solution are greater than their initial control values in Ringer's solution at pH 7.3. Therefore, measurements were taken only 3 to 4 min after a solution change, when the sodium currents had reached

a stationary level. This increase in sodium conductance after a treatment with a low pH solution was described by Hille (1968) and has been investigated in some more detail by Fox (1974). A possible explanation for this phenomenon is given in the Discussion and refers to a redistribution of surface charges on both sides of the membrane (McLaughlin and Harary, 1974). Sodium currents in Ringer's solution reached control values and, therefore, no after effect of the test solutions which were applied before could be detected. Thus, these effects of low sodium and low pH are reversible.

c) Effects on Capacitative Current Transients and on Leakage Currents. No systematic differences were found comparing a number of records of capacitive current transients obtained in one of the 10 test solutions used.

Leakage currents in Ringer almost were indistinguishable from those recorded bathing the test node with either test solution from 1 to 4 of reduced pH value but normal sodium ion activity. However, using solutions from No. 5 to 10, in which part of Na^+ was substituted by the Tris cation, the leakage conductance was $10-30\,\%$ lower than in Ringer's solution.

B. Evaluation of the Data in Terms of a Model

a) Outline of the Model. Within the pH range investigated (pH = 4.3—7.3) we postulate that unspecific and specific univalent negative fixed charges are operating and that they are located at the outer surface of the nodal membrane near the sodium channels. Unspecific charges are uniformly distributed at a surface density σ_0. The equilibrium constant for protonation of these charges will be designated by K. The unprotonated surface charges σ give rise to a negative surface potential ψ_0, and therefore the concentration of cations at the membrane surface would be higher than that in the bulk solution. Accordingly the pH value at the membrane surface would be lower than that in the bulk solution. For the specific charges we postulate a discrete distribution, one negative site per sodium channel, with equilibrium constants K_H and K_{Na} for their association with H^+ and Na^+ respectively. We assume that these charges bind temporarily the Na ions during their passage through the sodium channel. Whence, the sodium current will be proportional to the fraction of sites occupied by Na^+. If the sites are protonated, we assume that the channels would be blocked.

Let a' be the activity at the membrane surface and a the activity in the bulk of the solution. Application of Boltzmann distribution gives the following relations between surface and bulk activities

$$a'_{Na} = B\,a_{Na};\quad a'_H = B\,a_H;\quad a'_{Ca} = B^2\,a_{Ca} \tag{9}$$

where

$$B = \exp\left(-\frac{F\psi_0}{RT}\right) \tag{10}$$

is the Boltzmann factor for univalent cations; F = Faraday constant, R = gas constant, T = absolute temperature. Let us assume further that the binding of H^+ at the unspecific surface charges follows a Langmuir adsorption isotherm,

$$\frac{\sigma}{\sigma_0} = \frac{K}{K + a'_H}. \tag{11}$$

The screening of the unprotonated charges σ is described by the equation (Grahame, 1947):

$$\frac{\sigma^2}{2RT\varepsilon_0\varepsilon} = a_+(B-1) + a_-\left(\frac{1}{B} - 1\right) + a_{++}(B^2 - 1) \tag{12}$$

where

ε_0 : permittivity of free space ($= 8.854 \cdot 10^{-12}$ A sec V^{-1} m^{-1}),
ε : dielectric constant of water,
a_+ : activity of all univalent cations in bulk solution,
a_- : activity of all univalent anions in bulk solution,
a_{++}: activity of all divalent cations in bulk solution.

The activities a_S, $a_{\mathrm{Na}\,S}$, a_{HS} of free specific sites, S^-, and specific sites occupied by Na^+ and H^+ are related to the equilibrium constants as follows

$$K_{\mathrm{Na}} = \frac{a'_{\mathrm{Na}} \cdot a_S}{a_{\mathrm{Na}\,S}} \text{ (13a)}; \quad K_H = \frac{a'_H \cdot a_S}{a_{HS}}. \tag{13b}$$

