Anion Conductance of Frog Muscle Membranes: One Channel, Two Kinds of pH Dependence

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ABSTRACT Anion conductance and permeability sequences were obtained for frog skeletal muscle membranes from the changes in characteristic resistance and transmembrane potential after the replacement of one anion by another in the bathing solution. Permeability and conductance sequences are the same. The conductance sequence at pH = 7.4 is Cl⁻ > Br⁻ > NO₃⁻ > I⁻ > trichloroacetate ≥ benzoate > valerate > butyrate > propionate > formate > acetate > lactate > benzenesulfonate ≥ isethionate > methylsulfonate > glutamate ≥ cysteate. The anions are divided into two classes: (a) Chloride-like anions (Cl⁻ through trichloroacetate) have membrane conductances that decrease as pH decreases. The last six members of the complete sequence are also chloride like. (b) Benzoate-like anions (benzoate through acetate) have conductances that increase as pH decreases. At pH = 6.7 zinc ions block Cl⁻ and benzoate conductances with inhibitory dissociation constants of 0.12 and 0.16 mM, respectively. Chloride-like and benzoate-like anions probably use the same channels. The minimum size of the channel aperture is estimated as 5.5 × 6.5 Å from the dimensions of the largest permeating anions. A simple model of the channel qualitatively explains chloride-like and benzoate-like conductance sequences and their dependence on pH.

INTRODUCTION

Hutter and Warner (1967 a) showed that the Cl⁻ conductance (GCl) of frog skeletal muscle cell membranes depends on the pH of the bathing solution; a reduction of pH to 5.0 reduces GCl to about 0.1 of its value at pH = 9.0. The apparent pKₐ is near 7. Potassium and rubidium conductances are little affected by pH (Hutter and Warner, 1967 b, 1972). The effect of pH on GCl provides a method for separating currents flowing through “Cl⁻ channels” from other membrane currents. Any method which permits the
estimation of the current through one type of ion channel is useful for probing the channel’s structure: (a) The relative conductance of the channel for a different ion species can be determined and the resulting selectivity sequence gives some idea of the charge, size, and molecular structure of the channel. (b) Interactions between different ion species traversing the same channels may give more details of channel structure. (c) The effects of various reagents which chemically modify functional groups can help identify groups important for channel function. In addition, the pH sensitivity of Cl⁻ channels is one important clue to their nature, particularly since we show in this paper that the direction of the variation of membrane conductance with pH depends on the anion species in the bathing medium. Another attractive feature of studying chloride channels in frog skeletal muscle is that, in normal conditions, \( G_{Cl} \) is about twice \( G_K \), so that signals are relatively large (Hodgkin and Horowicz, 1959; Hutter and Noble, 1960; Adrian and Freygang, 1962 a).

There is good evidence that there are separate chloride and potassium channels in frog skeletal muscle. Hodgkin and Horowicz (1960) found that the change in transmembrane potential produced by a step change in \([Cl^-]\) is faster than that due to a step change in \([K^+]\), and suggested that Cl⁻ channels are situated closer to the outer surface of the cell than are K⁺ channels. From a study of current-voltage relations, Adrian and Freygang (1962 a) suggested that the surface membrane has a large and constant Cl⁻ permeability and low \( G_K \) and that most of \( G_K \) is in the walls of the sarcoplasmic reticulum. Eisenberg and Gage (1969) quantified this distribution by measuring membrane conductance in normal and detubulated (glycerol treated) muscles at normal and at low external pH values. They calculate that all of \( G_{Cl} \) and about one-third of \( G_K \) is in the surface membrane. The differential effects of external pH on \( G_{Cl} \) and \( G_K \) (Hutter and Warner, 1967 a) mentioned above are further evidence of the separability of Cl⁻ and K⁺ channels. Mashima and Washio (1964) found that 0.5 mM Zn⁺⁺ increases membrane resistance by about three times and Hutter and Warner (1967 c) found that Zn⁺⁺, Cu⁺⁺, and UO₂⁺⁺ in concentrations of about 10⁻⁴ M reduce \( ^{36}Cl^- \) efflux at normal pH and have no effect at low external pH’s when \( G_{Cl} \) is already low. More recently Stanfield (1970) has found that tetraethylammonium (TEA) ions reduce \( G_K \) and Zn⁺⁺ reduces \( G_{Cl} \) of muscle fibers with negligible cross effects.

Hodgkin and Horowicz (1959) found that membrane permeability to Cl⁻ as calculated from the constant field equation is about constant over a wide range of Cl⁻ concentrations and membrane potentials at normal pH, suggesting that the chloride channel is a relatively simple structure. However, Hutter and Warner (1972) have recently shown that the agreement with the constant field equation at normal pH is fortuitous; the steady-
state current-voltage relation at pH = 9.8 shows current saturation for large hyperpolarizations and a concave downward curvature at pH = 5.0. Both the pH 7.4 and 9.8 curves cross the pH = 5.0 curve. Furthermore, Warner (1972) shows that there is an exponential decrease in conductance during hyperpolarizing steps at pH = 9.8 and 7.4 and an increase at pH = 5.0.

This study began as a search for a completely "impermeant" monovalent anion in connection with attempts to measure the conductance of the membrane to HCO$_3^-$ ions and to determine if HCO$_3^-$ ions use Cl$^-$ channels (Woodbury, 1971). Much to our surprise, we found that the membrane conductance increases as pH is decreased when Cl$^-$ is replaced by some organic anions such as benzoate. Hence we attempted to answer some of the questions raised: (a) What types of anions show the reverse pH dependence? (b) Do these ions cross the membrane via the same channels as Cl$^-$? (c) If so, what can be deduced about the structure of Cl$^-$ channels? An abstract giving some of these results has appeared (Miles and Woodbury, 1971).

METHODS

Sartorius muscles from Rana pipiens were stored overnight at 4°C in a solution with all the Cl$^-$ replaced by glutamate or some other relatively impermeant anion being tested. Internal Cl$^-$ concentration was presumably reduced to zero. Experiments were performed at room temperature. The muscle was tied around three sides of a Lucite block, and mounted in a chamber. Solutions of the desired composition entered the chamber at the middle of the muscle and flowed toward both ends. The electrodes were inserted near the region of solution entry so that any changes in transmembrane voltage and resistance would appear quickly. The flow rate was about 3 cm$^3$/min but was increased to more than 15 cm$^3$/min for 15–30 s after a change in solution composition. Chamber volume is 1.5 cm$^3$. The indifferent electrode was a flowing KCl calomel electrode of the type used in commercial pH meters. The solution flowing past this electrode was diverted from the main stream to avoid elevating the K$^+$ and Cl$^-$ concentrations in the solution bathing the muscle.

Recording Technique

The electrical recording techniques are described by Woodbury (1971). Briefly, two micropipettes filled with half-saturated K$_2$SO$_4$ were inserted into the same cell at a separation of 100–200 μm. A constant current was passed through one electrode and transmembrane potential measured with the other. Since the high resistance (20–100 MΩ) micropipettes used did not pass sustained depolarizing currents, the applied current was a sine wave (0.2 Hz) superimposed on a steady hyperpolarizing current such that the current was zero at the peak of the sine wave: $I_{\text{applied}} = -I_0 + I_s \sin 0.4 \pi t; I_0 = 5 \times 10^{-9}$ A. Sine waves rather than square waves were used because a narrow band-pass filter can be used to eliminate noise and increase the sensitivity of membrane-conductance measurements (cf., top trace Fig. 1). How-
ever, the frequency of the sine wave must be low enough to make capacitative current negligible.

The characteristic or input impedance of a fiber is defined as $Z_e = \Delta V_m/I_0$, where $\Delta V_m$ is the change in transmembrane voltage at the current electrode. For an infinite cable $Z_e = 0.5 (\zeta_m r_i)^{1/2}$, where $\zeta_m$ is the membrane impedance for a unit length of fiber (ohm-centimeter) and $r_i$ is the myoplasm resistance per unit length (ohm-centimeter$^{-1}$). At low frequencies the tubular membrane can be lumped with the surface membrane with little error; hence $\zeta_m \approx r_m (1 + j \omega \tau)^{-1}$ and $|Z_e| = R_e (1 + \omega^2 \tau^2)^{1/4}$, where $\tau = r_m c_m$, $c_m$ is the capacitance per unit length (farad-centimeter$^{-1}$), and $R_e = 0.5 (r_m r_i)^{1/2}$ is $Z_e$ at $\omega = 0$. The highest $Z_e$'s were measured when the solutioining bathing the muscle was cysteate. From Table I, $Z_e$ (cyst) $1.5 \times 10^6 \Omega$ and $Z_e$ (Cl) $0.5 \times 10^6 \Omega$. If $\tau = 25 \text{ ms}$ in Cl (Gage and Eisenberg, 1969) then $\tau = 3.25 \times 225 \text{ ms}$ in cysteate. For $\omega = 2\pi \cdot 0.2 = 1.25$, $Z_e/R_e = (1 + 0.275)^{-1/4} = 0.98$ and even in this extreme case $R_e$ is negligibly different from $Z_e$.

Another important consideration is the time dependence of chloride conductance. Warner (1972) found an approximately exponential change in conductance with time after hyperpolarizing steps. The longest time constants reported are about 0.3 s and typically about 0.1 s. The corresponding angular frequencies are 3.3 and 10 rad/s. The applied sine wave frequency should be several times lower to assure a steady state. Although the existence of time-dependent conductance changes (Warner, 1972) had not been reported at the time these experiments were done, the frequency chosen (0.2 Hz, 1.25 rad/s) is 3–10 times lower than the equivalent angular frequency of the conductance changes; assuming Warner's data apply to our quite different experimental conditions, the measured conductances are close to steady-state values. Even if this is not so, the conductance sequences would be the same unless the various anions tested drastically affect the time constant of conductance change.

