Lyotropic Anions

Na Channel Gating and Ca Electrode Response

JOHN A. DANI, JORGE A. SANCHEZ, and BERTIL HILLE
From the Department of Physiology and Biophysics, University of Washington, Seattle, Washington 98195

ABSTRACT The effects of external anions on gating of Na channels of frog skeletal muscle were studied under voltage clamp. Anions reversibly shift the voltage dependence of peak sodium permeability and of steady state sodium inactivation towards more negative potentials in the sequence: methanesulfonate ≤ Cl− < acetate < Br− ≤ NO3 < SO4 < benzenesulfonate < SCN− < C104; approximately the lyotropic sequence. Voltage shifts are graded with mole fraction in mixtures and are roughly additive to calcium shifts. The peak PNa is not greatly affected. Except for SO4, these anions did not change the Ca++ activity of the solutions as measured with the dye murexide. Shifts of gating can be explained as the electrostatic effect of anion adsorption to the Na channel or to nearby lipid. Such adsorption is expected to follow the lyotropic series. Anions also interfere significantly with the response of a Ca-sensitive membrane electrode following the same sequence of effectiveness as the shifts of gating. The lyotropic anions decrease the Ca++ sensitivity and cause anomalously negative responses of the Ca electrode because these anions are somewhat permeant in the hydrophobic detector membrane.

INTRODUCTION

A wide variety of phenomena in biology and physical chemistry are affected by the anion in the bathing electrolyte solution. In many cases the effectiveness of the anions follows the Hofmeister or lyotropic or chaotropic series: SO4 < F− < Cl− < Br− < NO3 < I− < C104 < SCN−. Physicochemical examples include dissolving ("salting in") of small molecules and macromolecules; disaggregation, unwinding, and denaturation of macromolecules; and the tendency to bind or adsorb to proteins, gels, and air-water, lipid-water, or metal-water interfaces (Randles, 1963; Jencks, 1969; von Hippel and Schleich, 1969; Conway, 1977).

The lyotropic series is also found in work with excitable cells. For internally perfused squid giant axons, the longevity of excitability depends on the anion of the perfusion medium in the sequence SO4 < F− < Cl− < NO3 < Br− < I−.
For frog muscle fibers, sodium salts added to the external medium potentiate the strength of a twitch in the order Cl\(^-\) < Br\(^-\) < NO\(_3\) \(^-\) < I\(^-\) < SCN\(^-\) (Lillie, 1910; Kahn and Sandow, 1950, 1955; Hill and Macpherson, 1954; Horowicz, 1964). This twitch potentiation forms the background of our investigation.

Although other factors are also affected, the primary cause of twitch potentiation by anions such as nitrate or thiocyanate is a shift of the voltage dependence of contractile activation toward more negative potentials (Hodgkin and Horowicz, 1960; Kao and Stanfield, 1968). Thus, the muscle membrane is altered in such a way as to lower the threshold for mechanical activation. The effect of nitrate is complete within 10 s of a solution change and is equally readily reversed. Hodgkin and Horowicz (1960) suggested that lyotropic anions act by adsorbing to the muscle surface membrane, altering the electric field within the membrane. If anions can bind to some membrane components, then other voltage-dependent processes in the membrane might also be shifted. Indeed, Kao and Stanfield (1968) have demonstrated a shift of the voltage dependence of delayed rectifier K channels. Chao (1935) and Hutter and Padsha (1959) also found an increased electrical excitability of muscle, an effect which the latter authors ascribed partly to an observed increase of the membrane resistance when chloride was replaced by other anions and partly to an actual change of firing threshold. Kao and Stanfield (1968) reported that the "electrical threshold" for Na channel activation is not changed by lyotropic anions. We have studied this question again and have found that lyotropic anions do shift the voltage dependence of Na channel gating. Furthermore, as a consequence of control experiments to check for possible effects of these anions on calcium activity, we also discovered a strong effect of lyotropic anions on a Ca-sensitive, ion-selective electrode. Preliminary communication of these results has been made to the Biophysical Society (Dani et al., 1982; Sánchez et al., 1982).

**METHODS**

**Muscle Preparation and Voltage Clamp**

Segments of single muscle fibers from semitendinosus muscles of *Rana pipiens* were studied under voltage clamp as described by Hille and Campbell (1976). A fragment of twitch fiber, pinched off and pulled away from the edge of the muscle, was mounted with petroleum jelly and grease seals in the acrylic chamber containing a CsF internal solution (Table I) covering all pools and partitions. The fluid level was lowered to isolate electrically the four compartments, and the solution in the test pool (A pool) was replaced by the reference chloride solution. After a wait of 15–20 min to permit equilibration of temperature and ionic gradients in the fiber, voltage-clamp measurements of Na current (\(i_{Na}\)) were begun. The external test solutions given in Table I were designed to give small Na currents to reduce errors in the voltage clamp from large currents flowing in the external series resistance. This was accomplished by replacing most of the normal Na ion with impermeant tetramethylammonium ion (TMA), or by adding a small concentration of tetrodotoxin (TTX). The normal Ca\(^++\) concentration was 2 mM, but some other solutions were also used, differing only in the Ca\(^++\) concentration. For each solution change, 6–8 ml of precooled solution was
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injected through the 150-μl A pool. Recorded membrane potentials were corrected for junction potentials at the A-pool electrode measured with respect to a Beckman 29402 ceramic-junction, saturated-KCl reference electrode (Beckman Instruments, Inc., Fullerton, CA). Experiments were conducted at 15°C. A few experiments were done with very similar techniques on nodes of Ranvier from the sciatic nerve.

The voltage clamp, incorporating several improvements over that of Hille and Campbell (1976), was designed by Dr. W. Nonner (Nonner et al., 1979). A separate potential-measuring electrode was included in the A pool to reduce the effect of current flow in the external solution resistance. As before, however, electrical correction for most of the remaining external series resistance was still applied. Membrane currents were measured directly as the output of a current-to-voltage converter (200 kΩ recording resistance) placed after the clamp feedback amplifier, a circuit suggested to us by Dr. F. Sigworth (Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Federal Republic of Germany). The current signal was filtered with an active, four-pole, low-pass Bessel filter at 8 or 16 kHz and sampled digitally at 25- or 125-μs intervals by our LM² minicomputer (Kehl et al., 1965). Current and voltage were also monitored continuously on a strip chart recorder. All stimulus pulses and the digital sampling clock were generated by a locally built, digital stimulator,¹ which in turn was programmed by the LM² computer.

Protocol and Analysis

The experiments were designed to look for shifts in the voltage dependence of activation and inactivation gating in Na channels as the external anion was changed from chloride to methanesulfonate, benzenesulfonate, sulfate, acetate, bromide, nitrate, thiocyanate, and perchlorate. Sodium currents were elicited by 14-ms depolarizing test pulses. Leakage and capacity currents were removed by linear subtraction at two stages: first by a manually adjusted analog subtractor preceding the low-pass filter, and later by digital subtraction of the remaining waveform recorded in small steps made negative to the holding potential. Potassium currents were not a problem, as the CsF internal solution contained only blocking ions rather than permeant ions.

¹ Nonner, W., and T. H. Kehl. A stimulator complementing a computer in electrophysiological measurements. Manuscript submitted for publication.