After introducing the total activity of specific sites

$$a_{St} = a_S + a_{\mathrm{Na}\,S} + a_{HS} \tag{14}$$

the relative activity of NaS due to competitive inhibition of Na^+ and H^+ at S^- can be written as

$$\frac{a_{\mathrm{Na}\,S}}{a_{St}} = \frac{1}{1 + \frac{K_{\mathrm{Na}}}{a'_{\mathrm{Na}}}\left(1 + \frac{a'_H}{K_H}\right)}. \tag{15}$$

If $a'_{\mathrm{Na}} < K_{\mathrm{Na}}$, equation (15) simplifies to

$$\frac{a_{\mathrm{Na}\,S}}{a_{St}} = \frac{a'_{\mathrm{Na}}}{K_{\mathrm{Na}}} \cdot \frac{1}{1 + \frac{a'_H}{K_H}} = \frac{a_{\mathrm{Na}}}{K_{\mathrm{Na}}} \cdot \frac{B}{1 + B\frac{a_H}{K_H}}. \tag{16}$$

It should be emphasized that to describe screening and binding of ions at the membrane surface, equilibrium relations have been invoked. This is justifiable if the rate limiting step for the Na^+ transport is located in the interior of the membrane. Also, surface potentials have been calcu-

lated for a membrane phase of infinite thickness (biological membranes have a finite thickness). However, for a finite membrane thickness and surface charges at the interior surface only minor corrections have to be introduced (Neumcke, 1970; Läuger and Neumcke, 1973). Only unprotonated surface charges of the σ-type have been taken into account to determine the surface potential ψ_0 [equation (12)]. This is valid as long as the density of sodium channels is small compared with the density of surface charges and the contribution of free sites S^- to the surface charge σ then can be neglected.

b) Evaluation of Parameters. The model proposed contains 4 parameters: σ_0, $\mathrm{pK} = -\log \mathrm{K}$, $\mathrm{pK}_H = -\log \mathrm{K}_H$, and $\mathrm{pK}_{\mathrm{Na}} = -\log \mathrm{K}_{\mathrm{Na}}$. These parameters can be evaluated from the I_{Na}-V curves for solutions with various Na^+ and H^+ concentrations.

We need to assume that for a fixed potential difference between the outer membrane surface and the axoplasm, E_m, the sodium conductance is proportional to the fraction $a_{\mathrm{Na}\,S}/a_{St}$ of sites at the sodium channel which are occupied by Na^+. The voltage between the outside solution and the axoplasm, E, is the sum of the effective membrane potential, E_m, and the surface potential ψ_0 at the outer membrane surface; $E = E_m + \psi_0$.

When the pH value of the outer solution is lowered, ψ_0 becomes less negative due to protonation of negative surface charges, and a lower potential difference E is needed to give the same value of E_m. Therefore, the $g_{\mathrm{Na}}(V)$ curves are shifted towards more positive potentials. As a measure of this shift, $\Delta\psi$, we extrapolated the difference between the points of inflexion of experimental $g_{\mathrm{Na}}(V)$ curves where $g_{\mathrm{Na}}(V)$ is defined in the usual way by $g_{\mathrm{Na}}(V) = I_{\mathrm{Na}}/(V - V_{\mathrm{Na}})$. Gilbert and Ehrenstein (1970) equated $\Delta\psi$ with $\Delta\psi_0$ of the surface potentials. However, if one takes into consideration the voltage error due to the series resistance, $\Delta\psi_S = \Delta(u_3 - u_1)$, and the liquid junction potential, $\Delta\psi_{JP}$, between two test solutions I and II, one gets

$$\Delta\psi_0 = \Delta\psi - (\Delta\psi_S + \Delta\psi_{JP}). \tag{17}$$

The sign of these potential differences is as follows: $\Delta\psi_0 > 0$ if the pH value of test solution II is lower than that of test solution I; $\Delta\psi > 0$ if the point of inflexion of the $g_{\mathrm{Na}}(V)$ curve for solution II is located at a higher depolarization than for solution I; $\Delta\psi_S > 0$ for a lower current density in solution II than in solution I; and $\Delta\psi_{JP} > 0$ if solution II is positive with respect to solution I. For all solutions used, $\Delta\psi$ can be extrapolated from $g_{\mathrm{Na}}(V)$ curves, the determination of $\Delta\psi_S$ is described in the "method" section, and $\Delta\psi_{JP}$ can be obtained from Table 1.