The normal space constant of sartorius fibers is less than 3 mm; the space constant of a fiber bathed in cysteate would be less than 1 cm. The muscles used were 4 or more cm long so the fibers can be regarded as infinite cables to a good approximation.

Membrane-specific conductance is defined as $G_m$ (mho-centimeter$^{-2}$) = $1/R_m$ (ohm-centimeter$^2$), where $R_m = r_m \cdot 2\pi a$ and $a$ is a fiber radius. Only relative conductances are used in interpreting the results and it follows from the foregoing that $G_m(X)/G_m(Cl) = [R_e(Cl)/R_e(X)]^2$, where the symbol in parenthesis refers to the anion in the bathing solution.

Two tracings were obtained during each experiment (Fig. 1). The bottom tracing gives resting transmembrane potential (the maxima of the sine wave) and deviations from it produced by the current. The upper tracing is an amplified, filtered, AC coupled version of the lower tracing so that changes in $R_e$ are accentuated. The high- and low-pass filters both had time constants of 2 s.

The procedure was to insert the micropipettes, apply the current, and obtain steady values for transmembrane potential and $R_e$. Then the perfusion fluid was switched to one containing a different anion and observations made at least until
the voltage deviations leveled off. The perfusion was then switched back to the control or to another test solution.

**Current-Voltage Relations**

The current was always turned on in two steps, the 10 nA sine wave first and then the −10 nA hyperpolarizing current. The peak-to-peak amplitude of the sine wave was never noticeably altered by application of the steady current. This indicates that the current-voltage relation was approximately linear in the neighborhood of the resting potential. The peak-to-peak voltage swing depends on $R_e: \Delta V = 10 \text{nA} \cdot R_e$ and is about 4 mV when the anion is Cl− and about 15 mV when the anion is glutamate. Linearity was occasionally checked by turning the sine wave off and obtaining a steady-state current-voltage-relationship over a range of ±15 mV. The curves were approximately linear. The linearity may be somewhat surprising in view of the known characteristics of the potassium inward rectifier system (Adrian and Freygang, 1962 b; Adrian et al., 1970 b; Stanfield, 1970), the nonlinearity of the chloride current-voltage relation (constant permeability at pH ~ 7.0) (Hodgkin and Horowicz, 1959) and its change with pH (Hutter and Warner, 1972). Hutter and Padsha (1959) recorded current-voltage curves directly from frog muscle fibers, using two closely spaced microelectrodes, with the muscle bathed in chloride-Ringer's and in nitrate-Ringer's solutions. In both cases the curves are strikingly linear in the hyperpolarizing direction over the range covered, 20–30 mV. The possible reasons for linearity are considered in the Discussion. The important point here is that a linear relation is direct experimental evidence that $\Delta V/I_0$ is an accurate estimate of $R_e$ at the resting potential.

**Solutions**

In the experiments designed to measure the relative conductances and permeabilities of anions and pH effects on anion permeability, the solutions contained 115 mM of the monovalent anion being studied as the sodium salt, 2.5 mM potassium glutamate, 2 mM calcium glutamate, and 5 mM Tris-glutamate buffer. Sulfate was not used as a reference impermeant anion because there are appreciable concentrations of KSO₄ and NaSO₄ complexes when SO₄ concentration is high (Gordon et al., 1970). K⁺ ion activity is thus a complicated function of SO₄⁻ concentration and changes in voltage and conductance occurring when the bathing solution is changed from SO₄⁻ to some other anion are less certainly attributable to the anions. Tetrodotoxin (TTX), $10^{-7}$ M, was added to all solutions to block sodium channels (Adrian et al., 1970 a). Spontaneous muscle contractions were also abolished by TTX. In later experiments, TEA (115 mM) was substituted for sodium, and 5 mM sodium PIPES (piperazine-N,N-bis 2-ethane-sulfonic acid) used as a buffer. The advantage of having TEA present is that some kinds of potassium channels are partially blocked (Stanfield, 1970) thus increasing the fraction of current carried by anions. The PIPES buffer was used since it does not bind with Zn²⁺ (Good et al., 1966), and has a more appropriate pKₐ (6.9). The solutions used in studying the effects of Zn²⁺ on anion permeability were prepared by adding appropriate amounts of zinc acetate. When the concentration of Zn²⁺ was greater than 1 mM, an equivalent
amount of Na⁺ and anion was omitted to maintain normal osmolarity. The pH was kept somewhat below 7.0 to prevent precipitation of the Zn²⁺, presumably as the hydroxide (cf., Mashima and Washio, 1964; Stanfield, 1970).

Experimental Design

The conductance sequence experiments were designed to keep internal concentrations of the experimental anions at near-zero values. Since the muscles were soaked overnight in glutamate solution and resting potential was usually about -60 mV, internal glutamate had likely reached its equilibrium value of 12 mM. The half time for Cl⁻ efflux from frog fibers with \( V_m \approx 0 \) is about 5 min (Moore, 1969) and considerably shorter at \( V_m = -60 \) mV. Table I indicates that glutamate conductance is about \( \frac{1}{50} \) of Cl⁻ conductance, hence glutamate half time is considerably shorter than 250 min and is equilibrated in less than 12 h.

The fiber was perfused with glutamate, the perfusate was switched to another anion for 2–3 min, sufficient for the new anion concentration to reach a steady state near the fiber surface, and the conductance was measured. If fairly permeant ions were being studied the perfusate was switched back to glutamate for 10 or more min to allow any anion that had entered the cell to wash out. When relatively impermeant anions were being studied, internal accumulation was not a problem and it was possible to compare directly two anions \( X_1 \) and \( X_2 \) having closely similar conductances by switching the perfusate directly from \( X_1 \) to \( X_2 \) and back. The resultant changes in \( V_m \) and \( R_c \) gave unequivocal and reproducible evidence as to which anion is the more permeable and conducting.

The technique used for obtaining conductance sequences is not practicable for measuring the effects of pH on conductance primarily because anion changes, for example from glutamate to benzoate, frequently cause loss of electrode impalement. Other reasons appear below. Hence, the technique used by Hutter and Warner (1967 a) for Cl⁻ was adopted. The muscle was first immersed for about 30 min in a fluid of pH \( \approx 7.4 \) containing the anion to be tested. The perfusate was then changed to one of the same composition but different pH and the conductance measured. A typical sequence of pH's is 7.4, 8.0, 9.2, 7.4, 6.8, 6.0, 5.4, 6.8, 7.4. Even with the same anion, it is difficult to maintain the impalements through this series, possibly because of changes in cell volume. Few complete runs were obtained and the results are composed of runs of various lengths. The pH dependencies of most ions were established by this means but in a few cases these were determined by bracketing anions in conductance sequences obtained at normal and low pH's.

RESULTS

Permeability and Conductance Sequences

The change in transmembrane potential consequent on replacement of one anion by another can be interpreted as a measure of the ratio of the permeabilities of the two anions. Similarly the square of the ratio of the characteristic resistances is the inverse of the conductance ratio. These changes can be used to obtain anion permeability and conductance sequences, respectively.
Permeability and conductance are both measures of ease of ion penetration defined in terms of different driving forces, concentration, and voltage gradients, respectively. Nevertheless, conductance and permeability sequences are not necessarily the same. Hagiwara et al., (1971) found that the anion permeability sequence is nearly the reverse of the conductance sequence in barnacle muscle membranes, e.g., replacement of Cl\(^-\) by SCN\(^-\) causes a marked hyperpolarization, indicating that SCN\(^-\) is more permeant than Cl\(^-\), and also a decrease in conductance. Our results are more simply described; the conductance and permeability sequence are, with minor exceptions, the same. The results reported here are based almost entirely on conductance measurements because conductance measurements made with sinusoidal currents and a band-pass filter are more accurate than voltage changes with their inherent uncertainties in junction potentials.

Fig. 1 shows a record typical of all the results. The muscle, soaked overnight at 4°C in glutamate solution, was also perfused with a glutamate solution, pH = 7.4, for several minutes before the start of the record. The low resting potentials, -58 mV, is not unexpected. Cl\(^-\)-free muscle fibers often have two stable membrane voltages (Adrian and Freygang, 1962a; Woodbury, 1971). The low resting potential indicates that the K\(^+\) conductance is balanced by an inward current probably carried by Na\(^+\). When the perfusate was changed from glutamate to benzoate (left vertical bar, Fig. 1), the membrane hyperpolarized to -64 mV and \(R_c\) (proportional to width of the band) decreased from 1.4 to 0.88 MΩ. The hyperpolarization indicates that benzoate is more permeable than glutamate and the decrease in \(R_c\) in-
indicating that benzoate conductance is greater than that of glutamate. Similarly, there was a depolarization to \(-58\) mV and an increase in \(R_c\) to 1.05 M\(\Omega\) when the solution was changed from benzoate to benzenesulfonate, indicating that membrane permeability and conductance to benzenesulfonate are less than to benzoate. Comparison with the initial record indicates a benzenesulfonate conductance greater than glutamate and about the same permeability but this is less certain since the voltage is still changing at the end of the record. Only those changes in a given run which were reversible and reproducible were accepted. In a few instances, the results varied somewhat from fiber to fiber and muscle to muscle.

