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Chloride Conductance of the Amphiuma Red Cell Membrane

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Summary. Like most other red cells, the giant erythrocytes of *Amphiuma means* possess a system for rapid exchange of chloride across the membrane. Also, there are indications that the net transport of chloride in these cells is slow. The size of *Amphiuma* erythrocytes allows direct measurements of membrane potential with microelectrodes. The present work exploits the possibility that such measurements can be used to give a quantitative estimate of the chloride conductance (G_{Cl}) of the Amphiuma red cell membrane. The membrane potential was measured as a function of extracellular chloride concentration (5–120 mM), using an impermeant anion (Para-amino-hippurate) as a substitute. Furthermore, the effect of different pH values (6.0–7.2) was studied. For each extracellular chloride concentration the membrane potential was determined at a pH at which hydroxyl, hydrogen, and bicarbonate ions were in electrochemical equilibrium. From these membrane potentials and the corresponding chloride concentrations in the medium (at constant intracellular ion concentrations), the G_{Cl} of the membrane was calculated to be $3.9 \times 10^{-7}\,\Omega^{-1}\,cm^{-2}$. This value is some six orders of magnitude smaller than that calculated from the rate of tracer exchange under equilibrium conditions. The experimental strategy used gives the value for a "partial transference number" which takes into account only ions which are *not* in electrochemical equilibrium. Whereas this approach gives a value for G_{Cl}, it does not permit calculation of the overall membrane conductance. From the calculated value of G_{Cl} it is possible to estimate that the maximal value of the combined conductances of hydroxyl (or proton) and bicarbonate ions is $0.6 \times 10^{-7}\,\Omega^{-1}\,cm^{-2}$. The large discrepancy between the rate of exchange of chloride and its conductance is in agreement with measurements on human and sheep red cells employing the ionophore valinomycin to increase the potassium conductance of the membrane. The results in the present study were, however, obtained without valinomycin and an accompanying assumption of a constant field in the membrane. Therefore, the present measurements give independent support to the above mentioned conclusions.

It has long been known (e.g., Luckner, 1939; Tosteson, 1959) that a rapid exchange of monovalent inorganic anions such as Cl and HCO_3 takes place across the red cell membrane. This notion has been strongly confirmed and extended in recent years (Gunn, Dalmark, Tosteson & Wieth, 1973) by the demonstration that this exchange system is saturable and presumably

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occurs on a 1:1 basis. Because of the rapid exchange, most investigators were led to believe that the membrane conductance for chloride (G_{Cl}) was so large in comparison to the conductance for other ions (e.g., K and Na) that chloride was in electrochemical equilibrium under all conditions. A consequence of this assumption was that the membrane potential could be described in terms of the equilibrium potential for chloride ions. However, in studies using ionophores to increase the K permeability of the red cell, Harris and Pressman (1967) as well as Scarpa, Cecchetto and Azzone (1970) showed that the rate of KCl loss was orders of magnitude lower than the Cl exchange rate. These observations suggested that chloride ions were not necessarily in equilibrium. In 1971 Hunter used the K flux ratios in valinomycin-treated human red cells to calculate the membrane potential. From the calculated potential G_{Cl}, was estimated to be approximately four orders of magnitude smaller than expected from the maximal rate of exchange. Using a slightly different approach, Tosteson, Gunn, and Wieth (1973) arrived at a similar conclusion in the case of sheep red cells. In experiments with red cells from the salamander *Amphiuma means*, Hoffman and Lassen (1971, and *unpublished results; see* Lassen, 1972) reported the apparent membrane resistance to be greater than $2000\,\Omega cm^2$. This value should be compared to the equivalent resistance of less than $1\,\Omega cm^2$, as calculated from tracer exchange data (J. Brahm, *personal communication*). Since there is reason to believe that the exchange of chloride in *Amphiuma* red cells proceeds in a manner similar to that in human red cells, these findings are consistent with the proposal by Hunter (1971, 1977) and Tosteson *et al.* (1973) that the actual chloride conductance of the red cell membrane is much smaller than that predicted from tracer exchange studies.