Results for uncorrected and corrected shifts are compiled in Table 2. It is obvious that a substantial error would be introduced, if the change

Table 2. Measured and corrected shifts of curves relating sodium conductance and voltage (m: motor fibre, s: sensory fibre)

Experiment	Fibre type	Test solution No.	pH (15°C)	Measured shift $\Delta\psi$ [mV]	Correction $\Delta\psi_S + \Delta\psi_{JP}$ [mV]	Effective shift $\Delta\psi_0$ [mV]
17. 7. 73	s	1	6.4	0	− 1	1
GNR = 1		2	5.3	14	2	12
		3	4.9	21	2	19
18. 7. 73	m	1	6.4	0	− 2	2
GNR = 1, 2		2	5.3	14	1	13
		3	4.9	24	2	22
19. 7. 73	s	2	5.3	16	3	13
GNR = 2, 3						
19. 7. 73	s	2	5.3	16	1	15
GNR = 4, 5		3	4.9	28	1	27
20. 7. 73	m	1	6.4	− 2	− 2	0
GNR = 1, 2		2	5.3	12	2	10
		3	4.9	19	3	16
		4	4.3	27	3	24
24. 10. 73	m	8	5.5	20	8	12
GNR = 1, 2		9	5.0	28	8	20
		10	5.4	21	10	11
24. 10. 73	s	8	5.5	17	7	10
GNR = 20, 21		9	5.0	22	7	15
		10	5.4	18	9	9

$\Delta\psi_0$ in surface potential would be equated to the shift $\Delta\psi$ of the $g_{Na}(V)$ curves.

After having obtained the corrected shifts $\Delta\psi_0$ of the surface potential, the parameters σ_0 and pK can be determined in the same way as described by Gilbert and Ehrenstein (1970). Combining equations (9), (11) and (12), results in the following relationship between ψ_0 and pH which can be fitted to our experimental points $\Delta\psi_0$ (pH).

$$\frac{\sigma_0^2}{2RT\varepsilon_0\varepsilon}\left(\frac{K}{K + B\,a_H}\right)^2 = a_+(B-1) + a_-\left(\frac{1}{B} - 1\right) + a_{++}(B^2 - 1)\,. \quad (18)$$

Most of the univalent cations in Ringer's solution are Na^+ ions ($a_{Na} = 85$ mM), the activity of the remaining univalent cations (K^+, Tris cation) is estimated as 5 mM. In solutions with reduced Na^+ content,

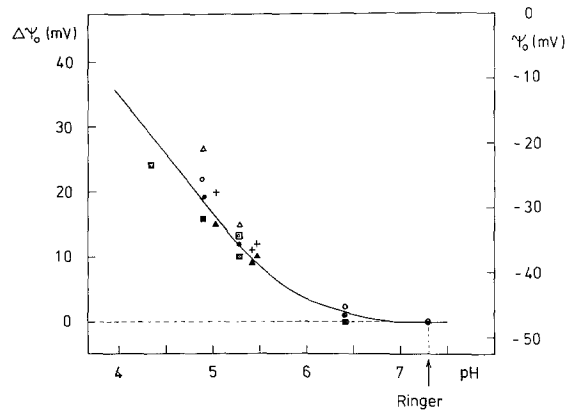

Fig. 5. Changes $\Delta\psi_0$ of membrane surface potential ψ_0, with respect to Ringer's solution as function of pH. Data taken from Table 2. The symbols refer to the following experiments: • 17. 7. 73 GNR = 1, ○ 18. 7. 73 GNR = 1, 2, □ 19. 7. 73 GNR = 2, 3, △ 19. 7. 73 GNR = 4, 5, ▪ 20. 7. 73 GNR = 1, 2, + 24. 10. 73 GNR = 1, 2, ▲ 24. 10. 73 GNR = 20, 21. Curve calculated with the parameters $\sigma_0 = -1\,e_0/(20\text{ Å})^2$, pK = 4.3. Righthand ordinate: Calculated surface potential ψ_0

equal amounts of NaCl are substituted by Choline chloride or Tris chloride. We therefore choose $a_+ = a_- = 90$ mM for all solutions. As Ca^{++} is the only divalent cation contained in the solutions: $a_{++} = a_{Ca} = 0.7$ mM.