The interpretation of the results in terms of anion conductances and permeabilities, although the simplest, is not the only one possible. Alternative explanations and justification for this interpretation are deferred to the Discussion since some of the data given below are relevant. The Results section is written on the assumption that changes in membrane conductance and voltage consequent on replacing one anion by another are due to changes in anion conductance and permeability rather than, for example, anion effects on potassium conductance. The results are valid and clear-cut regardless of the interpretation put on them.

**Membrane-specific resistance** Since no attempt was made to measure space constant and fiber diameter, no absolute values of membrane-specific resistance can be given. The mean \(R_c\) of 12 fibers from 4 muscles bathed in Cl\(^-\) solution at pH = 7.4 is 0.5 \(\pm\) 0.02 (SD) M\(\Omega\) (Table I). If these fibers had properties similar to those reported in the literature, then \(R_m \approx 4\) k\(\Omega\)-cm\(^2\) (cf., Mashima and Washio, 1964; Gage and Eisenberg, 1969). If so the mean radius of our fibers is about 35 \(\mu\)m, a not unreasonable value since Eisenberg and Gage (1960) reported a mean of 26 \(\mu\)m and Mashima and Washio (1964) a mean of 45 \(\mu\)m.

The \(R_c\)'s of fibers bathed in benzoate and glutamate at pH = 7.4 are 1.03 \(\pm\) 0.1 M\(\Omega\) (25 cells, 8 muscles) and 1.42 \(\pm\) 0.3 M\(\Omega\) (23 cells, 7 muscles). These translate to \(R_m\)'s of 17 and 32 k\(\Omega\)-cm\(^2\) for benzoate and glutamate. The membrane conductance in glutamate, \(G_m = 1/32 \text{k}\Omega\text{-cm}^2 = 30 \mu\text{mho/cm}^2\) is the sum of glutamate plus cation conductances. However, 30 \(\mu\text{mho/cm}^2\) is less than half of the values reported in the literature for resting \(G_K\) (cf., Hodgkin and Horowicz, 1959; Eisenberg and Gage, 1969), suggesting that glutamate conductance is near zero and that in these depolarized fibers \(G_K\) is considerably reduced. Hutter and Warner (1972) measured membrane conductances of a muscle fiber bathed in a solution where potassium was replaced by rubidium and the anion was methylsulfate. The conductances were about 40 \(\mu\text{mho/cm}^2\).

**Anion conductance sequence at pH = 7.4** 17 anions which had reversible effects on conductance and voltage at pH = 7.4 are ranked in
order of decreasing conductance in Fig. 2. The structures of the organic anions and their pKₐ's where known are also given to facilitate discussion of the possible relationships between an anion's conductance and structure. The sequence is unequivocal despite wide variations in absolute conductances and resting potentials from fiber to fiber with the same anion in the perfusate. Woodbury (1971) gives the tentative sequence benzoate ≈ propionate ≈ isethionate ≈ glutamate.

\begin{align*}
\text{Conductance sequence for anions at } pH = 7.4. \text{ Numbers in parentheses below formulae are pK}_{a}'s. \text{ Numbers above formulae give relative conductance with respect to } Cl^- (1.0) \text{ and cysteate (0.0) (from Table I). The dashed vertical lines to the left of benzoate and to the right of acetate demarcate the benzoate-like ions. See text. Other anions tested but not included in the sequence. monochloroacetate, slightly lower conductance than trichloroacetate; methylsulfate, slightly lower conductance than methylsulfonate; and aspartate, about the same as glutamate. Salicylate, acetyl-salicylate, and perchlorate were tested but not included because they produced irreversible changes.}
\end{align*}
to muscle. Although it is probable that the membrane conductance to a given anion depends on membrane voltage, the voltage dependence is likely to be about the same for all anions. In any case, the range of voltages encountered was not large enough to affect the sequence. For example, the conductance sequence for Br\(^-\), benzoate, valerate, butyrate, propionate, and acetate were obtained in five different fibers in the same muscle. In fiber one, the resting potential varied from \(-37\) to \(-63\) mV depending on the ion in the bath; in fiber two, from \(-28\) to \(-58\) mV; in fiber three, from \(-20\) to \(-48\) mV; in fiber four, from \(-24\) to \(-50\) mV; and in fiber five, from \(-30\) to \(-54\) mV. In all five fibers the conductance sequence was the same despite the spread in membrane potentials. Most resting potentials measured fell between \(-25\) and \(-65\) mV; the full range was \(-7\) to \(-80\) mV.

The lack of dependence of the anion conductance sequence on voltage indicates that voltage-dependent changes in K\(^+\) flow in the inward rectifier cannot account for the observed changes in conductance due to changes in the anion. However, the possibility that K\(^+\) conductance is altered to differing degrees by the various anions is not excluded.

The anion conductance sequence shown in Fig. 2 can be split into three classes based on similarities and differences in the chemical properties of the anions. (I) Highly conducting class: Anions of strong acids having zero or negative pK\(_a\) values (Cl\(^-\), Br\(^-\), NO\(_2\)\(^-\), I\(^-\), and trichloroacetate). Hutter et al. (1969) obtained the same sequence for the halides and NO\(_2\)\(^-\). Conductance is inversely related to ionic radius and directly to mobility in water. (II) Moderately conducting class: Anions of weak carboxylic acids (benzoate through lactate). The pK\(_a\) values (shown in Fig. 2) are high, ranging from 3.75 to 4.87. Conductance is not obviously correlated with pK\(_a\). (III) Poorly conducting class: Organic anions (benzenesulfonate through cysteate) whose charges are due either to a sulfonate group or to two carboxyls and one amino group. The pK\(_a\)s are lower in most cases than those of class II and higher than those of class I.

**Permeability sequence at pH = 7.4** Only occasionally did the permeability change in the direction opposite to the conductance when one anion was replaced by another. In one cell benzenesulfonate was slightly less permeant and slightly more conducting than glutamate. There were several cells in which the positions of adjacent anions in class II were interchanged in the permeability as compared with the conductance sequences. The conductance and voltage changes are small and the interchange may have been due to differences in junction potentials.

**Conductance ratios** The conclusions about the nature of anion permeation which can be drawn from the conductance sequence do not de-
pend on the absolute values of anion conductance. Nevertheless, it is desirable to make a quantitative comparison between our results and those available in the literature. Conductances are usually given in the form of ratios with respect to chloride.

The data on absolute and relative conductances are summarized in Table I. The anions are listed in order of decreasing conductance in column 1.

| Anion          | no. of muscles | no. of cells | $R_c$ (MΩ) ± SD | $G_m(X)$ | $G_m(glut)$ | $G(X)$ | $G(Cl)$ |
|----------------|---------------|--------------|-----------------|----------|-------------|--------|---------|
| Cl⁻            | 4             | 12           | 0.5±0.02        | 8.1      | 1.0         |
| Br⁻            | 2             | 8            | 4.7±1.1         | 4.0      | 0.53        |
| NO₃⁻           | 2             | 6            | 2.98±0.29       | 2.1      | 0.17        |
| I⁻             | 2             | 6            | 2.1±0.17        | 1.94     | 0.15        |
| Trichloroacetate⁻ | 2     | 2            | 4.7±1.1         | 1.77     | 0.13        |
| Benzoate⁻      | 2             | 6            | 4.7±1.1         | 1.94     | 0.15        |
| Valerate⁻      | 2             | 6            | 4.7±1.1         | 1.94     | 0.15        |
| Butyrate⁻      | 2             | 6            | 4.7±1.1         | 1.94     | 0.15        |
| Propionate⁻    | 3             | 7            | 4.7±1.1         | 1.94     | 0.15        |
| Formate⁻       | 2             | 5            | 4.7±1.1         | 1.94     | 0.15        |
| Acetate⁻       | 3             | 6            | 4.7±1.1         | 1.94     | 0.15        |
| Lactate⁻       | 2             | 4            | 4.7±1.1         | 1.94     | 0.15        |
| Benzenesulfonate⁻ | 2    | 7            | 4.7±1.1         | 1.94     | 0.15        |
| Isethionate⁻   | 2             | 6            | 4.7±1.1         | 1.94     | 0.15        |
| Methylsulfonate⁻ | 2     | 5            | 4.7±1.1         | 1.94     | 0.15        |
| Glutamate⁻     | 7             | 23           | 1.42±0.3        | 1        | 0.02        |
| Cysteate⁻      | 1             | 4            | 0.86±0.2        | 1        | 0.02        |

Values for valerate through lactate overlap considerably so all values are lumped and the range given in column 5. Benzenesulfonate ≥ isethionate > methylsulfonate values are also lumped. Values in the last column calculated on assumption that $G(cyst) = 0$ from the equation

$$G(X) = \frac{G_m(X) - G_m(cyst)}{G_m(Cl) - G_m(cyst)}$$

where $G_m(X)$ is the measured membrane conductance ($= 1/R_c$) with muscle bathed in anion X.

and the numbers of muscles and cells in columns 2 and 3. The mean absolute $R_c$ values for Cl⁻, benzoate, and glutamate are given in column 4. Values of $R_c$ are not given for the other ions; the small sample size means that the variances due to the range of fiber diameters and the relative contributions of the individual ionic conductances from fiber to fiber are much larger than the absolute differences between adjacent anions. Since it takes a long time to wash Cl⁻ out of a muscle, Cl⁻ was not used in most experiments.
Instead conductance ratios with respect to glutamate were calculated individually for each cell. The mean values are given in column 5 under the heading $G_m(X)/G_m(\text{glut})$, where $G_m$ refers to total membrane conductance and the symbol in parentheses to the anion. The values for valerate through lactate are lumped and the range given (1.77 and 1.39) because of the small sample and large variation of conductance ratios from fiber to fiber. Similarly the values for benzene-sulfonate, isethionate, and methylsulfonate are lumped and averaged.