The values for G_{Cl} obtained by Hunter (1971, 1977) are based on the assumption that, in the presence of valinomycin, the flux ratio for K in red cells is solely dependent on concentration gradients and the membrane potential. The existence of a complicated transport kinetics involving the valinomycin-induced K transport (Ting-Beall, Tosteson, Gisin and Tosteson, 1974; Läuger and Stark, 1970) may bring into question this simple assumption. On the other hand, the direct resistance measurements in *Amphiuma* red cells reported by Hoffman and Lassen (1971; *see also* Lassen, 1972) suffer from the technical errors associated with incomplete sealing around the penetrating electrode (Lassen, Nielsen, Pape and Simonsen, 1971). The leak resulting from such an incomplete sealing would lead to a gross underestimation of the membrane resistance.

Ideally, the experimental determination of membrane conductance implies the measurement of a complete I–V characteristic. While this has

been done for a number of cell types, it is at present not possible in red cells for technical reasons. An alternative is to measure the membrane potential directly and from a determination of the changes in potential related to alterations in chloride concentration gradients, calculate the chloride conductance. One is still faced with the difficulty that direct membrane potential measurements in human red cells are not as yet feasible. Hoffman and Lassen (1971) demonstrated that such measurements are possible in the much larger red cells of *Amphiuma means* using para-amino-hippurate as an impermeable substitute for chloride. Since, as discussed above, these cells exhibit a rapid chloride exchange and apparent low conductance in common with mammalian red cells, the present study was undertaken in order to more directly measure G_{Cl}. From the present data it is concluded that G_{Cl} for *Amphiuma* red cells is even smaller than previously suggested, and is in magnitude comparable to G_{Na} and G_K. As a consequence, it is clear that unless chloride ions redistribute via the exchange pathway, chloride disequilibrium can be encountered under certain experimental conditions. Part of this work has been presented elsewhere (Lassen, Pape and Vestergaard-Bogind, 1975).

Materials and Methods

Red cells from anaesthetized specimens of *Amphiuma means* were obtained by cardiac puncture and immediately suspended in large volumes of Ringer's. The cells were then washed twice in "normal" Ringer's and stored at 17 °C until used. For experiments in which the membrane potential was measured under altered conditions a small sample of blood was centrifuged and the supernatant discarded. The cells were then carefully resuspended in 50–100 volumes of the new medium. In time-dependent studies, the instant the cells were transferred to the appropriate test medium was taken as zero time. After recentrifugation the cell pellet was resuspended in the experimental medium so as to obtain a hematocrit of 0.1–0.5%. This suspension was then suitable for introduction into the microelectrode measuring chamber. The time interval from the initial change in suspension medium until the first potential measurements could be obtained was typically 60–80 sec.

In the case of Ringer's solutions with different Cl^- concentrations, para-amino-hippurate (PAH) (Merck, Darmstadt, W. Germany) was used as the substitute impermeant anion, and added in an amount calculated to maintain constant osmolarity. Composition of incubation media are given in Table 1.

All media contained 1 g of bovine serum albumin (Calbiochem, San Diego, Calif.) per liter and were buffered with 10 mM morpholino-propane sulphonic acid (MOPS) (Sigma Chemical Co., St. Louis, Mo.). Media were adjusted to the appropriate pH by titration with 1 M NaOH.

Membrane potentials were measured with conventional KCl (2.5 M) filled microelectrodes with tip diameters of 0.2 μm. The electrodes were mounted in a holder attached to a piezoelectric electro-mechanical transducer allowing rapid advancement of the microelectrode. The membrane potentials are taken as the peaks of potential change in immediate relation to the penetration of the cell membrane with the microelectrode tip. The validity of

Table 1

mM	"Normal" Ringer's	Reduced Cl^- Ringer's
Na^+	118	118
K^+	2.5	2.5
Cl^-	124	x
Ca^{++}	1.8	1.8
PAH	0	$124-x$

this procedure has received strong support from the study of Stoner and Kregenow (1976, and *personal communication*) who also measured membrane potential of *Amphiuma* red cells employing a method which leads to a very slow decay of the potentials. The experiments of Hoffman and Laris (1974) using a fluorescent dye to monitor membrane potentials also support the validity of the direct measurements of *Amphiuma* membrane potentials. [For a general discussion of these and related problems, *see* Lassen (1977) and Lassen and Rasmussen (1977).]

Extra- and intracellular concentrations of Na, K, and Cl were measured as described by Lassen, Pape, Vestergaard-Bogind and Bengtson (1974). Fluxes were calculated from a simple two-compartment model.