The best fit to our experimental points occurs for the values $\sigma_0 = -1\,e_0/(20\text{ Å})^2$ (e_0: elementary charge), pK = 4.3 and is represented by the curve in Fig. 5. With these parameters, $\psi_0 = -47.6$ mV is calculated for pH = 7.3 (Ringer solution). Thus the absolute surface potential ψ_0 is known for arbitrary pH values, and this scale is drawn on the right hand side of Fig. 5.

As pointed out by Gilbert (1971), an increase of the surface charge density results in a higher maximum slope of the curve $\Delta\psi_0$ (pH), whereas an increase in pK mainly causes a shift of this curve to the right. From the uncertainty of slope and location of the fitted curve we then estimate that σ_0 lies in the range between $-1\,e_0/(18\text{ Å})^2$ and $-1\,e_0/(22\text{ Å})^2$ and pK between 4.2 and 4.4.

We now turn to the question of estimating the constant K_{Na} for the binding of Na^+ ions at the sites S^-. At increasing concentrations of sodium ions in the solution, the flux of Na^+ ions through the membrane should saturate when the activity a_{Na}' of sodium ions at the membrane surface exceeds the binding constant K_{Na} [see equation (15)]. To test this point we calculated maximum g_{Na} values from experiments with Ringer and solutions 5, 6, 7 of reduced sodium ion content. For Ringer's

Table 3. Maximum sodium conductance as function of a_{Na}.
(m: motor fibre, s: sensory fibre, R: Ringer's solution, $\bar{g}_{Na}$: relative maximum sodium conductance with respect to Ringer)

Experiment	Fibre type	Solution	a_{Na} [mM]	Maximum g_{Na} [Ω^{-1} cm^{-2}]	$\bar{g}_{Na}$
9. 8. 73	m	R	85	1.94	1
GNR = 3, 4, 5		5	44	0.975	0.502
		6	23	0.58	0.299
		7	12	0.36	0.186
16. 8. 73	s	R	85	1.60	1
GNR = 1, 2, 3		5	44	0.78	0.487
		6	23	0.49	0.306
		7	12	0.33	0.206
12. 10. 73	m	R	85	1.74	1
GNR = 1, 2		5	44	0.75	0.431
		6	23	0.41	0.236
		7	12	0.33	0.190
12. 10. 73	m	R	85	1.22	1
GNR = 3, 4		5	44	0.57	0.467
		6	23	0.37	0.303
		7	12	0.26	0.213
12. 10. 73	s	R	85	2.06	1
GNR = 20, 21		5	44	0.90	0.437
		6	23	0.46	0.223
		7	12	0.35	0.170

solution the function $g_{Na}(V)$ has a clear maximum which is located between $V = 50-60$ mV. Solutions 5, 6, 7 have rather flat maxima at higher depolarizations. But even for solution 7, with the lowest Na^+ activity, the maximum occurs within the voltage range covered by our experiments.

Table 3 represents values for absolute and relative maximum sodium conductances from 5 experiments. Due to the flat maxima of the conductance voltage curves for solutions with low sodium ion activities, almost the same values for $\bar{g}_{Na}$ follow if the maximum g_{Na} in Ringer and conductance values for solutions 5, 6, 7 at identical effective membrane potentials E_m are taken.

In Fig. 6 average $\bar{g}_{Na}$ values are plotted as function of the activity a_{Na} of sodium ions in the bulk solution. The values almost lie on the straight line connecting the origin with the reference point of Ringer's solution. From this linearity we conclude that no saturation of the sites S^- with