The ratio of the conductance of ion X to Cl is given by $G(X)/G(Cl) = [G_m(X) - G_0]/[G_m(Cl) - G_0]$; $G_0$ is $G_m$ when the muscle is bathed in a hypothetical impermeant anion. There is no way of determining if an ion has zero conductance using this method so $G_0$ is not known accurately. Ignorance of the exact value of $G_0$ makes little difference in estimating the conductance ratio for a high conductance anion but the calculation becomes meaningless where $G(X) \approx G_0$ as is true for the less permeant anions. Column 6, Table I gives $G(X)/G(Cl)$ calculated on the assumption that $G_0 = G_m(\text{cysteate})$. The value of 0.05 for benzenesulfonate and isethionate is based on their near equality (Fig. 2). The value for methylsulfonate is probably about 0.04. The range of 0.07-0.13 for the six ions lactate through valerate indicates that their conductances are approximately 0.01 $G_{Cl}$ apart.

The uncorrected ratios given in column 5, Table I can be compared with the data of Hutter and Padsha (1959). They measured the membrane time constant of frog muscle with various anions in the bath. Their results, converted to conductance ratios are $\text{Cl}^-:\text{Br}^-:\text{NO}_3^-:\text{I}^- = 1.0:0.67:0.5:0.43$. Our ratios calculated from column 5 are much the same: $\text{Cl}^-:\text{Br}^-:\text{NO}_3^-:\text{I}^- = 1.0:0.58:0.49:0.37$.

**Effects of pH on Membrane Conductance**

The diversity in sizes and shapes of monovalent anions which affect membrane conductance raises two questions: Do all these anions traverse the membrane through Cl$^-$ channels or are there one or more additional types of anion channels? Do some of the anions exert their effects indirectly such as by affecting the conductance of some other ion? Experimental approaches to the answers are afforded by the findings of Hutter and Warner (1967 a, b, c) that $G_{Cl}$ and $^{36}\text{Cl}$ efflux are reduced by lowering the pH or adding the foreign cations Zn$^{++}$, UO$^{2+}$, or Cu$^{++}$ to the bathing medium. If all anions use Cl$^-$ channels then it would be expected that their conductances should be affected by pH and foreign cations similarly to the way that Cl$^-$ is.

The effects of pH on anion conductance were unexpected. Many, but not all, of the anions show a dependence of conductance on pH like that of Cl$^-$, i.e., a decrease in pH decreases conductance; but many other anions, such as benzoate, have conductances which increase as pH is decreased. As a
check on our technique, we repeated the experiments of Hutter and Warner (1967a) and confirmed their results for Cl\(^-\). Conductance vs. pH titration data with Cl\(^-\) as the anion obtained from one cell in Cl\(^-\) are shown in Fig. 3 (open circles). Hutter and Warner modeled the effects of pH on Cl\(^-\) conductance by assuming that one H\(^+\) ion can block one channel by combining with some functional group in the channel with a pK\(_a\) of about 7. Their data have pK\(_a\)'s in the range 6.5–7.5. A theoretical titration curve having a pK\(_a\) = 7.0 (solid line, Fig. 3) gives a reasonable fit to the data. Moore (1969) measured the \(^{36}\)Cl efflux from small bundles of muscle fibers as a function of pH. The apparent pK\(_a\) is about 6.6.

**CHLORIDE-LIKE ANIONS** Many of the anions tested showed a direct dependence of conductance on pH like that of Cl\(^-\). These anions are called chloride-like anions for convenience. All have lower conductances than Cl\(^-\). Chloride-like anions, in order of decreasing conductance at pH = 7.4 are: Cl\(^-\) > Br\(^-\) > NO\(_3\) > I\(^-\) > trichloroacetate > lactate > benzenesulfonate > isethionate > glutamate > cysteate. Comparison of this sequence with the overall sequence (Fig. 2) shows that chloride-like anions all belong either to class I (high-conductance small anions of strong acids) or to class III (low-conductance anions containing sulfonate or three charges) with the exception of lactate. The conductances of class III anions are so low that the variation of membrane conductance with pH could be due to effects on other (e.g., K\(^+\)) channels.
Spurway (1972) quotes unpublished work of G. Stenhouse as showing that the peak conductances of "weakly hydrated" ions (presumably Br\textsuperscript{–}, I\textsuperscript{–}, and NO\textsubscript{3}\textsuperscript{–}) usually occur at a pH slightly above neutral and decrease at higher and lower pH's. All our data on Cl\textsuperscript{–}-like ions show a direct, monotonic relation between conductance and pH. The source of the disagreement cannot be ascertained without further details of Stenhouse's results.

BENZOATE-LIKE ANIONS Our findings that the membrane conductance varies inversely with pH when the bath anion is either benzoate or a "benzoate-like" anion is new. Hence the supporting evidence is given in detail. The "benzoate-like" anions in order of decreasing conductance at pH = 7.4 are benzoate > valerate > butyrate > propionate > formate > acetate. Reference to Fig. 2 shows that with the exception of formate, conductance goes directly with the size of the hydrophobic moiety.

Complete and reversible conductance vs. pH titration curves for one cell are difficult to obtain for three reasons: (a) No matter what benzoate-like anion is used, large changes in pH usually cause a loss of impalement. (b) The conductances of these ions are low and the observed effects are small. (c) Benzoate-like anions irreversibly increase membrane conductance at low pH's on the time scale of these experiments. The lower the pH, the more rapidly the irreversible increase appears. Irreversible effects probably occur at pH = 7.4 also but the time scale is much longer; reliable, reproducible results can be obtained for several hours from muscles soaked in benzoate at pH = 7.4 but muscles soaked overnight at 4°C in benzoate at pH = 7.4 are dead.

PROPRIONATE Few complete runs where pH was increased, returned to normal, decreased, and returned to normal were obtained in one cell. The two best sets of results were obtained for propionate and are plotted in Fig. 4 a. The abscissa is pH and the ordinate is $R_c$ in megohms. The numbers by the points give the order in which they were taken. The results for cell 2 show dramatically the irreversible effects of low pH. Points 1–7 show that relatively reversible changes in $R_c$ are obtained when the pH is changed progressively from 6.8 to 7.2, to 8.0, 9.2, and then backward through the sequence to 6.8. The agreement is even better if only points 2 through 6 are considered. Points 7, 8, and 9 show a large decrease in $R_c$ as pH is decreased to 6.0 and 5.1. The reverse sequence of pH changes caused an increase in $R_c$ (points 10, 11, and 12) but the final $R_c$ (point 12) is 50 percent less than the $R_c$ for point 6. The results for cell 1 are the same except that the impalement was lost while the muscle was in a solution having pH = 5.1.

Similar data were obtained for benzoate, acetate, and formate. In every case, there were irreversible and large decreases in $R_c$ at pH's below about 6.0–6.5. Thus it was deemed necessary to exclude all data for pH's below
6.0–6.5 in determining the effects of pH on the conductances of benzoate-like ions.

Fig. 4b is a replot of the data in Fig. 4a in terms of relative conductances of points 1 through 5 of cell 1 (filled squares) and 1 through 7 for cell 2 (open squares). The conductance of the first reading at pH 7.2 is used as the reference value for cell 2. Relative membrane conductance = \([R_c(7.2)/R_c(pH)]^2\). The large open circles are the mean values. The conductances at pH 6.0 and 5.3 are too large to be shown. The solid line is a theoretical titration curve. The pKa and the change in relative conductance between high and low pH's were chosen to give a good fit to the average values. The pKa is 7.4. The fitted curve is unique in the sense that only a small range of conductance change amplitudes and pKa's give curves that come as close to the experimental points. Nevertheless, the data cannot be regarded as showing that propionate conductance is an S-shaped function of pH because the average points are still concave upward at the lowest pH's shown. The irreversible effects appearing rapidly at low pH's obscure any leveling off of the curve that may occur. The points from cell 1 (filled squares, Fig. 4b) do show a distinct S-shape and a titration curve with about the same pKa as the one shown is a good fit.
benzoate Despite many attempts to measure the effects of pH on benzoate conductance, useful results (two or more points) were obtained from only eight cells in three different muscles. The relative conductance values are plotted as a function of pH in Fig. 5 for values above 6.0. Each symbol represents the results from a single cell with the exception that the open circles and open squares are results from the same cell which were renormalized because of a moderate, irreversible change in conductance ("change in impalement") that occurred at high pH. There is a large scatter in the size of the conductance changes with pH from cell to cell, but there is clearly a significant relationship having a negative slope between conductance and pH. The data were divided into groups by the reference pH of 7.36 and least square straight lines through point (1.0, 7.36) fitted (solid lines, Fig. 5). The slope of the left-hand line is significantly different from zero ($P < 0.01$) and the slope of the right-hand line is just not significant ($0.05 < P < 0.10$). Hutter and Warner (1967 a) and Warner (1972) report large variations in the size of the pH-dependent membrane conductance when cells are bathed in Cl$^-$ solution. The variability of our results may have the same, unknown basis.

The combined data on benzoate-like ions leave little doubt as to the existence of an inverse dependence of conductance on pH. The benzoate data, like the propionate data, indicate but do not establish that the conductance-pH data is S shaped. There can be little doubt that the relationship is up-
wardly concave at high pH. In order to establish a downwardly concave conductance-pH relationship at low pH's, at least three points must be obtained there. Such data were obtained from seven of the eight cells represented in Fig. 5. Four of these cells (crosses; open, upright triangles; filled, upright triangles; open squares) show downward concavity; two show upward concavity (crosses; filled, upside-down triangles); and one shows first an increase, then a decrease as pH is lowered (solid squares). The best that can be said is that the data on benzoate-like ions are consistent with, or possibly indicative of, an S-shaped relationship between conductance and pH and the apparent pKₐ may be about 7.