### Table I

| Internal       | External A | External B | External C |
|---------------|------------|------------|------------|
| mM            | mM         | mM         | mM         |
| 110 CaF       | 2 CaX²*    | 2 CaSO₄   | 2 CaY²$   |
| 5 NaF         | 30 NaX*    | 15 NaSO₄  | 115 NaY²$ |
| 4 Na/MOPS§    | 8 Na/MOPS  | 8 Na/MOPS  | 8 Na/MOPS |
| 85 TMA-X*     | 60 (TMA)₂SO₄ | 3–6 nM TTX |

* X is Cl, Br, NO₃, methanesulfonate, acetate, or benzenesulfonate.
$ Y is Cl, SCN, or ClO₄⁻.
§ Morpholinopropanesulfonic acid buffer brought to pH = 7.4 with NaOH. Where possible, external solutions were made by adding cation hydroxides to the corresponding acids.
for K channels. To keep the resting state of Na channels constant despite shifts of
gating properties caused by the different anions, we readjusted the holding potential
in each solution to set steady state inactivation at approximately $h_m = 0.6$. The
protocols given here refer to voltage deviations from this adjusted holding potential.
In the Results, voltages are given on an absolute scale of inside minus outside.

The voltage dependence of activation was studied for depolarizing test pulses of
16–160 mV from holding, preceded by a 40-ms, 40-mV hyperpolarizing prepulse to
remove ordinary sodium inactivation. Peak $I_{Na}$ was measured at each voltage and
converted to peak sodium permeability ($P_{Na}$) by the Goldman (1943), Hodgkin and
Katz (1949) current equation. Finally, the cube root of the peak permeability
($P_{Na}^{1/3}$) was fitted by a function $Y$ related to the Boltzmann equation

$$Y(E) = Y_{max} \{1 + \exp[(E_m - E)/k_m]\}^{-1}, \tag{1}$$

where $E$ is the test-pulse potential, $E_m$ is the potential where $Y = 0.5 Y_{max}$, and $k_m$
is a measure of the steepness of the curve. The fitting was done with a nonlinear,
weighted, least-squares method. This procedure, related to the Hodgkin and Huxley
(1952) $m^3$ description of Na activation, gave a simple, empirical description of the
observations. Shifts were calculated as the change of the midpoint potential $E_m$ on
changing the solution and are considered negative when $E_m$ was more negative with
the test anion than with the chloride reference solution.

The voltage dependence of sodium inactivation was measured with 200-ms hyper-
polarizing and depolarizing prepulses followed by a 90-mV depolarizing test pulse.
Peak $I_{Na}$ was measured again and fitted to a function of the same form as Eq. 1 but
with $(E - E_h)/k_h$ in the exponential, where $E$ is the prepulse potential, $E_h$ is the
midpoint of the inactivation curve, and $k_h$ is a measure of its steepness. Shifts were
calculated as the change of $E_h$.

Measurements in a test solution were bracketed by measurements in the chloride
reference solution. If the bracketing reference values of $E_m$ and $E_h$ differed by >2–3
mV, the measurement was rejected. Final shift values were the difference of the test
measurement and the mean of the bracketing reference measurements. Values given
in the tables are expressed as means ± standard errors with the number of observations
in parentheses.

**Measurements with the Ca-sensitive Electrode**

To check whether the Ca ion activity in our solutions was affected by anions, we
made potential measurements with an Orion 93-20 Ca electrode (Orion Research
Inc., Cambridge, MA) with a freshly purchased 93-20-01 Ca-sensing module (Orion
Research Inc.) against a Corning calomel-saturated KCl fiber-junction reference
electrode (Corning Glass Works, Corning, NY). All of the solutions used on the muscle
fibers were assayed at 12 ± 1.5°C. The electrode was calibrated with solutions
containing 100 mM NaCl and 0.5, 2, 5, or 20 mM added CaCl$_2$ and with solutions
containing mostly TMA·Cl with some NaCl and known amounts of CaCl$_2$. The
readings were the same whether the solutions contained mostly TMA or NaCl. The
potentials were displayed on a digital pH meter and recorded on a strip chart recorder.
Solutions were well stirred and measurements with the standards were made repeat-
edly to guard against drift or possible irreversible effects of the anions. There were no
irreversible effects, but in the first solution, after an exposure to SCN$^-$ and ClO$_4^-$, the
electrode responded severalfold slower than usual.

When the electrode reported large apparent Ca activity decreases (i.e., very negative
electrode potentials) with lyotropic anions, we also studied the properties of the
electrode per se in a wider variety of solutions. In addition, we made a test for possible effects of anions on the reference electrode. In this test, 10 mM potassium biphalate was added to solutions of pure 100 and 500 mM NaCl and 100 and 500 mM NaSCN. Potassium biphalate is the primary pH standard for pH 4, so each solution should have been well buffered near pH 4. Assuming that pH glass is not affected by lyotropic anions, we used a Beckman 39500 pH electrode (Beckman Instruments, Inc.) measuring against the same reference electrode that had been used with the Ca electrode to record the pH of each solution. The two 100-mM solutions read pH 3.923 and 3.937, corresponding to a difference of 0.9 mV, and the two 500-mM solutions read pH 3.720 and 3.772, a difference of 2.7 mV. Hence, even at high concentrations, changing from Cl\(^-\) to SCN\(^-\) does not make large junction potential differences at the reference electrode.

Spectrophotometric Determination of Free Ca\(^{++}\)

Possible binding of Ca\(^{++}\) by the different anions was also studied spectrophotometrically using the metallochromic Ca\(^{++}\) indicator murexide (Ohnishi and Ebashi, 1963; Scarpa et al., 1978). A reference solution was prepared for each anion with 100 mM of its sodium salt, 50 μM murexide (J. T. Baker Chemical Co., Phillipsburg, NJ), and 10 mM Na-MOPS buffer at pH 7.4. Test solutions were identical except that various amounts of the Na ion were replaced with Ca\(^{++}\) ion. Murexide was used at a low concentration to avoid any significant reduction of free Ca\(^{++}\) by the formation of Ca-murexide complexes. A Varian Super Scan 1 recording spectrophotometer (Varian Associates, Inc., Palo Alto, CA) was used to obtain difference spectra between test solutions and the reference solution with the same anion (to control against effects of the anion on murexide). These difference spectra were all well behaved with the expected isosbestic point at 507 nm and a minimum at 540 nm (Scarpa et al., 1978). In the figures we plot the difference between these two values as an index to compare the free Ca\(^{++}\) in solutions with different anions.

RESULTS

Anions Shift Sodium Activation and Inactivation

The primary action of anions was to shift the voltage dependence of gating with little effect on the steepness or maximum sodium permeability. Fig. 1 illustrates in a semilogarithmic plot the effect of replacing all the Cl\(^-\) by SCN\(^-\) on the peak PN8 - E curve. Open symbols are measurements in Cl\(^-\), filled symbols are measurements in SCN\(^-\), and insets show some original records. The smooth curves are the fitted function, Eq. 1, raised to the third power. For this illustration only, the before and after reference points were considered together in generating the fitted curve. The experiment reveals that SCN\(^-\) shifts Na activation -13 mV relative to Cl\(^-\), after correction for junction potentials, while decreasing the maximum PN8 by 12% and changing the steepness of the voltage dependence of activation by <5%. Fig. 2 shows the change of the inactivation curve in the same experiment. Again there is a negative shift with SCN\(^-\), in this case -17 mV, and the steepness of the inactivation curve (k\(_{h}\)) is changed by only 2%.