To determine H/OH fluxes, cells were suspended in a medium containing 16 mM chloride and adjusted to various initial pH values. The change in extracellular pH was monitored for a period of 10–12 min during which changes in the extracellular concentration of chloride were also followed. Changes in pH during this period ranged from −0.02 to 0.25 pH units. Net fluxes of $OH + HCO_3$ per liter cells were then calculated from the changes in pH and the known buffering capacity of the suspension medium (5.3 mmoles/liter extracellular phase/pH unit at pH 6.70).

Results

Representative oscilloscope traces obtained on impalement of *Amphiuma* red cells with microelectrodes are shown in Fig. 1. The upper trace (*A*) is characteristic of cells suspended in "normal" Ringer's solution containing 120 mM Cl. As discussed previously (Lassen *et al.*, 1971), the peak negative value is taken as a measure of the membrane potential of the unperturbed cell. This value, which is approximately −15 mV in "normal" Ringer's, does not remain constant during the recording but, as seen in the figure, decays to a less negative value. This decay results from the leak induced in the membrane by the mechanical damage caused by penetration by the electrode. If the extracellular chloride concentration is reduced to 10 mM by substituting chloride with para-amino-hippuric acid (PAH) to which the membrane is essentially impermeable, the typical potential trace is altered to that shown in Fig. 1*B*. This replacement by PAH reverses the equilibrium potential for chloride; and, as expected, the change in chloride

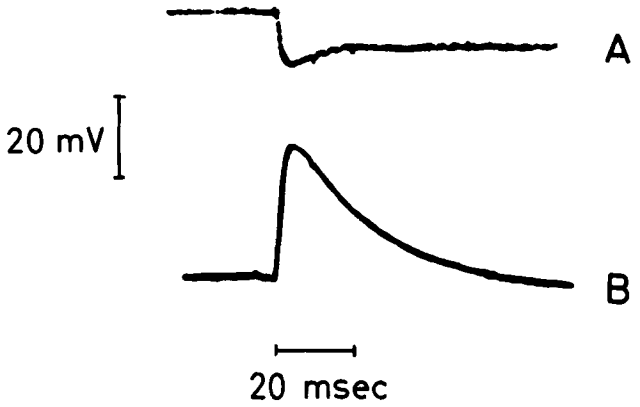

Fig. 1. Representative oscilloscope traces following micropuncture of *Amphiuma* red cells. (*A*): Normal Ringer's, pH 7.2 (120 mM Cl); (*B*): 10 mM Cl-Ringer's, pH 7.2 (110 mM PAH). *Ordinate:* in mV (vertical bar); *abscissa:* time in msec (horizontal bar). 17 °C

gradient results in a change in membrane potential. The rate of decay of the potentional in Fig. 1*B* is somewhat slower than that in Fig. 1*A*. This difference is not consistent but rather reflects variation between individual measurements. Fig. 2 is a plot of the measured membrane potential vs. log concentration of extracellular chloride.

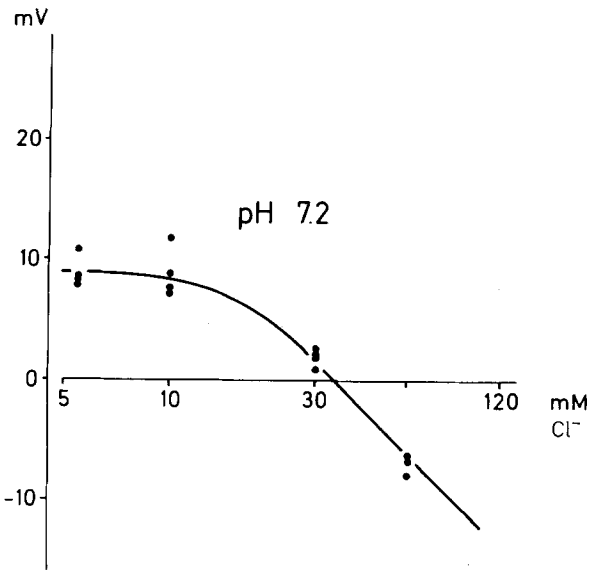

Fig. 2. Membrane potential of *Amphiuma* red cells as a function of extracellular chloride concentration. *Ordinate:* membrane potential in mV; *abscissa:* Cl concentration in mM (log scale). Points indicate separate experiments. pH 7.2, 17 °C