Possible Nature of Irreversible Conductance Increase

One possible basis for explaining the pH dependence of the irreversible effects of benzoate-like ions on conductance is their relatively high pKₐ values (3.75-4.87). It is a well-known phenomenon that many weak acids readily cross the membrane in the undissociated, unionized form and then dissociate in the intracellular fluid. The weak acid thereby added to the cell fluid is almost completely dissociated at cell pH and titrates cell buffers in the acid direction, i.e., H⁺ combines with some of the impermeant internal anions, leaving the weak acid anions in their place. If it is supposed that benzoate-like anion conductance depends on both external and internal pH, then changing the pH of a bathing solution containing a benzoate-like ion from 7.4 to 6.0 or lower would cause an immediate increase in conductance due to the fall in external pH and a delayed, only slowly reversible further increase in conductance due to the slow fall in internal pH.

Two lines of evidence support a fall in internal pH as the cause of the irreversible effects: (a) The rate of onset of irreversible effects is inversely related to the pH of the bathing solution. The concentration of undissociated benzoic acid in the bath is directly proportional to bath [H⁺]: [benzoic acid] = ([benzoate] · [H⁺])/K, where K is the dissociation constant. It follows that a low pH and the high pKₐ favor the formation of benzoic acid in the bath. The flux of benzoic acid into the cell, being proportional to external concentration, will increase as pH decreases, and the irreversible effects will have a faster onset. (b) The irreversible conductance increase may well be a specific increase in benzoate conductance. Since benzoate is permeant, its internal concentration should be somewhere between the external concentration and the concentration in equilibrium with the membrane voltage. Hence, if the irreversible increase in conductance is specific to benzoate, the potential should tend toward a constant depolarization as pH is reduced from normal. Some evidence for this view is shown in Fig. 6 where the change in resting potential, ΔV, at some pH, with respect to the potential at 7.36, is plotted against relative conductance when both are altered by pH changes. The symbols are the same as used in Fig. 5. The values
for pH's below 6.0 are also included. It can be seen that increasing the membrane conductance by decreasing pH depolarizes the membrane, and, despite the scatter, it appears that the value of $\Delta V$ tends toward a limiting value of about 15 mV at large conductances. This relationship indicates that the membrane voltage is approaching a benzoate equilibrium potential 15 mV positive to the resting potential. The three rightmost points were obtained at pH's between 5.2 and 5.6. The conductances are much larger (note gap in and change in scale of the abscissa) than at pH's above 6.0 but the $\Delta V$'s are little if any larger. The plateauing of $\Delta V$ indicates that the irreversible increase in conductance found below pH = 6.0 is a specific increase in benzoate conductance.

Venosa et al. (1972) report that the addition of benzoate, salicylate, or acetylsalicylate to the bath causes a marked increase of $^{36}$Cl efflux from sartorius muscles bathed in a Ringer's solution made hypertonic with 100 mM KCl. For example, 10 mM benzoate increases $^{36}$Cl efflux 1.41 times. They found no increase in membrane conductance or in $^{42}$K efflux under the same circumstances and tentatively concluded that benzoate and related "... compounds stimulates an exchange-diffusion type of mechanism for chloride." An alternative explanation, suggested by the possibility that benzoate-like anions lower internal pH, is that a fall in internal pH stimulates an active extrusion of $\text{H}^+$ which is accompanied by $\text{Cl}^-$ rather than benzoate, since internal $\text{Cl}^-$ concentration was about 100 mM in their experiments.
OTHER BENZOATE-LIKE ANIONS The benzoate-like nature of acetate and formate were established by the same means as described for benzoate and propionate. A different approach was used for valerate and butyrate. Sequences were first measured at pH = 7.4 in four cells of one muscle and then at pH = 5.4 in another muscle. The muscles were equilibrated in Br\(^{-}\) and only briefly exposed to the benzoate-like anions in order to minimize irreversible effects. Since the sequences were the same in all four cells in each experiment and independent of the order of exposure, the irreversible effects did not affect the results. The sequence at pH = 7.4 is Br\(^{-}\) > benzoate > valerate > butyrate > propionate > acetate and at pH = 5.4 is benzoate > valerate > butyrate > propionate = acetate > Br\(^{-}\). The sequences are the same except that Br\(^{-}\) changes from most conducting at 7.4 to least conducting at 5.4. Also, the differences between adjacent ions at low pH are considerably smaller. Since valerate and butyrate are bracketed by benzoate and propionate at 7.4 and 5.4, the former two must have the same type of pH dependence as the latter two.

CONDUCTANCE SEQUENCE AT pH = 5.4 The conductance sequence at pH = 5.4 was extended in a few experiments. The combined data give the probable sequence: benzoate > valerate > butyrate > propionate ≈ acetate > Br\(^{-}\) > trichloroacetate > benzenesulfonate > glutamate > cysteate.

Hutter et al. (1969) report that the permeability sequence Cl\(^{-}\) > Br\(^{-}\) > NO\(^{-}\) > I\(^{-}\) at pH = 9.8 is reversed to I\(^{-}\) > NO\(^{-}\) > Br\(^{-}\) > Cl\(^{-}\) at pH = 5.0. The conductance sequence for benzoate-like ions is unaltered by pH, but, like the halides and NO\(^{-}\), the differences are smaller. All benzoate-like ions have higher conductances at pH = 5.4 than Br\(^{-}\) and Cl\(^{-}\) and probably greater than I\(^{-}\) and NO\(^{-}\). The sequence for the low conductance Cl\(^{-}\)-like (benzenesulfonate et seq.) ions is not altered.

The opposite effects of pH on the conductances of chloride-like and benzoate-like ions suggest the possibility that the conductance sequence at pH > 9.0 may differ slightly from the one at pH = 7.4 (Fig. 2). This possibility was not recognized at the time these experiments were done and we have no information on this point.

Effects of Zn\(^{++}\) on Benzoate and Chloride Conductance

The markedly different structural characteristics of Cl\(^{-}\)-like and benzoate-like anions and the opposite effects of pH on their conductances suggest that Cl\(^{-}\)-like anions use Cl\(^{-}\) channels and benzoate-like anions use a different set of channels or have other effects on the membrane. A multichannel hypothesis is testable by comparing the effects of Zn\(^{++}\) on benzoate and Cl\(^{-}\) conductances. Mashima and Washio (1964) and Hutter and Warner (1967 c)
showed that Zn\(^{++}\) in low millimolar concentrations reduces membrane conductance when Cl\(^{-}\) is the anion. If benzoate-like anions do not use Cl\(^{-}\)-channels then Zn\(^{++}\) likely would not reduce membrane conductance when benzoate is the anion. Hence, conductance vs. [Zn\(^{++}\)] curves were obtained for Cl\(^{-}\) and for benzoate. Zn\(^{++}\) concentrations were varied from 0.13 to 5 mM and were corrected for a Zn-benzoate association constant of 8.0 M\(^{-1}\) (Martell, 1964). The data are plotted in Fig. 7 with the conductance scales normalized to aid comparison and are inverted to put the curve in the standard form. The points are the mean values of relative membrane conductance. The solid curve is drawn assuming that one zinc ion blocks one anion channel with a dissociation constant of 0.125 mM. The mean value of the dissociation constant with Cl\(^{-}\) as the anion (four cells, three muscles) is 0.12 mM and for benzoate is 0.16 (four cells, three muscles). Mashima and Washio (1964) measured the dependence of the membrane time constant of frog muscle on Zn\(^{++}\) concentration in a chloride containing bathing solution. The dissociation constant estimated from their graph is about 0.15 mM. Hutter and Warner (1967 c) give the constant as about 0.1 mM.

In most experiments a small hyperpolarization (2-5 mV) accompanied the change in resistance caused by the addition of Zn\(^{++}\) over the entire range of Zn\(^{++}\) concentrations. Occasionally, however, the addition of Zn\(^{++}\) caused very large changes in \(R_e\) and transmembrane potential with either Cl\(^{-}\) or benzoate as the anion, particularly when the cell was exposed to Zn\(^{++}\) for the first time. Typical values are a 10-fold increase in \(R_e\) and a hyperpolarization of 40-50 mV. These effects are transient and a new steady

![Figure 7](https://example.com/figure7.png)

**Figure 7** Effects of Zn\(^{++}\) concentration on relative membrane conductance with chloride (open circles) and benzoate (filled circles) as the anion. Ordinate is 1 minus the ratio of conductance at experimental [Zn\(^{++}\)] to conductance with [Zn\(^{++}\)] = 0. Abscissa is concentration of Zn\(^{++}\) in bathing solution corrected for a Zn-benzoate association constant of 8 M\(^{-1}\) (Martell, 1964). Solid curve is drawn assuming that one Zn\(^{++}\) blocks one channel, the reaction having a dissociation constant of 0.125 mM. Each point is the mean value from four cells in three muscles. Error bars are ± SD. See text for details.
state is attained in 10–15 min. This effect was not investigated further but data from cells in which these large effects were seen are not included in Fig. 4.