All anions tested, except for methanesulfonate, gave negative shifts of activation and inactivation relative to Cl\(^-\). Table II summarizes the mean
observed shifts. The sequence of effectiveness was methanesulfonate \( \leq \) Cl\(^-\) \( \leq \) acetate \( \leq \) Br\(^-\) \( \leq \) NO\(_3\) \( \leq \) SO\(_4^{2-}\) \( \leq \) SCN\(^-\) \( \leq \) ClO\(_4^\)\(^-\), with benzenesulfonate being less effective than SO\(_4^{2-}\) and NO\(_3\) on inactivation and more effective on activation.\(^2\) Fig. 3 plots the shifts of \( E_m \) vs. the shifts of \( E_h \). These data, neglecting benzenesulfonate, fall within \( \pm 1.8 \text{ mV} \) of the line \( \Delta E_m = 0.7 \Delta E_h - 0.5 \), which suggests that in general anions shift activation less than inacti-

![Figure 1](image-url)

**Figure 1.** Shift of the voltage dependence of opening of sodium channels by full replacement of Cl\(^-\) by SCN\(^-\). Symbols are measured peak \( P_{\text{Na}} \) for step depolarizations from holding potentials of \(-90 \text{ mV} \) for Cl\(^-\) and \(-103 \text{ mV} \) for SCN\(^-\). The open triangles were recorded after exposure to SCN\(^-\). Smooth curves are drawn from Eq. 1 raised to the third power with \( E_m = -34.2 \text{ mV} \) and \( k_m = 12 \text{ mV} \) for Cl\(^-\), and \( E_m = -49.1 \text{ mV} \) and \( k_m = 13 \text{ mV} \) for SCN\(^-\). Insets are the original current records recorded with pulse potentials spaced at 9 mV from \(-42 \) to \(+86 \) for Cl\(^-\) and from \(-55 \) to \(+73 \) for SCN\(^-\). Calibration bars: 23 ms and 2 mA/cm\(^2\).

The results also show that the commonly used methanesulfonate anion is relatively inert with respect to shifts and that inorganic anions act on Na channel gating approximately in accordance with the classical lyotropic series Cl\(^-\) < Br\(^-\) < NO\(_3\) < ClO\(_4^\)\(^-\) < SCN\(^-\). Perchlorate has not been used in the recent muscle membrane literature but was suggested to us by Dr. S. G.

\(^2\) In the Discussion, Ca\(^{++}\) binding by SO\(_4^{2-}\) is shown to account for the low position of SO\(_4^{2-}\) in this series.
Figure 2. Shift of the voltage dependence of Na channel inactivation with full replacement of Cl\(^-\) by SCN\(^-\). Symbols are relative amplitudes of peak Na current measured with a 90-mV test pulse after different prepulse potentials. Smooth curves are drawn as described in the text with \(k_h = 6.3 \text{ mV}\) and \(E_h = -55.7 \text{ mV}\) for Cl\(^-\), and \(k_h = 6.4 \text{ mV}\) and \(E_h = -68.7 \text{ mV}\) for SCN\(^-\). Same fiber and holding potentials as in Fig. 1. Insets are the original current records recorded with prepulse potentials spaced at 8-mV intervals from \(-138\) to \(-42\) for Cl\(^-\) and from \(-151\) to \(-55\) for SCN\(^-\). Calibrations bars: 2 ms and 2 mA/cm\(^2\).

**TABLE II**

| Anion          | Activation shift | Inactivation shift |
|----------------|------------------|--------------------|
| \(\text{CH}_3\text{SO}_3^-\) | \(+0.1\pm0.4\ (8)\) | \(+1.5\pm0.3\ (7)\) |
| \(\text{CH}_3\text{COO}^-\)  | \(-2.6\pm0.5\ (5)\) | \(-0.7\pm0.8\ (5)\) |
| \(\text{Br}^-\)            | \(-3.7\pm0.4\ (7)\) | \(-3.5\pm0.5\ (8)\) |
| \(\text{NO}_3^-\)          | \(-3.6\pm0.6\ (14)\) | \(-6.6\pm0.6\ (13)\) |
| \(\text{SO}_4^-\)          | \(-4.2\pm0.7\ (6)\) | \(-7.9\pm0.4\ (6)\) |
| Benzenesulfonate           | \(-10.1\pm0.4\ (5)\) | \(-4.4\pm0.2\ (5)\) |
| SCN\(^-\)                  | \(-11.9\pm0.5\ (7)\) | \(-17.0\pm0.1\ (6)\) |
| Cl\(^-\)                   | \(-12.7\pm0.7\ (7)\) | \(-17.4\pm0.5\ (7)\) |

Shifts are defined as change of \(E_m\) (Eq. 1) or \(E_h\) (see text) when all the chloride is replaced by the given anion.
McLaughlin as a prototypical "chaotropic" ion in the chemical literature. Indeed, our results show that it even has a slightly stronger effect than SCN\(^-\), rather than the classic sequence \(\text{ClO}_4^- < \text{SCN}^-\). Another such ion, PF\(_6\), was also tried but it destroyed the muscle membrane before we could make a measurement.

Control experiments were done to test whether a high Na concentration is important to get the large shifts seen in SCN\(^-\). For this test, the Cl\(^-\) and SCN\(^-\) solutions were prepared as in solution A of Table I, except that Na was reduced all the way to 10 mM and tetraethylammonium was used in place of TMA. The shifts obtained on switching from Cl\(^-\) to SCN\(^-\) in these very low-Na solutions were $-11.1 \pm 1.3$ mV (10) for activation and $-12.9 \pm 0.9$ mV (8) for inactivation. Therefore, high Na is not necessary for a large shift. However, in the 115 mM Na experiments (Table II) the shift for inactivation was 4.1 mV larger.

The small effects of the tested anions on the relative maximum sodium permeability and on the steepness of the activation and inactivation curves are summarized in Table III. None of these parameters is changed by $>22\%$. The anions are listed in the sequence of increasing shift, but the effects on

![Graph showing \(\Delta E_m\) and \(\Delta E_n\) for different anions.](image)
maximum $P_{Na}$ values and steepness evidently do not follow this lyotropic sequence.

Several similar experiments had been done in this laboratory with Na currents of nodes of Ranvier. In observations repeated on two nerve fibers, the sodium activation curve was shifted an average of $-3.3$ mV in $Br^-$ and $-16.9$ mV in $NO_3^-$ relative to $Cl^-$. In other measurements done on a single myelinated fiber together with Dr. Philip Miles (West Virginia University, Morgantown, WV), the corresponding shifts of sodium inactivation were: $-2.0$ formate, $-5.4$ acetate, $-6.8$ lactate, $-11.3$ butyrate, $-11.9$ propionate, $-13.7$ benzenesulfonate, $-15.8$ valerate, $-16.4$ trichloroacetate, and $-19.5$ mV benzoate. These values were not corrected for junction potentials but suggest that Na channels of vertebrate nerve are even more sensitive to lyotropic and organic anions than those of muscle.

| Anion (A)       | $P_{max}^A/P_{max}^{Cl^-}$ | $k_m$  | $k_h$  |
|-----------------|---------------------------|--------|--------|
| $Cl^-$          | 1.00                      | 11.7±0.1 (106) | 7.1±0.1 (102) |
| $CH_3SO_3^-$    | 0.84±0.02 (8)             | 10.6±0.2 (8) | 7.4±0.3 (6) |
| $CH_3COO^-$     | 0.84±0.02 (5)             | 10.2±0.1 (5) | 7.4±0.3 (5) |
| $Br^-$          | 1.03±0.02 (7)             | 12.3±0.4 (7) | 6.8±0.2 (8) |
| $NO_3^-$        | 0.95±0.03 (14)            | 13.6±0.4 (14) | 5.8±0.1 (13) |
| $SO_4^2-$       | 0.85±0.01 (6)             | 10.7±0.2 (6) | 7.3±0.1 (6) |
| Benzenesulfonate| 0.78±0.02 (5)             | 10.3±0.4 (5) | 7.0±0.2 (5) |
| SCN$^-$         | 0.86±0.03 (7)             | 11.8±0.4 (7) | 6.3±0.1 (6) |
| $ClO_4^-$       | 0.92±0.03 (7)             | 12.9±0.7 (7) | 6.5±0.1 (7) |

Values were determined by fitting Eq. 1 to observations. $P_{max}$ is the cube of the $Y_{max}$ values determined in activation experiments.