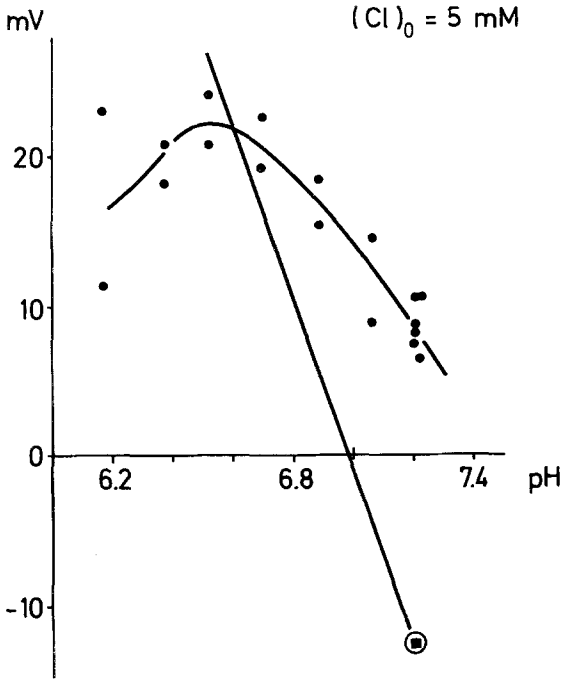

Fig. 3. Membrane potential of *Amphiuma* red cells as a function of extracellular pH at an extracellular chloride concentration of 5 mM (115 mM PAH). *Ordinate:* membrane potential in mV; *abscissa:* extracellular pH. Points represent separate experiments. 17 °C. Cells were preequilibrated at pH 7.2 (normal Ringer's)

Tosteson *et al.* (1973) have shown that G_{OH} is of the same order of magnitude as G_{Cl} in sheep red cells. If this is true of *Amphiuma* red cells as well, then changes in potential resulting from a shift in chloride concentration will give rise to a rapid net flux of OH(H) which in turn will play a role in determining the membrane potential. In particular, the hydroxyl ion flux would be expected to cause a reduction in the positive change in potential produced by the shift in extracellular chloride. In order to ascertain whether this occurred, membrane potentials were measured as a function of extracellular pH for various concentrations of chloride in the medium. The results are shown in Figs. 3 to 6. It is clear from these results that a reduction in pH leads to a positive change in membrane potential with a maximum potential at a pH value from 6.4 to 6.6. However a further reduction in extracellular pH results in a smaller positive change. Both the magnitudes of membrane potentials at given extracellular chloride ($[Cl]_o$) concentrations and pH, as well as the overall shape of these curves will be dealt with in detail in the discussion.

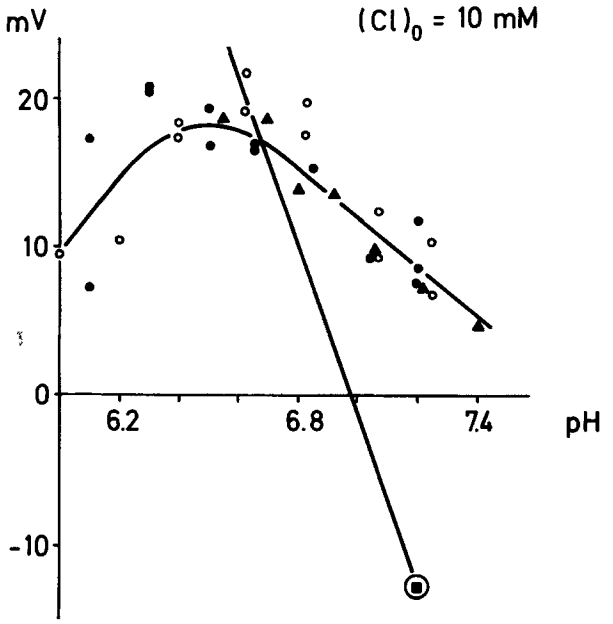

Fig. 4. Membrane potential of *Amphiuma* red cells as a function of extracellular pH at an extracellular chloride concentration of 10 mM. *Filled circles:* measurements in Ringer's containing 110 mM PAH as the substitute for chloride. *Triangles:* same medium plus 0.9 mM acetazolamide. Open circles: 110 mM MOPS substituted for chloride. *Ordinate:* membrane potential in mV, *abscissa:* extracellular pH. 17 °C. Cells were preequilibrated at pH 7.2 (normal Ringer's)