The near identity of the effects of zinc on relative conductance when the anion is either Cl− or benzoate casts considerable doubt on the multichannel hypothesis; Zn++ could have nearly identical effects on two types of channels but this seems improbable. On the other hand, the one channel hypothesis that all permeant anions use chloride channels is consistent with the Zn++ data and with the pH data in the sense that the apparent pKₐ's are about the same for Cl−-like and benzoate-like anions.

DISCUSSION

Most of the results obtained in these experiments were unexpected: (a) The conductance of large anions such as benzoate and trichloroacetate are surprisingly high compared to chloride. (b) The discovery of the class of benzoate-like anions, anions whose conductances vary inversely with pH; in contrast, the conductances of Cl−-like ions vary directly with pH. (c) The conductances of benzoate-like ions increase with the size of the hydrophobic moiety attached to the carboxyl group (Fig. 2). (d) Some evidence indicating that benzoate-like ions traverse the membrane via chloride channels. Discussion is directed first at the reliability of the results and the validity of our interpretations of them and then to implications of these and other results for the structure of anion channels in muscle membranes.

Reliability of Measurements

Two criticisms can be directed at the experimental techniques: (a) Membrane voltage was not controlled and varied widely. (b) Characteristic resistance, Rₑ, was measured only in the hyperpolarizing direction. The first criticism has already been considered in the results section: The anion conductance and permeability sequences obtained are independent of the transmembrane potential over the wide range of values (-7 mV to -80 mV) encountered. Hutter and Warner (1972) show that Cl− conductance varies with voltage and pH. Thus it seems likely that the conductance of any anion species does depend on voltage but it is clear that the differential effects of voltage between ion species are not large enough to cause inversions in the sequence. Space clamp or voltage clamp experiments aimed at elucidating the effects of voltage on conductance such as those of Hutter and Warner (1972) will increase understanding of channel structure but would have been an unnecessary complication at the descriptive level of our experiments.

Accuracy of Rₑ Measurements

Rₑ measurements were made with hyperpolarizing currents because the current electrode would not pass sustained
depolarizing currents. The measurements might be in error if the current-voltage ($I-V$) curve has appreciable curvature at the resting potential. Even if this were so, our sequences would be invalid only if changing the anion in the bath changed the curvature of the $I-V$ relation of some other ion species (e.g., K$^+$). This possibility is discussed below. However, current-voltage relations were approximately linear over a $\pm 15$ mV range even when the anion had a low apparent conductance.

The approximately linear $I-V$ relation deserves some comment since the $I-V$ relation for the inwardly rectifying K$^+$ channel curves sharply in the region of the K$^+$ equilibrium potential (Adrian and Freygang, 1962b) and the current-voltage relation for Cl$^-$ is curved (Hutter and Warner, 1972). Three considerations show that our results are expected: (a) Measurements at one point on an infinite cable tend to smooth out nonlinearities in the $I-V$ curve. (b) For voltages more than 20 mV positive to the K$^+$ equilibrium potential, the $I-V$ relation for K$^+$ is nearly linear. Adrian and Freygang (1962b) dissected the $I-V$ curve into the sum of currents due to a constant K$^+$ conductance and a K$^+$ conductance that decreases exponentially with voltage (the inward rectifier). The size of the inward rectifier-specific conductance is relatively constant from fiber to fiber, whereas the size of the constant conductance component varies considerably. The voltage-dependent conductance is negligible for $V-V_K > 20$ mV, where $V_K$ is the K$^+$ equilibrium potential and the conductance is constant and low. (Adrian et al., 1970b, show that the situation with respect to K$^+$ currents is much more complicated than indicated here but further discussion is not warranted.) Since our resting potentials were usually more positive than $-60$ mV, then $V-V_K > 20$ mV if $V_K < -80$ mV and the contribution of K$^+$ to the $I-V$ curve is likely small and linear. (c) The curvature of the $I-V$ relation for Cl$^-$ is slow enough to appear approximately linear for deflections of 15 mV or less except possibly at pH = 9.8 where the $I-V$ curve begins bending appreciably at hyperpolarizations of 10 mV. Cable properties obscure this curvature.

**Interpretation of Conductance and Voltage Changes**

Our results are interpreted using the assumption that the various anion species permeate the membrane in varying degrees and do not have any affect on the permeabilities of other ion species. Although this is the simplest assumption, it is not the only one. For example, benzoate might actually be impermeant but produces the hyperpolarization and decrease in $R_s$ seen in Fig. 1 by acting on K$^+$ channels to increase their conductance and permeability, or more accurately, benzoate might depress K$^+$ conductance less than does glutamate. This type of second-order effect hypothesis applies only to K$^+$ channels; indirect effects on Na$^+$ channels could not produce
both the observed conductance and voltage changes. Although it is not possible to exclude indirect effects of this type, a number of points argue strongly against it:

(a) Edwards et al (1957) found that Br\textsuperscript{−}, NO\textsubscript{3}\textsuperscript{−}, and I\textsuperscript{−} do not greatly influence the movements of \textsuperscript{42}K in frog muscle. Hence the effects of these anions on conductance and permeability are very likely direct.

(b) Stanfield (1970) showed that replacing NaCl with 115 mM TEA-Cl increases membrane resistance from about 3,500 to 5,600 \(\Omega\) cm\textsuperscript{2} and that TEA acts to block one type of K\textsuperscript{+} channel with little affect on Cl\textsuperscript{−} channels. Our conductance and permeability sequences are the same independent of whether the principal cation is Na\textsuperscript{+} or TEA, indicating that K\textsuperscript{+} currents are not greatly altered by the anions used.

(c) There is good evidence that Zn\textsuperscript{++} in millimolar concentrations blocks Cl\textsuperscript{−}-channels with no detectable affect on K\textsuperscript{+} channels (Mashima and Washio, 1964; Hutter and Warner, 1967; Stanfield, 1970). Fig. 7 shows that the shape of the curve relating relative conductance and Zn\textsuperscript{++} concentration is the same shape when the anion is Cl\textsuperscript{−} and when it is benzoate. Furthermore, the residual conductances (mostly K\textsuperscript{+} conductance) in Cl\textsuperscript{−} and benzoate are about the same at high Zn\textsuperscript{++} concentrations.

Not only does this evidence indicate that benzoate does not alter potassium conductance but also that benzoate ions use Cl\textsuperscript{−} channels. It will be assumed hereafter that the effects of anions on membrane conductance are direct and reflect the passage of anions through the membrane.

Do Benzoate-Like Ions Use Chloride Channels?

The effects of pH on the conductances of chloride-like and benzoate-like anions and of Zn\textsuperscript{++} on Cl\textsuperscript{−} and benzoate conductances indicate that all the anions tested use the same channel. We attempted to test this one-channel hypothesis by looking for evidence of interactions between Cl\textsuperscript{−} and benzoate. Hutter and Padsha (1959) found that the addition of NO\textsubscript{3}\textsuperscript{−} and particularly thiocyanate to the bath increases the membrane time constant more than expected by assuming the anion movements are independent of each other. Similarly, Hutter and Warner (1967) showed that addition of I\textsuperscript{−} to the bathing medium greatly reduces the \textsuperscript{42}Cl efflux at alkaline pH's and reduces it at acid pH's. These actions may be explained by supposing that I\textsuperscript{−} can bind to sites in the Cl\textsuperscript{−} channel and thereby block the channel to the passage Cl\textsuperscript{−}.

Interactions between ions, e.g., Cl\textsuperscript{−} and benzoate, can be detected by measuring membrane conductance as a function of Cl\textsuperscript{−} or benzoate concentration with the sum of benzoate and Cl\textsuperscript{−} concentrations being kept constant. Our early attempts were unsuccessful but recent experiments done in this laboratory (J. Hestenes and J. W. Woodbury, 1972, unpublished)
establish that the conductance vs. Cl⁻ concentration curve with benzoate as the replacement anion is a straight line, showing that these two ion species do not interfere with each other as they traverse the membrane. This result is compatible both with the one-channel and multi-channel hypotheses. However, another type of experiment has produced convincing evidence supporting the one-channel hypothesis (Hestenes and Woodbury, 1973): The normalized conductance vs. Cl⁻ concentration curve with p-toluene-sulfonate as the replacement anion is strongly curved in a manner consistent with the hypothesis that toluenesulfonate binds to Cl⁻ channels and blocks them to the passage of Cl⁻. The apparent dissociation constant is 48 mM. The same experiment was repeated for p-toluene-sulfonate vs. benzoate. Again the apparent dissociation constant for p-toluene-sulfonate is 48 mM. Thus, toluenesulfonate blocks to the same degree channels that are used by both Cl⁻ and benzoate. This result is exactly analogous to the effects of Zn⁺⁺ which also blocks to the same degree Cl⁻ and benzoate channels. It is unlikely that two substances as diverse as Zn⁺⁺ and toluenesulfonate could have identical effects on distinct chloride channels and distinct benzoate channels, indicating that both ions use the same channels.

A Model of the Anion Channel

Perhaps it should be emphasized here that the remainder of the discussion is highly speculative. Such speculation is included for two reasons: (a) The discovery of the class of benzoate-like anions is unexpected and it is thus important to develop any testable hypothesis that can explain this puzzling phenomenon. (b) The hypothesis developed permits testable predictions and is thus a useful guide to future experimentation.

The results obtained for toluenesulfonate make the case for the one-channel hypothesis so strong (though not proven) that we felt obligated to adopt it tentatively and seek a simple, detailed model that explains the inverse pH dependences of Cl⁻-like and benzoate-like anions and the other known characteristics of chloride channels, hereafter called anion channels. A satisfactory model has been developed by one of us (J. W. Woodbury). Since some of the postulates are based on unpublished results and results published only in abstract form (Hestenes and Woodbury, 1972, 1973), only a brief description of the aspects concerned with conductance sequences and their pH dependence will be given here. The objective is to show that a simple model of a single type of channel can account for the behavior of Cl⁻-like and benzoate-like ions. The model is an extension of the model of Hutter and Warner (1967b, c).