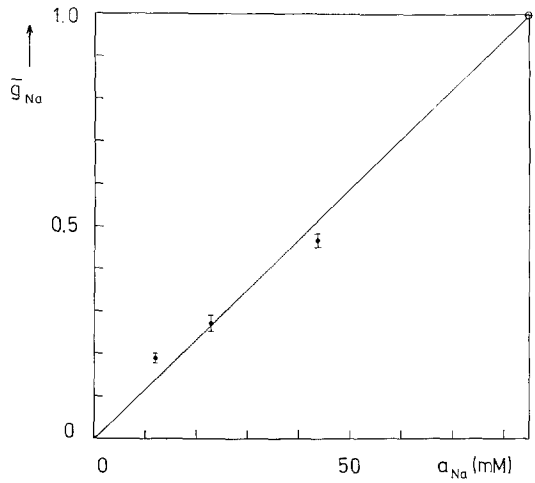

Fig. 6. Maximum sodium conductance $\bar{g}_{Na}$ as function of sodium ion activity a_{Na} in test solution normalized at $a_{Na} = 85$ mM (reference Ringer's solution). Average values and standard deviations from data of Table 3

Na^+ ions occurs up to the sodium ion activity $a_{Na} = 85$ mM in the bulk solution. The corresponding activity a_{Na}' at the membrane surface is:

$$a_{Na}' = 85 \text{ mM} \cdot \exp\left(\frac{F}{RT} \cdot 47.6 \text{ mV}\right) \cong 570 \text{ mM}.$$

This result indicates that

$$K_{Na} > 0.57 \text{ M}.$$

Therefore, the exact value of K_{Na} could be determined from experiments using solutions with sodium concentrations exceeding that for Ringer's solution. We did not perform such experiments because we wanted to avoid a drastic increase of the osmolality of the test solutions. But we can refer to results of Dubois and Bergman (1971) after which a half saturation of the inward Na^+ flux occurs at a sodium ion concentration of 170 mM in the bulk solution. With the same Boltzmann factor as above, the Na^+ concentration at the membrane surface then would be approximately 1 M. The binding constant K_{Na} is equal to the sodium ion activity at the membrane surface with half saturation of the Na^+ flux and will be lower than 1 M. A comparison between our results and those of Dubois and Bergman suggests that the actual value of K_{Na} is in the range 0.57 M and 1 M. It should be mentioned, however, that Woodhull (1972) claims that sodium currents increase linearly with the sodium concentration up to the highest concentrations used by Dubois and Bergman, thus suggesting that K_{Na} may be even greater than 1 M.

Table 4. Determination of parameter pK_H.
(m: motor fibre, s: sensory fibre)

Experiment	Fibre type	Test solution No.	pH (15°C)	pK_H
17. 7. 73	s	2	5.3	4.4
GNR = 1		3	4.9	4.5
19. 7. 73	s	2	5.3	4.6
GNR = 2, 3				
19. 7. 73	s	2	5.3	4.5
GNR = 4, 5		3	4.9	4.6
20. 7. 73	m	2	5.3	4.3
GNR = 1, 2		3	4.9	4.4
		4	4.3	4.4
24. 10. 73	m	8	5.5	4.5
GNR = 1, 2		9	5.0	4.3
		10	5.4	4.1
24. 10. 73	s	8	5.5	4.6
GNR = 20, 21		9	5.0	4.2
		10	5.4	4.1

The last parameter of our model to be determined is the equilibrium constant, K_H, for the binding of protons at the specific sites S^-. This constant was evaluated by comparing sodium conductances at several identical effective membrane potentials E_m for solutions of physiological (I) and reduced pH value (II). As $a_{Na}' < K_{Na}$, for our test solutions, formula 16 may be used for calculating the ratio $g_{Na}^{(I)}/g_{Na}^{(II)}$ as follows:

$$\frac{g_{Na}^{(I)}}{g_{Na}^{(II)}} = \frac{a_{Na}^{(I)} B^{(I)}}{a_{Na}^{(II)} B^{(II)}} \cdot \frac{K_H + B^{(II)} a_H^{(II)}}{K_H + B^{(I)} a_H^{(I)}} \tag{19}$$

$g_{Na}^{(I)}/g_{Na}^{(II)}$, $a_{Na}^{(I)}$, $a_{Na}^{(II)}$, $a_H^{(I)}$, $a_H^{(II)}$ are experimentally measured, and the surface potentials, necessary to calculate the Boltzmann factors $B^{(I)}$, $B^{(II)}$ can be read off from Fig. 5. Therefore, equation (19) can be solved for the parameter K_H.