The Shifts Are Graded with Concentration

To test whether anions act by binding to high-affinity sites at the membrane surface, we looked for evidence of saturation as increasing amounts of SCN$^-$ were added to the solution. Fig. 4 is a semilogarithmic plot of normalized peak $P_{Na} - E$ curves with $Cl^-/SCN^-$ mixtures containing 0, 10, 50, and 100% SCN$^-$. Shifts of activation and of inactivation from such curves are summarized in Fig. 5. Although the dose-response relationship may fall more steeply at low SCN$^-$ concentrations, it continues to fall at the highest concentration and has not leveled off. If the shift is due to binding of SCN ions to a single class of membrane binding sites, the apparent dissociation constant of the sites would probably be $>60$ mM.

Anion Shifts and Calcium Shifts Are Additive

Negative shifts of gating similar to those seen with lyotropic anions also occur when the calcium ion activity of the external solution is lowered (Franken-
haeuser and Hodgkin, 1957; Campbell and Hille, 1976). Thus, the shifts with anions would be understandable if the anions lowered the bulk Ca\(^{++}\) activity or displaced Ca ions from sites near Na channel voltage sensors. Direct measures of Ca\(^{++}\) activity are reported in later sections. This section approaches the possible interactions between SCN ions and Ca ions using physiological experiments. We measured shifts induced by Ca\(^{++}\) concentration changes in 100% Cl\(^{-}\) and in 100% SCN\(^{-}\) Ringer solutions. As has previously been reported for these muscles (Campbell and Hille, 1976), raising the [Ca\(^{++}\)] in Cl\(^{-}\) Ringer (open symbols, Figs. 6A and B) gives positive shifts, and lowering it gives negative shifts that level off near \(-15 \text{ mV}\) at very low [Ca\(^{++}\)].

![Figure 4](image)

**Figure 4.** Shifts of the voltage dependence of opening of sodium channels in mixtures of SCN\(^{-}\) with Cl\(^{-}\). Symbols are peak normalized P\(_{Na}\)-E curves. Smooth lines were calculated according to Eq. 1 raised to the third power with shifts of \(-2.7 \text{ mV}\) in 10% SCN\(^{-}\), \(-6.9 \text{ mV}\) in 50% SCN\(^{-}\), and \(-11.9 \text{ mV}\) in 100% SCN\(^{-}\).

This negative limit is determined by the weak, calcium-like effect of the remaining monovalent cations (Hille et al., 1975). However, whether the [Ca\(^{++}\)] is high or low, replacing all the chloride with thiocyanate gives an additional large negative shift (filled symbols, Fig. 6). The total shift in low-Ca, SCN\(^{-}\) Ringer is 10 mV more negative than can be obtained by removing all the Ca\(^{++}\) from Cl\(^{-}\) Ringer. Therefore, SCN\(^{-}\) could not simply be lowering the activity or the membrane binding of Ca ions. In the concentration range studied, lyotropic anions and Ca ions seem to have nearly additive effects.

Although the Ca\(^{++}\) effects must be attributed to some combination of Ca\(^{++}\) binding or screening of charges in the neighborhood of Na channels, no
experiment has revealed the relative contribution of these two mechanisms. To see the effects of screening alone, Dr. S. G. McLaughlin suggested to us that we replace $Ca^{++}$ by a larger divalent organic ion that might not bind, ethylene bis(trimethylammonium), which we shall call ethamethonium. We did this using a sample of ethamethonium bromide that he kindly provided, keeping $30 \text{ mM Na}^+$ and a constant $40 \text{ mM Br}^-$ in all solutions. In two experiments with $20 \text{ mM}$ of this salt and no $CaCl_2$, activation and inactivation were shifted $-14$ and $-19 \text{ mV}$ (with respect to $2 \text{ mM CaCl}_2$), whereas with

![Graph showing shifts of activation and inactivation at various SCN concentrations.](image)

The relative $Ca^{++}$ activities of the test and reference solutions were studied first with the Orion Ca electrode. The solid symbols in Fig. 7 define the $20 \text{ mM CaCl}_2$ and no ethamethonium they were shifted $+24$ and $+22 \text{ mV}$ in the same fibers. Effectively, the fibers acted as if no divalent ion were in the solution when there was $20 \text{ mM}$ ethamethonium. The experiments were complicated by a remarkable suppression of outward Na currents in all ethamethonium solutions.

**Measurements of Relative $Ca^{++}$ Activities**

The relative $Ca^{++}$ activities of the test and reference solutions were studied.
FIGURE 6. A. Voltage shift of activation in Cl\(^-\) (open symbols) and SCN\(^-\) solutions (filled symbols). Shifts are plotted against the concentration of added Ca\(^++\), with the horizontal line indicating the value for 2 mM Ca\(^++\). Each point is the average of at least five observations. The open triangles are values taken from Campbell and Hille (1976). The curves are calculated with the model discussed in the text and used in Fig. 5. B. Voltage shifts of inactivation in Cl\(^-\) and SCN\(^-\). The smooth lines are the same model as in A except \(K_{SCN} = 10\) mM. Each point is the average of at least five observations. When not shown, the standard error was less than the size of the symbols.
calibration curve obtained with the stated CaCl₂ concentration added to 100 mM NaCl (or NaCl plus TMA·Cl), and the solid line indicates the expected Nernstian slope, 28.3 mV per 10-fold increase of calcium activity. Open symbols give the electrode output for the test solutions, which contained different anions and 2 mM added calcium. Changing the anion does change the electrode output with a sequence of effectiveness (CH₃SO₃⁻ < Cl⁻ < Br⁻ < CH₃COO⁻ < benzenesulfonate < NO₃⁻ < SO₄²⁻ ≫ SCN⁻ ≫ ClO₄⁻) similar to that for shifting gating of Na channels. Another lyotropic anion, PF₆⁻, also gave very negative electrode readings like those in SCN⁻ and ClO₄⁻. According to the electrode, the Ca²⁺ activity falls to 3 and 0.3 µM in SCN⁻ and ClO₄⁻ test solutions, respectively. Minor differences in activities would not have been surprising, but such a large drop with SCN⁻ and ClO₄⁻ was quite unexpected. Tables of association constants do not suggest any strong Ca²⁺ complexation by SCN⁻ or ClO₄⁻. The Ca(ClO₄)₂ salt is soluble up to 8 M, and the activity coefficient of the salt is actually higher than that of CaCl₂ at all concentrations. For example, at 0.1 M, the activity coefficient is 0.557 compared with 0.518 for CaCl₂ (Robinson and Stokes, 1965). Therefore, we hypothesized that
lyotropic anions have special effects on the Ca electrode, and we tried another method to compare Ca$^{++}$ activities of our solutions.