The anion channel is at least 5.5 × 6.5 Å. If all anions use the same channel, then the minimum dimensions of the channel can be obtained from the sizes of the largest permeating anions. Molecular scale models were
made of all the ions shown in Fig. 2 and an estimate mode of the minimal dimensions of a rectangular hole which allows passage of the ion. In some cases these estimates were checked by direct calculations from bond lengths and atomic radii (Pauling, 1960). The largest anions are trichloroacetate, 5.5 \(\times\) 6.0 Å; benzoate, 3.4 \(\times\) 6.5 Å; benzenesulfonate, 5.0 \(\times\) 6.5 Å; and glutamate, approximately 4.5 \(\times\) 5.5 Å. Since the largest anions, trichloroacetate and benzoate, have high conductances (Table I), we conclude that an anion channel is at least 5.5 \(\times\) 6.5 Å and that size plays at best a minor role in determining the conductance of the anions tested.

Hille (1971) measured the relative permeabilities of the sodium channels in myelinated nerve fibers to organic cations. He explained the permeability sequence by assuming that a sodium channel consists of oxygen atoms arranged to make a roughly rectangular opening of 3 \(\times\) 5 Å and the associated molecular structure necessary to hold the oxygens in place. The aperture of an anion channel appears to occupy more than twice as much area as a sodium channel.

**Assumptions**

The conductance sequences and pH dependences of Cl\(^-\)-like and benzoate-like ions can be qualitatively explained by a model based on four simple assumptions about the structure of the anion channel. There is experimental evidence for each assumption:

(a) The channel is thin in the sense that the rate of passage of an anion through the channel depends only on the rate at which the anion passes one site (rate-limiting step). The identity of the permeability and conductance sequences is expected of thin membranes with neutral (dipolar) sites (Barry and Diamond, 1971).

(b) The rate-limiting step is located near a group that is dipolar at high pH and which can accept a proton, the reaction having a pK\(_a\) of about 7. Hutter and Warner (1967 c) give convincing evidence that the dipole is an imidazole group.

(c) There is a hydrophobic region adjacent to the site of the rate-limiting step. The direct relationship between the conductance of benzoate-like ions and the sizes of their hydrophobic groups reported here leads directly to this assumption. Armstrong (1969) measured the potency of analogs of TEA ions in blocking K\(^+\) channels when injected into squid giant axons. He found that the binding constant for blocking goes directly with the length of the hydrocarbon chain which replaced one of the ethyl groups, and concluded there is a hydrophobic region adjacent to the inside entrance to the K\(^+\) channel.

(d) The rate-limiting step is a potential energy valley (binding site) for chloride-like anions and a potential energy hill or barrier for benzoate-like anions. The potential energy valley for Cl\(^-\) is shallow or perhaps flat at high pH. The distinction is observable: Anions that bind to the channel block
it to the passage of other anions. Anions that see the channel as a potential energy barrier spend a very short time in the region of this rate-limiting step and hence do not interfere with the passage of other anions. Evidence that Cl$^-$-like anions bind to sites is good. Hutter and Padsha (1959) found that membrane resistance increases more than expected on the basis of independence when part of the Cl$^-$ is replaced by thiocyanate, I$^-$ and perhaps NO$_3^-$. Hutter and Warner (1967 c) report that external I$^-$ slows $^{36}$Cl efflux. Hestenes and Woodbury (1973) report that NO$_3^-$, Br$^-$, I$^-$, and trichloroacetate all have blocking effects on chloride conductance. As mentioned above, Cl$^-$ and benzoate do not interfere with each other.

**CL$^-$-LIKE ANIONS** Hutter and Warner (1967 b, c) model the effect of pH on Cl$^-$ conductance by assuming that protonation of a channel site (presumably imidazole) reduces flow of Cl$^-$ through the channel. This is reasonable because protonation increases the field strength of the imidazole group, increases the binding energy of Cl$^-$ to the group, and reduces the Cl$^-$ conductance by decreasing the rate of dissociation of Cl$^-$ from the group. The same argument holds for all Cl$^-$-like anions. The important quantity according to Eisenman’s equilibrium-binding theory (Eisenman, 1961, 1965; Diamond and Wright, 1969) is the difference in water-anion and site-anion binding energies. Small anions with intense fields (e.g., F$^-$) prefer the strongly polar water molecules to the weak dipole of imidazole at high pH. Larger anions with weaker fields may prefer the imidazole group and hence may bind to it in preference to water. These are the Cl$^-$-like anions. Protonation increases the binding energy between ion and site. For example, Hestenes and Woodbury (1973) found that reducing pH from 8.4 to 5.9 increases the association constant of p-toluene sulfonate (a Cl$^-$-like anion) from about $20 \text{M}^{-1}$ to $250 \text{M}^{-1}$, a greater than 10-fold increase.

On the basis of the model, Cl$^-$-like anions are characterized at the molecular level as having relatively weak field strengths (Cl$^-$ or weaker) such that they bind to unprotonated and protonated imidazole groups in preference to water. The higher the binding energy the lower the conductance.

The halide conductance sequence we find at normal pH is the reverse of the one expected from Eisenman’s equilibrium-binding theory for weak sites in a thick membrane. However, Barry and Diamond (1971) using Eisenman’s theory calculate that conductance and permeability sequences are the same in thin membranes with neutral (dipolar) sites and that conductance goes inversely with binding energy.

**BENZOATE-LIKE ANIONS** The functional definition of benzoate-like anions—that increasing pH decreases membrane conductance—can be explained in terms of the energetics of ion penetration through a channel. The benzoate-like anions are all carboxylic acids with attached hydrophobic moie-
ties. The pKₐ's are 4–5, indicating that the field strength of the carboxyl group is high, i.e., carboxyl has a high affinity for H⁺. The binding energy between the carboxyl and H₂O is assumed to be greater than between carboxyl and an imidazole dipole. It is thus energetically unfavorable for a benzoate-like ion to leave the solution and enter an anion channel, the ion "sees" the channel as a potential energy hill. The higher the barrier is, the lower the frequency that an ion leaves the solution, surmounts the barrier, and crosses the membrane, and the lower the conductance.

The conductance of benzoate-like ions increases as pH is lowered because protonation of the imidazole increases its field strength, thereby lowering the height of the potential energy barrier and increasing the frequency at which ions cross the barrier. If the height of the potential energy barrier were determined solely by the differences in binding energies of the carboxyl group to water and to site, then the conductance sequence would be expected to be the reverse of the pKₐ sequence, i.e., a high pK means that the anion has a high affinity for H⁺, a high field strength, a high potential energy barrier, and a low conductance. Inspection of Fig. 2 shows little correlation between conductance and pKₐ for benzoate-like anions; the pKₐ's in order of decreasing conductance are 4.20, 4.82, 4.8, 4.87, 3.75, and 4.76. The good correlation between the size of the hydrophobic moiety and conductance strongly suggests that there is a hydrophobic region adjacent to the site of the potential energy barrier. Binding of the anion's hydrophobic moiety to the hydrophobic region reduces the height of the barrier (cf., Jencks, 1969). The larger the hydrophobic binding energy is, within limits, the greater the reduction in the height of the barrier and the higher the conductance.

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**SULFONATE** The replacement of the carboxyl group by a sulfonate group in a benzoate-like anion (e.g., benzoate to benzenesulfonate) reduces its conductance and makes it Cl⁻-like (Fig. 2). This behavior is contained in the model: Since the field intensity of a sulfonate group (pKₐ ~ 0) is much weaker than that of a carboxylate group (pKₐ ~ 4.5), the potential energy barrier is greatly reduced, possibly reversed, and hydrophobic bonding is the dominant energy term at both high and low pH's. Sulfonate ions are thus expected to and do have low conductances, bind to the channel site, and interfere with the movements of higher conductance ions. Protonation of the channel further increases binding energy and decreases conductance, the defining characteristic of the Cl⁻-like ions.

**FORMATE AND BICARBONATE** Formate, HCOO⁻, has a slightly greater conductance than acetate and is thus the only exception to the rule that conductance of benzoate-like ions varies directly with the size of the hydrophobic moiety. Formate is small, has a pKₐ of 3.75 (about one unit smaller
than the most of the benzoate-like ions), and can hardly be regarded as having a hydrophobic region. No satisfactory explanation for the anomalous behavior of formate has been developed. Regardless of the exact explanation of the behavior of formate, it can be predicted with some confidence that replacing the H of HCOO\(^-\) by an OH to give HOOCOO\(^-\) (bicarbonate) will produce a Cl\(^-\)-like ion with a lower conductance than formate. This is in strict analogy to the relation between propionate CH\(_3\)CH\(_2\)COO\(^-\) (benzoate-like) and lactate CH\(_3\)(CHOH)COO\(^-\) (Cl\(^-\)-like). Further, the conductance of bicarbonate, HOOCOO\(^-\), should be about 0.05 \(G_{Cl}\), just lower than lactate in the sequence in Fig. 2. This figure is in reasonable agreement with Woodbury’s (1971) rough estimate of 0.1 for the value of \(P_{HOOC}/P_{Cl}\).