Table 4 contains our results. From experiments, in which the test solutions 2, 3, 4, and 8 were used in addition to Ringer's solution, the average value for $pK_H = -\log K_H$ is $pK_H = 4.5$. With solutions 9 and 10 a lower value for pK_H was obtained. This difference was presumably due to the effects of various errors in the measurement of the small sodium currents recorded under these conditions.

At a fixed sodium ion activity in the solution, the maximum sodium conductance $\bar{g}_{Na}$ predicted by formula 16 should be proportional to

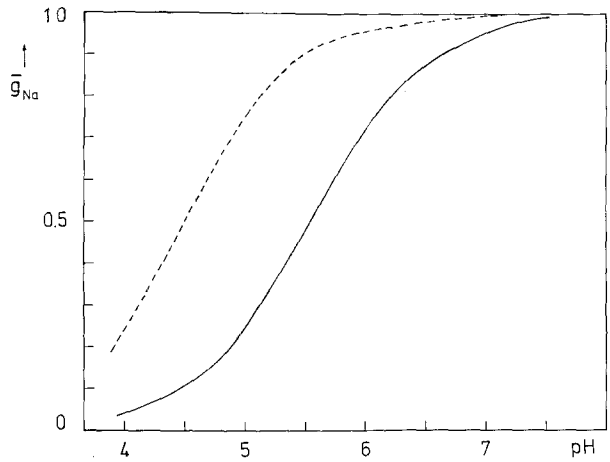

Fig. 7. Full curve: Calculated maximum sodium conductance $\bar{g}_{Na}$ as function of pH with intrinsic $pK_H = 4.5$ and normalized at high pH values. Interrupted curve: Calculated dissociation curve of an acid with $pK_a = 4.5$

$B/(1 + B\,a_H/K_H)$. In Fig. 7 this function (normalized at high pH values) is drawn for $pK_H = 4.5$ together with the dissociation curve $1/(1 + a_H/K_H)$ of an acid with the same pK value.

The most striking difference between the two curves in Fig. 7 is the displacement of the $\bar{g}_{Na}$ (pH) relation towards higher pH values. Nevertheless, the intrinsic pK value of the site S^- at the sodium channels is 4.5 as obtained from our analysis. In addition, a comparison between the full and interrupted curves reveals that the edges of the function $\bar{g}_{Na}$ (pH) are a bit smoother than those of the dissociation curve. Location and shape of our calculated $\bar{g}_{Na}$ (pH) curve are in agreement with other experimental results (Hille, 1968). Thus our model gives a good quantitative description not only for the shifts of conductance voltage curves, but also for the blocking of sodium channels at reduced pH values.

Discussion

Other authors have reported higher surface charge densities at the nerve membrane than those estimated in this work. However, only a few investigations refer to the surface charge near the sodium channels of the nodal membrane and can be compared with our results. Gilbert and Ehrenstein (1970), for example, using experimental data of Hille (1968) obtain an average spacing of 15 Å between univalent negative surface charges. Brismar (1973) arrives at 18 Å and Vogel (1973) at distances less than 10 Å. Hille, Woodhull, and Shapiro (1974) postulate three types of surface groups near the sodium channel in order to explain experiments with mono- and divalent cations. With Ringer as external solution, their

results can be fitted with a net surface charge corresponding to a charge separation in the range 10 to 14 Å. The average distance of 20 Å found in this work is the result of using ion activities instead of ion concentrations (and also of correcting the voltage shifts for changes in voltage drop across the series resistance). Without these refinements, higher surface charge densities near the sodium channel are obtained. Since the actual arrangement of surface charges is unknown, for simplicity we treated them as uniformly distributed over the nerve membrane. As pointed out by Cole (1969), to obtain a given surface potential, a discrete distribution of charges requires a higher density. Gilbert and Ehrenstein (1970) report a pK of 4.6 for the binding of protons at the unspecific membrane surface charges. Our pK is 4.3. This difference could be due to the use of activities and corrected voltage shifts in our calculations. For the binding of protons at the specific sites S^- at the sodium channel we have obtained a value pK_H of 4.5.