Murexide solutions change color visibly as calcium salts are added in the range 0.5 to 20 mM calcium. The filled circles in Fig. 8 show the differential absorbance change ($A_{540} - A_{507}$) of Cl$^{-}$ solutions containing different amounts of calcium. The signal, caused by calcium binding to the dye, is nicely graded with concentration and could be used as a sensitive index of calcium activities at these relatively high concentrations. The other symbols in Fig. 8 show the murexide signals for solutions with other test anions. Within the scatter of the results, the majority of points fall on the curve for Cl$^{-}$. Thus, the Ca$^{++}$ activity is approximately the same in Cl$^{-}$ solutions as it is in solutions with ClO$_4^{-}$.
SCN\(^-\), NO\(_3^-\), CH\(_3\)SO\(_3^-\), Br\(^-\), CH\(_3\)COO\(^-\), benzenesulfonate, or propionate. Three other anions did reduce the activity appreciably. These were F\(^-\), which reduced it to near zero, and SO\(_4^{2-}\) and maleate, both of which reduced the activity to 27–34% of the Cl\(^-\) values in the concentration range studied. Calcium binding by these ions is well known. Although they were not used in the external solutions for our muscle experiments, fluoride, maleate, and propionate were included in the murexide measurements because of their use in physiology as Cl\(^-\) substitutes. In conclusion, of all the anions we studied, only SO\(_4^{2-}\) should have bound Ca ions and caused negative shifts of gating and a more negative potential in the Orion Ca electrode. The pronounced effects of the other lyotropic cations therefore have another explanation.

The Electrode Is Selective for Lyotropic Anions

A direct effect of anions on the Ca electrode was easy to demonstrate with Ca-free solutions. Table IV compares the electrode potentials recorded in NaCl, NaNO\(_3\), and NaSCN solutions containing a small quantity of EGTA to chelate any residual Ca\(^+\). As the NaCl concentration is increased from 0.1 to 2 M, the electrode potential becomes less negative, reflecting the slight Na sensitivity of the Ca electrode (documented in the instructions received with the electrode). With an increasing concentration of NaNO\(_3\), the potential again goes more positive (although it remains more negative than in NaCl). However, with NaSCN, the potentials start much more negative and go still more negative with increasing concentration. The electrode is responding as if it were more permeable to SCN ions than to Na ions. The hypothesis that the electrode has a selectivity for some anions is also suggested by the measurements of Table V, where 0.5-M, Ca-free solutions of NaCl and NaSCN are mixed, keeping the Na\(^+\) concentration constant. As little as 10 mM SCN\(^-\) makes the electrode output more negative. The hypothesis was tested more quantitatively by studying the Ca sensitivity in NaCl, NaSCN, and mixtures of the two. The results, shown in Figs. 9A and B, can be fitted fairly well by a theory (solid lines) given in the Discussion based on a finite permeability to SCN ions in the hydrophobic membrane of the Ca electrode.

The Orion electrode uses a thick hydrophobic support membrane saturated with decanol containing a Ca ionophore. To test whether lyotropic ions have a measurable permeability in an undoped layer of liquid decanol, we placed
TABLE V
ANION SENSITIVITY OF Ca ELECTRODE IN Ca-FREE SOLUTION

| [SCN⁻] (mM) | [Cl⁻] (mM) | Electrode output (mV) |
|-------------|-------------|----------------------|
| 0           | 500         | -80.6                |
| 1           | 499         | -80.5                |
| 10          | 490         | -84.2                |
| 50          | 450         | -104.1               |
| 200         | 300         | -107.8               |
| 400         | 100         | -129.3               |
| 500         | 0           | -135.0               |

Most potentials are the means of two measurements. Solutions contained NaSCN, NaCl, and 0.3 mM EGTA.

Figure 9. Demonstration of SCN⁻ responses in the Ca electrode. A. Electrode response in solutions of NaCl or NaSCN. Aliquots of 0.1 M CaCl₂ were added to an initial solution of 0.1 M NaCl (Δ). Aliquots of 0.1 M Ca(SCN)₂ were added to an initial solution of 0.1 M NaSCN (▲). B. Electrode response in mixtures of NaCl and NaSCN. Aliquots of 0.1 M CaCl₂ were added to 0.5-M solutions containing mixtures of NaCl and NaSCN as indicated. The solid lines in A and B are theoretical curves, obtained from Eq. 3 with $K_{Ca} = 0.08$ mM, $K_{Na} = 1$ M as apparent dissociation constants, and $P_{SCN}/P_{Ca} = 1/4$, $P_{Na}/P_{Ca} = 1/2,800$. Other combinations of constants also gave reasonable fits.
a small drop of 99% decanol across a hole drilled in a Teflon cup and measured potential changes as the ions bathing the two sides were changed. Dilution potentials showed the decanol to be more permeable to SCN\(^{-}\) or Cl\(^{-}\) than to Na\(^{+}\), and bionic potentials showed SCN\(^{-}\) to be about 15 times more permeant than Cl\(^{-}\). For example, with 0.1 M NaCl on one side and 0.1 M NaSCN on the other, the potential was -63 mV, NaCl-side negative. With 2 M NaCl on both sides, the decanol drop had a resistance of \(~100 \text{ M}\Omega\) and with 2 M NaSCN it fell to \(~10 \text{ M}\Omega\). Clearly, the decanol is permeable to SCN ions.

**DISCUSSION**

*Comparison with Previous Work on Excitable Membranes*

We found that lyotropic anions, particularly SCN\(^{-}\) and ClO\(_4\), shift the voltage dependence of Na channel activation and inactivation in frog muscle and nerve. The effect on inactivation is generally somewhat stronger than that on activation. Our work agrees well with Koppenhöfer's (1965) study of nodes of Ranvier in *Xenopus* nerve fibers. Using the maximum rate of rise of action potentials as a criterion, he found shifts of the inactivation curve following the sequence Cl\(^{-}\) < pyroglutamate < NO\(_3\) < acetylglycinate < SCN\(^{-}\) with the shift in SCN\(^{-}\) being -15.5 mV (see also Hashimura and Osa, 1963). The firing threshold for action potentials was also decreased in NO\(_3\) and SCN\(^{-}\), but only by a few millivolts.

These observations fit generally with the long history of findings that lyotropic anions potentiate the twitch (Lillie, 1910; Kahn and Sandow, 1950, 1955; Hill and Macpherson, 1954) and the electrical and chemical irritability of skeletal muscle (Chao, 1935; Hutter and Padsha, 1959; Hodgkin and Horowicz, 1960; Kao and Stanfield, 1968). The older literature, reviewed by Horowicz (1964), is difficult to interpret mechanistically, however, since external anions also have profound transient effects on membrane potential and lasting effects on resting membrane resistance in muscle (Hodgkin and Horowicz, 1959, 1960; Hutter and Padsha, 1959; Horowicz, 1964), in addition to effects on voltage-dependent processes. Two studies with two-microelectrode techniques disagree on whether anions affect Na channels. Hutter and Padsha (1959) report that NO\(_3\) facilitates "subthreshold regenerative potential changes," whereas Kao and Stanfield (1968) report that 115 mM NO\(_3\) or 58 mM SCN\(^{-}\) gives insignificant shifts (-1 and -1.5 mV) of the "electrical threshold" for an inward sodium current. In our hands these two solutions shift Na channel activation by -3.6 and -6.0 mV. However, Na inactivation is shifted even more, and in a threshold experiment, if the holding potential is not changed to compensate, the resulting inactivation of channels would tend to obscure the changes in activation (cf. Hashimura and Osa, 1963; Koppenhöfer, 1965; Horowicz, 1964). In Kao and Stanfield's work, the fibers actually depolarized 5-6 mV in SCN\(^{-}\), which despite a constant holding potential of the point clamp could further increase the already enhanced resting inactivation in the neighboring membrane.