**Other Predictions** The model presented is sufficiently detailed to make several classes of testable predictions: (a) The sulfonate analogs of benzoate-like ions (e.g., benzenesulfonate) should bind to channels at high pH and more tightly at low pH as shown by Hestenes and Woodbury (1973). Binding constants should, within limits, increase with the size of the hydrophobic group. (b) Benzoate-like anions with sufficiently large hydrophobic groups should bind to channel sites. This prediction is supported by the findings of Bryant and Morales-Aguilera (1971) that monocarboxylic acids with huge hydrophobic groups, e.g., phenanthrene-9-carboxylic acid, lower membrane Cl\(^-\)-conductance at concentrations of \(10^{-5}\) to \(10^{-4}\) M. (c) Hydrophilic anions with field strengths equal to or weaker than chloride’s should bind to channel sites and will be Cl\(^-\)-like; ions with higher field strengths will not bind and will be benzoate-like and may have quite low conductances. Hestenes and Woodbury (1972) find that the high field-strength fluoride ion does not interfere with Cl\(^-\) and has a lower conductance than I\(^-\).

**The Completely “Impermeant” Monovalent Anion** The desirable properties of a completely impermeant anion, A\(^-\), can now be specified more completely: A\(^-\) should not bind to channel sites, i.e., be benzoate-like and should be so large that it cannot enter the channel. Unfortunately, benzoate-like ions whose hydrophobic groups are considerably larger than benzene are not very soluble and probably Cl\(^-\)-like because they block channels in low concentrations (Bryant and Morales-Aguilera, 1971). Presumably hydrophobic binding is greater than the carboxyl’s preference for water. The other alternative for A\(^-\) is a Cl\(^-\)-like, hydrophilic ion too large to enter the channel and/or one whose electric field is so weak that the binding energy to the site is negligibly small. Likely candidates are gluconate (six carbon straight chain, one carboxyl, and five hydroxyl groups) and glucuronate (pyranose ring, one carboxyl, and four hydroxyl groups).

The detailed model that forms the basis for the preceding discussion was developed before the publication of results of Hutter and Warner (1972) on
the $I-V$ relations of chloride channels and by Warner (1972) of the time dependence of Cl$^-$ conductance. Hence, their results provide a stringent test of the model. Preliminary considerations indicate that the model has implicit properties that predict their results at least qualitatively. Regardless of whether the model is adequate or has to be modified or replaced, it makes testable predictions. In addition, a wealth of data are presently available for deducing the essential molecular features of the anion channels of muscle membranes.

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REFERENCES

ADRIAN, R. H., W. K. CHANDLER, and A. L. HODGKIN. 1970 a. Voltage clamp experiments in striated muscle fibers. J. Physiol. (Lond.). 208:607.

ADRIAN, R. H., W. K. CHANDLER, and A. L. HODGKIN. 1970 b. Slow changes in potassium permeability in skeletal muscle. J. Physiol. (Lond.). 208:655.

ADRIAN, R. H., and W. H. FREYGANG. 1962 a. The potassium and chloride conductance of frog muscle membranes. J. Physiol. (Lond.). 163:61.

ADRIAN, R. H., and W. H. FREYGANG. 1962 b. Potassium conductance of frog muscle membrane under controlled voltage. J. Physiol. (Lond.). 163:104.

ARMSTRONG, C. M. 1969. Inactivation of the potassium conductance and related phenomena caused by quaternary ammonium ion injection in squid axons. J. Gen. Physiol. 54:553.

BARRY, P. H., and J. M. DIAMOND. 1971. A theory of ion permeation through membranes with fixed neutral sites. J. Membrane Biol. 4:295.

BRYANT, S. H., and A. MORALES-AGUILERA. 1971. Chloride conductance in normal and myotonic fibers and the action of monocarboxylic aromatic acids. J. Physiol. (Lond.). 219:567.

DIAMOND, J. M., and E. M. Wright. 1969. Biological membranes: The physical basis of ion and non-electrolyte selectivity. Annu. Rev. Physiol. 31:581.

EDWARDS, C., E. J. HARRIS, and K. NISSE. 1957. The exchange of frog muscle Na$^+$ and K$^+$ in the presence of anions Br$^-$, NO$_3^-$, I$^-$ and CNS$^-$. J. Physiol. (Lond.). 135:560.

EISENBERG, R. S., and P. W. GAGE. 1969. Ionic conductances of the surface and transverse tubular membranes of frog sartorius fibers. J. Gen. Physiol. 53:279.

EISENMAN, G. 1961. On the elementary atomic origin of equilibrium ionic specificity. In Membrane Transport and Metabolism. A. Kleinzeller and A. Kotyk, editors. Academic Press Inc., London. 163.

EISENMAN, G. 1965. Some elementary factors involved in specific ion permeation. 29th Int. Congr. Physiol. Sci. Lect. Symp., Tok. 489.

GAGE, P. W., and R. S. EISENBERG. 1969. Capacitance of the surface and transverse tubular membrane of frog sartorius muscle fibers. J. Gen. Physiol. 53:265.

GOOD, N. E., G. D. WIDGERT, W. WINTER, T. N. CONNOLLY, S. IZAWA, and R. M. M. SINGH. 1966. Hydrogen ion buffers for biological research. Biochemistry. 5:467.

GORDON, A. M., R. E. GODT, and J. W. WOODBURY. 1970. Ionic strength as a determinant of calcium-activated tension in skinned muscle fibers in various salt solutions. Fed. Proc. 29:656.

HAGIWARA, S., K. TOYAMA, and H. HAYASHI. 1971. Mechanisms of anion and cation permeations in the resting membrane of a barnacle muscle fiber. J. Gen. Physiol. 57:408.

HESTENES, J. D., and J. W. WOODBURY. 1972. Anomalous position of fluoride in the halide conductance sequence of frog sartorius muscle membrane. Physiologist. 15:166.

HESTENES, J. D., and J. W. WOODBURY. 1973. Anion binding to anion channels of frog sar-
torius: estimates of dissociation constants. Abstracts of the Biophysical Society, 17th Annual Meeting, Columbus, Ohio. 243a.

Hille, B. 1971. The permeability of the sodium channel to organic cations in myelinated nerve. *J. Gen. Physiol.* 58:599.

Hodgkin, A. L., and P. Horowicz. 1959. The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol. (Lond.)* 148:127.

Hodgkin, A. L., and P. Horowicz. 1960. The effect of sudden changes in ionic concentrations on the membrane potential of single muscle fibres. *J. Physiol. (Lond.)* 153:370.

Hutter, O. F., W. C. Mello, and A. E. Warner. 1969. An application of the field strength theory. In The Molecular Basis of Membrane Function. D. C. Tosteson, editor. Prentice-Hall, Inc., Englewood Cliffs, N. J. 391.

Hutter, O. F., and D. Noble. 1960. The chloride conductance of frog skeletal muscle. *J. Physiol. (Lond.)* 151:89.

Hutter, O. F., and S. M. Pashka. 1959. Effect of nitrate and other anions on the membrane resistance of frog skeletal muscle. *J. Physiol. (Lond.)* 146:117.

Hutter, O. F., and A. E. Warner. 1967 a. The pH sensitivity of the chloride conductance of frog skeletal muscle. *J. Physiol. (Lond.)* 189:403.

Hutter, O. F., and A. E. Warner. 1967 b. The effect of pH on the 36Cl efflux from frog skeletal muscle. *J. Physiol. (Lond.)* 189:427.

Hutter, O. F., and A. E. Warner. 1967 c. Action of some foreign cations and anions on the chloride permeability of frog muscle. *J. Physiol. (Lond.)* 189:445.

Hutter, O. F., and A. E. Warner. 1972. The voltage dependence of the chloride conductance of frog muscle. *J. Physiol. (Lond.)* 227:275.

Jencks, W. P. 1969. Catalysis in Chemistry and Enzymology, McGraw-Hill Book Company, New York. 393.

Marrelli, A. E. 1964. Organic ligands. In Stability Constants of Metal-Ion Complexes. Special publ. no. 17, The Chemical Society, Burlington House, London.

Mashima, H., and H. Washio. 1964. The effect of zinc on the electrical properties of membrane and the twitch tension in frog muscle fibers. *Jap. J. Physiol.* 14:538.

Miles, P. R., and J. W. Woodbury. 1971. Anion permeability sequence for frog muscle fiber membranes. Abstracts of the Biophysical Society, 15th Annual Meeting, Columbus, Ohio. 142a.

Moore, L. E. 1969. Anion permeability of frog skeletal muscle. *J. Gen. Physiol.* 54:33.

Pauling, L. 1960. The Nature of the Chemical Bond. Cornell University Press, Ithaca, N. Y. 3rd edition.

Spurway, N. C. 1972. Mechanisms of anion permeation. In Biomembranes, Volume 3, Passive Permeability of Cell Membranes. F. Kreuzer and J. F. G. Slegers, editors. Plenum Publishing Corporation, New York.

Stanfield, P. R. 1970. The differential effects of tetraethylammonium and zinc ions on the resting conductance of frog skeletal muscle. *J. Physiol. (Lond.)* 209:231.

Venosa, R. A., A. C. Ruarte, and P. Horowicz. 1972. Chloride and potassium movements from frog's sartorius muscle in the presence of aromatic anions. *J. Membrane Biol. 9*:37.

Warner, A. E. 1972. Kinetic properties of the chloride conductance of frog muscle. *J. Physiol. (Lond.)* 227:291.

Woodbury, J. W. 1971. Fluxes of H+ and HCO3- across frog skeletal muscle membranes. In Ion Homeostasis of the Brain (Alfred Benzon Symposium III). B. K. Siesjo and S. C. Sorensen, editors. Munksgaard, A/S, Copenhagen.