Since we estimated an uncertainty of 0.1 for pK values the difference between the pK values (4.3 and 4.5) may not be significant. The binding constants K, K_H are defined by equations (11) and (13) with the proton activity a'_H at the membrane surface. Therefore, pK and pK_H represent intrinsic pK values. The corresponding apparent pK values are equal to the bulk pH of the external solution at which half of the surface charges or sites S^- are protonated and are 0.5 units higher. An identification of chemical groups from derived pK values certainly is premature because the pK value may change considerably if free groups are incorporated into an interface (Overbeek, 1948; Yap, 1973). Only the application of specific reagents could clarify this point. With this method Shrager and Profera (1973) presented evidence for a carboxyl group near the TTX binding site of the sodium channels.

In this work the specific site S^- of the sodium channel was assumed to be on the outer membrane surface. With an intrinsic $pK_H = 4.5$ for this group the decrease of sodium currents at reduced pH values then could be described for all voltages investigated. Woodhull (1972, 1973) postulates a similar site in the interior of the sodium channel. These different interpretations probably are due to the formalisms which were employed to describe sodium currents. While in this work conductances were preferred, Woodhull used permeabilities.

One important complication inherent in the method used to evaluate K values (K_{Na} for example) arises from the possibility that in our experiments (with test solutions 5, 6, 7) not only Na^+ but also the $Tris^+$ could compete for S^-. For equal binding constants of Na^+ and $Tris^+$ at S^- no nonlinearity of the function $\bar{g}_{Na}$ (a_{Na}) could then be expected though most of the sites S^- would be occupied by Na^+ or $Tris^+$. More experiments with different sodium ion substitutes are needed to clarify this point. In addi-

tion we want to emphasize that a linear relation between $\bar{g}_{Na}$ and a_{Na} does not necessarily imply that the Na^+ ions pass the membrane according to the independence principle (Hodgkin and Huxley, 1952). A linear relation between I_{Na} and a_{Na} is compatible with this principle only for small variations of a_{Na} and at potentials V near V_{Na}.

In the determination of the density σ_0 of unspecific surface charges at the outer membrane surface we tacitly have assumed that there is no exchange between inner and outer membrane surface charges after a change of the external solution. Such a redistribution of surface charges recently has been proposed by McLaughlin and Harary (1974) and could be a possible explanation for the observed increase of sodium conductance after a treatment at low pH. As McLaughlin and Harary point out, charged phospholipids will traverse the membrane until a stationary state is reached. In extracellular solutions of low pH part of the external negative surface charges are being protonated and more internal negatively charged lipids will move outward by "phospholipid flip-flop." Shortly after returning to Ringer, therefore, additional negative charges are located at the external membrane surface and the surface concentration of Na^+ ions will be higher than normal. This could explain the observed excess sodium currents which relax within a few minutes.

In conclusion we summarize the range of parameters which were varied in our experiments: Depolarizations were applied up to $V = 140$ mV, the pH was varied between 4.3 and 7.3, and the effect of sodium ions was studied between $a_{Na} = 12$ mM and 85 mM. Therefore, no conclusions of this work can be applied to conditions exceeding this range. For example, experiments with different amounts of divalent cations may lead to a different picture of the nerve membrane. The measured voltage shift at a tenfold increase of the external Ca^{++} concentration ranges between 20 and 24 mV (Hille, 1968). However, only a 6.6 mV drop of surface potential follows with our parameters if the activity of Ca^{++} ions is raised by a factor 10 with respect to Ringer's solution and no binding of Ca^{++} ions occurs at the membrane. Therefore, a very tight binding of Ca^{++} had to be postulated to correspond with experimental results. It then seems to be more realistic to assume different types of surface charges with different binding properties as it was done by Hille, Woodhull, and Shapiro (1974).

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Part of the numerical evaluation was performed on the CD 3150 university computer in Homburg/Saar.

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Dr. H. Drouin
Dr. B. Neumcke
I. Physiologisches Institut
der Universität des Saarlandes
D-6650 Homburg/Saar
Federal Republic of Germany