The muscle literature documents large negative shifts of two other voltage-dependent membrane functions: the delayed rectifier K channel, and the
excitation-contraction coupling mechanism of transverse tubules. Thus, Kao and Stanfield (1968) report shifts of \(-9.7\) and \(-12.3\) mV for these two functions in 115 mM NO\(_3^-\), and \(-18.7\) and \(-17.0\) mV in 58 mM SCN\(^-\). Hodgkin and Horowicz (1960), using a less quantitative method, estimate a shift of contraction threshold of about \(-5\) mV both in 120 mM NO\(_3^-\) and in 12 mM SCN\(^-\). The shifts of delayed rectification and contraction found by Kao and Stanfield (1968) are about twice those we found for Na inactivation in muscle. To summarize, all voltage-dependent membrane functions that have been tested are shifted in the negative direction by lyotropic anions, although the magnitude of the effect varies among functions and tissues.

The shifting actions of SO\(_4^2^-\) must be compounded from at least two effects: direct actions on the membrane, and reduction of the free-Ca\(^{++}\) concentration. The Ca\(^{++}\)-related component can be estimated from our measurements. According to the murexide experiments (Fig. 8) and also to the Ca-sensitive electrode experiments (Fig. 7), a SO\(_4^2^-\) Ringer with 2 mM Ca\(^{++}\) added has the same Ca\(^{++}\) activity as a Cl\(^-\) Ringer with 0.6 mM Ca\(^{++}\) added. Changing from 2 mM Ca\(^{++}\) to 0.6 mM Ca\(^{++}\) in a Cl\(^-\) Ringer would shift Na channel activation by approximately \(-8.5\) mV and inactivation by \(-7.5\) mV (Fig. 6). Subtracting these shifts from the total shifts in SO\(_4^2^-\) (\(-4.2\) and \(-7.9\) mV, Table II) gives the shifts attributable to the membrane actions of SO\(_4^2^-\) of \(+4.3\) and \(-0.4\) mV. Thus, on average, SO\(_4^2^-\) probably would give a positive shift if it did not complex with Ca\(^{++}\), placing it in the appropriate position for the classical lyotropic sequence SO\(_4^2^-\) < Cl\(^-\) < Br\(^-\), etc.

Many previous investigators have used isotonic SO\(_4^2^-\) Ringer in studies of skeletal muscle, usually adding extra Ca\(^{++}\) to bring the Ca\(^{++}\) activity closer to that in Cl\(^-\) Ringer. Hodgkin and Horowicz (1959) initiated the practice of using 8 mM Ca\(^{++}\) and suggested that this would be equivalent to 1 mM free Ca. We measured murexide spectra only up to 5 mM Ca\(^{++}\) in SO\(_4^2^-\), but estimate by extrapolation that 8 mM Ca\(^{++}\) is equivalent to 2.1 mM Ca\(^{++}\) in Cl\(^-\) solutions. Rather than attempting to use a dissociation constant for CaSO\(_4\) (which requires making assumptions about activity coefficients for mixtures of monovalent and divalent salts), we suggest the rule of thumb that \(~30\%\) of the Ca\(^{++}\) is free in isotonic SO\(_4^2^-\) over the range of 0.5–8 mM added Ca\(^{++}\).

Other investigators have used Ca-sensitive electrodes to determine Ca\(^{++}\) binding by anions used to replace Cl\(^-\) in Ringer (e.g., Christofferson and Skibsted, 1975; Pollard et al., 1977; Kenyon and Gibbons, 1977). An Orion electrode was used in the latter two papers. Our work shows, however, that such electrodes report artifactually low Ca\(^{++}\) activities in the presence of lyotropic anions, so they should be used with caution. For example, our murexide measurements confirm Kenyon and Gibbons's estimate of \(~60\%\) Ca\(^{++}\) binding by maleate and no binding by methanesulfonate, but disagree with their suggestion of significant Ca\(^{++}\) binding by propionate.

**Adsorption of Lyotropic Anions Is Well Known in Physical Chemistry**

In order to cause shifts, anions must interact with components of the membrane. Indeed, there are many precedents for selective adsorption of lyotropic
anions with consequent electrostatic effects at interfaces and macromolecules. We review three of many examples in the colloid chemical literature that will be useful in our later analysis.

Hofmeister (1890) first recognized the lyotropic series of anions and cations in his studies of the salting out of ovalbumin. Subsequent work showed that the solubility, folding, and solution interactions of many proteins, indeed of almost any small or large molecule with apolar groups, are affected by the lyotropic series (Jencks, 1969; Record et al., 1978). There must, therefore, be a special interaction between the anions and the dissolved molecule. Scatchard's laboratory made a clear analysis of anion binding to serum albumin. As Na salts of different anions are added to a salt-free solution of serum albumin the solution pH increases by up to a pH unit because anions bind to the protein making it more negative, raising the apparent pKₐ of its ionizable groups and drawing protons from the solution (Scatchard and Black, 1949). The sequence of effectiveness at low concentration is Cl⁻ > F⁻ > Br⁻ > NO₃⁻ > I⁻ > SCN⁻ > ClO₄⁻ > p-toluenesulfonate > trichloroacetate. For 118 mM of added salt, 6 mol of an acid (per mole of protein, 69,000 daltons) must be added to restore the original solution pH with NaCl, and 24 mol of acid with NaSCN. The results can be summarized by a model postulating 1 high-affinity, 4 medium-affinity, and 22 low-affinity binding sites on serum albumin with intrinsic dissociation constants of 0.9, 14, and 100 mM for Cl⁻ and 0.008, 0.5, and 26 mM for SCN⁻ (Scatchard et al., 1957; Scatchard and Yap, 1964).

Lyotropic anion adsorption to lipid bilayers and lipid vesicles are also well documented. The adsorption gives a net negative surface charge to bilayers made from the zwitterionic phospholipid phosphatidylethanolamine (McLaughlin et al., 1975). The development of a negative surface potential on switching from NaCl to NaClO₄ can be observed in three ways: as an increase in the membrane conductance to a positive permeant species (the K⁺-nonactin complex); as a decrease of the conductance to a negative permeant species (the anion of a hydrophobic weak acid); and as an increased tendency for phospholipid vesicles to migrate toward the anode in an electrophoretic experiment. The order of increasing effectiveness is Cl⁻ < SCN⁻ < ClO₄⁻ < PF₆⁻, and the effects are graded continuously with concentration, at least up to 390 mM salt. For example, surface potential changes on adding NaClO₄ are 0, -10, -25, and -35 mV for 25, 100, 250, and 390 mM ClO₄⁻. Similar effects are seen with the zwitterionic phospholipid phosphatidylcholine, and much smaller effects are seen with the neutral lipid glycerol monooleate. Dr. Stuart McLaughlin was kind enough to measure for us the zeta potential changes with a negatively charged phospholipid, brain phosphatidylserine, using the electrophoretic methods of McLaughlin et al. (1975). In 100 mM NaCl with 10⁻⁴ M EDTA to remove traces of divalent ions, the zeta potential was -58.0 ± 1.4 (n = 30). In similar solutions containing NaSCN, NaN₃, or NaClO₄ instead, the potentials were insignificantly different and ranged from -56.8 to -58.6 mV. Evidently, the existing negative surface potential of the negative lipid is enough to prevent significant binding of lyotropic anions from the 100 mM solutions of lyotropic salts.

Lyotropic anion effects even occur at air-water interfaces in the absence of
added solutes. When low concentrations of almost any salt are added to pure water, the surface tension increases (Randles, 1963), which indicates from the Gibbs adsorption isotherm that the ionic concentration near the air-water interface is less than in the bulk solution. As the concentration of added salt is increased, the surface deficit grows, but it grows much more for salts such as KCl and K$_2$SO$_4$ than for KSCN or KClO$_4$. Thus, although almost all ions are desorbed from the surface, lyotropic anions are desorbed less or even adsorbed. They enter the interfacial layer preferentially where they set up a surface potential that can be measured with an ionizing electrode in the air adjacent to the interface. For example, the addition of 300 mM KSCN to pure water changes the surface potential by 25 mV, air-side negative, whereas KCl changes it at most 1 mV (Frumkin, 1924; Randles, 1957). The sequence of effectiveness is F$^-$ < Cl$^-$ < Br$^-$ < I$^-$ < ClO$_4^-$ < SCN$^-$ < PF$_6^-$.

These three examples, serum albumin, phospholipid bilayers, and the air water interface, all show that lyotropic anions in the 100 mM concentration range have a graded adsorption in boundary layers where they can produce electrostatic effects equivalent to tens of millivolts. Many similar, well-studied examples are available in the literature. Therefore, one would expect all membrane properties that are sensitive to the membrane electric field to be shifted by lyotropic anions. The question would not be whether they are shifted, but only how much they are shifted. In addition, the lyotropic anions could favor processes that lead to more unfolding of proteins and that expose more macromolecule surface to the solution. The existence of lyotropic effects does not, however, distinguish whether lipids, proteins, or both are involved in a response.

Despite its ubiquitous significance, lyotropic adsorption is not yet completely explained from first principles. The discussions hinge on water structure, entropy, and energy in interfacial zones around hydrophobic groups, and in the hydration sphere of anions (Randles, 1963; Jencks, 1969; von Hippel and Schleich, 1969). Measurements with neutral model compounds show that for binding to follow the lyotropic series requires a combination of two molecular attributes (a) appropriate anion-attracting groups, like an amide dipole, and (b) neighboring hydrophobic groups. Thus, polystyrene columns, which have neutral benzene side chains, show no interactions with salts, whereas polyacrylamide columns, which have neutral amide side chains on a hydrocarbon backbone, retard salts in accordance with the lyotropic series (von Hippel et al., 1973). Similarly, formamide discriminates weakly among salts, whereas its methyl derivatives acetamide, N-methylformamide, N-methylacetamide, etc., have well-developed lyotropic selectivity (Hamabata and von Hippel, 1973). The stronger anion binding sites of serum albumin are thought to be at arginine groups in a hydrophobic cavity as well (Record et al., 1978).

To summarize, then, the affinity for adsorption most strongly favors the lyotropic series when cationic groups or dipoles act to pull anions into water near hydrophobic groups. Molecular dynamics simulations of a dipeptide in water show changes of water structure in the immediate neighborhood of
hydrophobic groups. The translational and rotational mobility of water molecules in contact with the polar atoms of the dipeptide are normal (like bulk water), whereas mobility next to the methyl and methylene groups is reduced severalfold (Rossky and Karplus, 1979). This conformationally and dynamically restricted water, in combination with the neighboring region of low dielectric constant, is evidently more favorable for the approach of large anions of low charge density and weak hydration ($\text{ClO}_4^-$) than of small anions with strong hydration ($\text{Cl}^-$).

A related property of lyotropic anions that we will come back to is that they are less hydrophilic than small anions. They have lower hydration energy and more favorable free energies of transfer from water into nonaqueous media as compared with $\text{Cl}^-$ (Cox et al., 1974). The anions SCN$^-$, $\text{ClO}_4^-$, and PF$_6^-$ all have a small but measurable electrical conductance in undoped phospholipid (plus decane) bilayer membranes (McLaughlin et al., 1975; Gutknecht and Walter, 1982; O. S. Andersen, personal communication). We have shown that SCN$^-$ has a relatively high conductance in thick membranes of the relatively more polar solvent decanol.

The Mechanism of Shifts with Lyotropic Anions

Hodgkin and Horowicz (1960), already well aware of the literature on adsorption of anions to macromolecules, suggested that lyotropic anions shift the contractile threshold of muscle because they adsorb selectively to the membrane, and because of their negative charge, decrease the steepness of the local potential gradient within the resting cell membrane. Shifts of Na channel gating caused by changes in monovalent and divalent cation concentrations have previously been described as surface potential changes in a Gouy-Chapman-Stern model of nerve and muscle membranes (Hille et al., 1975; Campbell and Hille, 1976). We now show that anion adsorption can also be incorporated into such a model. First, however, we give our philosophy of such models: Membrane proteins and lipids contain charged groups and sites of adsorption that attract screening counterions and ligands. These attracted ions will necessarily have a local electrostatic effect on any field-sensitive mechanism in the neighborhood in addition to other allosteric influences that might occur. The number of groups and the electrostatic effects would vary from region to region on the surface of channel macromolecules, as individual effects can extend only over a few Debye lengths. Although it assumes an unrealistic planar geometry and a hypothetical uniform density of charge, we regard the Gouy-Chapman-Stern theory as an elementary approximation for calculating local potentials of collections of ionized groups in the immediate vicinity of the Na channel voltage sensors. We specifically do not believe that the membrane as a whole, or even different points on a channel, are electrostatically uniform.

The double-layer model can be extended to describe anion effects by adding to the usual equations for screening and cation binding an additional term for charge contributed by anions adsorbed to the membrane surface. For simplicity, we represent adsorption as binding to neutral sites with a surface density
of $-\sigma_{An}$ and an intrinsic dissociation constant $K_{An}$. If $[An^*]$ is the surface free anion concentration, then the bound surface charge from anions would be $\sigma_{An}/(1 + K_{An}/[An^*])$.

We used the parameters of model II in Hille et al. (1975) to describe shifts observed in Cl$^-$ solutions, assuming no binding of Cl$^-$, and then picked values of $K_{SCN}$ and $\sigma_{SCN}$ to approximate the shifts in 119 mM SCN$^-$. The density of adsorption "sites," $-\sigma_{SCN}$, had to be $>1/(150 \text{ Å}^2)$ to obtain enough shift of activation. Somewhat arbitrarily we chose $-\sigma_{SCN} = 1/(60 \text{ Å}^2)$ so that the anion binding required at 119 mM SCN$^-$ would occupy less than half the sites, and the dose-response curve in SCN$^-$/Cl$^-$ mixtures would not show much saturation. A reasonable fit with this site density and $K_{SCN} = 20$ mM is drawn as smooth curves in Fig. 6A. If instead we had started with model I of Hille et al. (1975), which has a higher standard surface potential, $-91$ mV rather than $-65$ mV, a fair fit would have been obtained with $K_{SCN} = 3$ mM. Either model assumes $\sim40\%$ occupancy of anion sites in SCN Ringer. Thus, even if the local potential near a Na channel is quite negative, the required intrinsic anion dissociation constants fall in the range between the medium- and low-affinity SCN-binding sites characterized in serum albumin (Scatchard and Yap, 1964). Adding a few sites with higher affinity (as in albumin) would help to imitate the steeper initial fall of the SCN$^-$ dose-response curve (Fig. 5).

We take the agreement of the surface-potential model as a confirmation of Hodgkin and Horowicz’s (1960) adsorption hypothesis that Hodgkin and Horowicz’s (1960) adsorption hypothesis is compatible with what is known about the adsorption of lyotropic ions on macromolecular surfaces. Again, we regard our calculations as only a crude approximation of the electrostatic effects of charges within a few Debye lengths of the voltage sensors of the Na channel. If the Na channel really has as high a local surface potential as our two models assume, then (as McLaughlin’s measurements with phosphatidylserine suggest) probably only protein can provide sites of the affinity required. On the other hand, the complete lack of Ca$^{2+}$-like actions of the divalent ethamethonium might imply that there is almost no negative surface potential. Even low-potential Gouy-Chapman-Stern models will fit our observations reasonably. We obtained an adequate description with only $-20$ mV of surface potential, very strong binding of Ca ions, and weak binding of anions. Although low surface potentials and strong Ca$^{2+}$ binding have been demonstrated for phospholipid membranes (McLaughlin et al., 1981), we are reluctant to accept this conclusion for Na channels solely on the basis of our ethamethonium experiments, as the molecule may be too large to penetrate to charged groups close to the voltage sensor. Unhydrated ethamethonium measures $5.5 \times 6.1 \times 9.6 \text{ Å}$, whereas the Ca ion has a crystal diameter of 2.0 Å.

The three voltage-dependent physiological functions that have been studied, Na channels, delayed rectifier K channels, and excitation-contraction (E-C) coupling, are not equally sensitive to divalent cations or to anions. Anions bathing muscle fibers shift with the sequence Na channel activation < inactivation < K channel activation = E-C coupling (Kao and Stanfield,
Divalent cations on nerve or muscle shift with the sequence K channel activation < Na channel inactivation ≤ Na channel activation = E-C coupling (Mozhayeva and Naumov, 1970; Dörrscheidt-Käfer, 1976; Chiarandini et al., 1980; Hille, 1968; this paper). These differences imply that the effective local charges and site densities near the voltage sensors for these processes are not identical. As Gilly and Armstrong (1981; López-Barneo and Armstrong, 1982) have pointed out, an ionic double layer is not a static structure but should alter as charged groups from the protein macromolecule are removed or exposed during the conformational changes of gating. Then individual rate constants in the gating processes can be altered differently. A related possibility with similar consequences is that binding of anions or cations anywhere on the channel can change the stability of different conformations. A necessary test of such hypotheses is to study kinetics, which we did not do. In addition, if the shape or steepness of the steady state activation or inactivation curves are changed, then one also knows at once that a pure voltage shift of all kinetic parameters has not occurred. In our experiments the steepness of these curves was changed little and not according to the lyotropic series (Table III), but this does not rule out selective changes of certain rate constants as opposed to pure shifts of all of them.

The general occurrence of shifts with lyotropic anions in the extracellular medium needs to be considered in other experiments. Thus, when one studies the anion selectivity of a membrane or when one is testing cations that are not supplied as a chloride salt, the assumption that cation permeabilities are unaffected by changing the anions of the medium will not hold. It would be interesting also to test whether lyotropic anions shift when applied inside excitable cells. Although anions can cross the muscle membrane, we do not believe that the effects we describe are due to internal actions. Our measurements were completed within 5 min of the solution change, and our fibers had short cut ends bathed in a large volume of internal solution. Furthermore, in fast-flow experiments with uncut fibers, NO₃ increases twitch tension with a time constant of only 3 s, whereas internal Cl⁻ concentration equilibrates with a time constant of 4 min (Hodgkin and Horowicz, 1959, 1960). Poorly permeant ions such as NO₃ should equilibrate much more slowly.

Loss of Ca Selectivity of the Ca Electrode

If the Orion Ca electrode were unaffected by anions, the response would follow the Nernst equation for Ca ions with a small deviation given by the known slight interference of Na ions. From the conventional Goldman (1943), Hodgkin and Katz (1949) theory, the output would satisfy the zero-current condition for the electrode membrane:

\[
\frac{4 \{[\text{Ca}]_i - [\text{Ca}]_o v^{-2} \}}{v^{-2} - 1} + \frac{P_{\text{Na}}}{P_{\text{Ca}}} \frac{[\text{Na}]_i - [\text{Na}]_o v^{-1}}{v^{-1} - 1} = 0,
\]

where \( P_{\text{Na}}/P_{\text{Ca}} \) is an apparent permeability ratio, the subscript i refers to the filling solution, the subscript o refers to the test solution, and \( v = \exp(EF/RT) \) where \( E \) is the electrode potential and \( F/RT \) is 24.6 mV.
In the presence of the more lyotropic anions, the output of the Orion electrode was more negative and less responsive to $[Ca^{++}]$ than expected. Qualitatively, the electrode was responding to anion concentration, and indeed we have shown that the decanol barrier in the electrode should be permeable to lyotropic anions. However, when we tried to account for measurements with SCN$^-$ by adding an additional term with a fixed permeability ratio $P_{SCN}/P_{Ca}$ to Eq. 2, the theory did not show even approximately the near complete loss of Ca sensitivity seen at high SCN$^-$ concentrations. The discrepancy suggested that anions and cations cross the membranes by different mechanisms. If anions move by free diffusion and cations by a saturating carrier, then nonsaturating SCN$^-$ fluxes at high concentrations could overwhelm a small saturated Ca flux.

As a first approximation, we represented anion fluxes by a simple Goldman-Hodgkin-Katz flux expression and we represented the cation fluxes by the left-hand side of Eq. 2 multiplied by a term expressing competition of Na$^+$ and Ca$^{++}$ for a common carrier:

$$W = \frac{W}{1 + [Ca]_o/K_{Ca} + [Na]_o/K_{Na}} \cdot \frac{P_{SCN}}{P_{Ca}} \cdot \frac{v}{v - 1} = 0,$$

where $W$ is the left-hand side of Eq. 2. The smooth curves in Fig. 9, drawn from this theory, fit the observations reasonably with the parameters given in the legend. The specifics of the theory are ad hoc, but the agreement with the observations supports our contention that lyotropic anions pass through the electrode membrane by a different and less saturable mechanism than Ca ions. The possibility of this kind of interference should probably be considered when using any type of ion-selective electrode manufactured with a hydrophobic membrane or liquid interface. Also, if the interfering ion permeates by solution and the assayed ion by a saturating carrier, a correction based on the Goldman-Hodgkin-Katz equation alone will not be adequate. It should be noted that several cation carriers with peptide- or imide-containing ligands also carry lyotropic anions (Margalit and Eisenman, 1979). Presumably such interactions reflect the special lyotropic affinities of amide groups in a hydrophobic environment.

Note added in proof: Two more papers have come to our attention. M. Gomolla, G. Gottschalk, and H. Ch. Lüttgau (manuscript submitted for publication) found similar shifts to ours in the spike threshold of skeletal muscle when most of the chloride was replaced with perchlorate. Perchlorate caused still larger shifts in mechanical activation. Interference in the response of cation-selective, membrane electrodes by thiocyanate has been discussed by W. E. Morf, D. Ammann, and W. E. Simon (1974. Chimia. 28:65-67).

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