An additional condition for obtaining G_{Cl} from these data is that the membrane potential at any given $[Cl]_o$ remain constant within the measuring period. In order to verify whether this condition was maintained, membrane potentials at a given $[Cl]_o$ were measured as a function of time. The results are shown in Figs. 7 and 8. At pH 6.4 (Fig. 7) this condition is obviously fulfilled. At pH 6.0 (Fig. 8) this is not the case. There is a tendency for the positive potential to decay with time, and in addition a small fraction of the cells have highly negative potentials. The presence of a small number of hyperpolarized (negative) cells at the lowest pH values was characteristic of all extracellular chloride concentrations. Furthermore, the number of hyperpolarized cells decreased with time. This finding is both unexpected and, at present, unexplainable.

A prerequisite for the application of these data to the calculation of G_{Cl} is that the equilibrium potentials for all ions remain essentially constant during the period of membrane potential measurement for all extracellular chloride concentrations. However, changes in $[Cl]_o$ cause a change in the

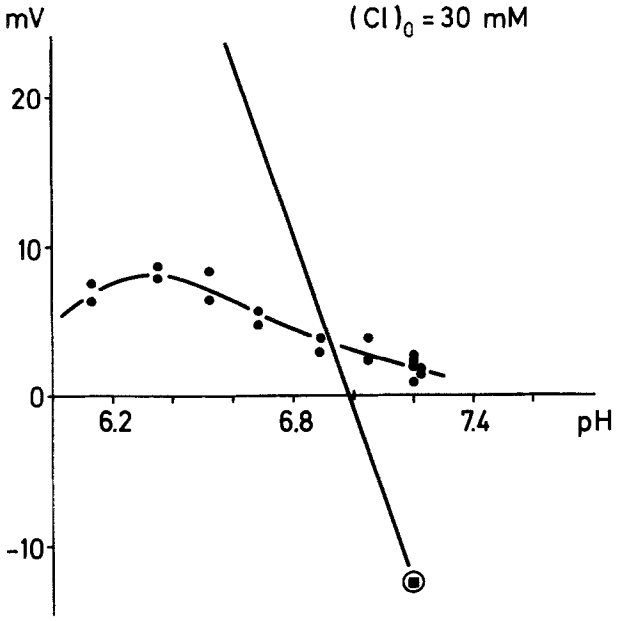

Fig. 5. Membrane potential of *Amphiuma* red cells as a function of extracellular pH at an extracellular chloride concentration of 30 mM (90 mM PAH). *Ordinate:* membrane potential in mV; *abscissa:* extracellular pH. Points represent separate experiments. 17 °C. Cells were preequilibrated at pH 7.2 (normal Ringer's)

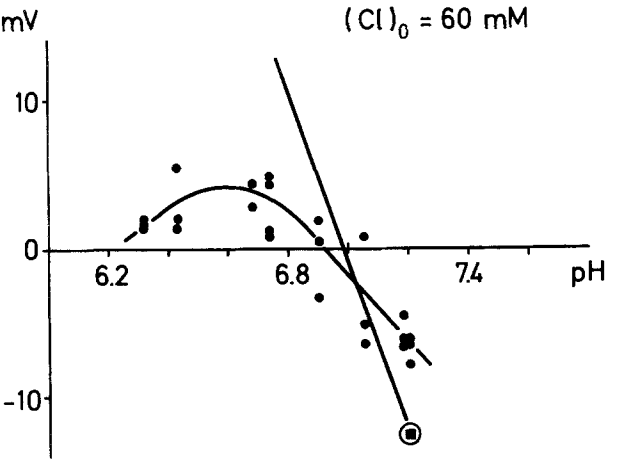

Fig. 6. Membrane potential of *Amphiuma* red cells as a function of extracellular pH at an extracellular chloride concentration of 60 mM (60 mM PAH). *Ordinate:* membrane potential in mV; *abscissa:* extracellular pH. Points represent separate experiments. 17 °C. Cells were preequilibrated at pH 7.2 (normal Ringer's)

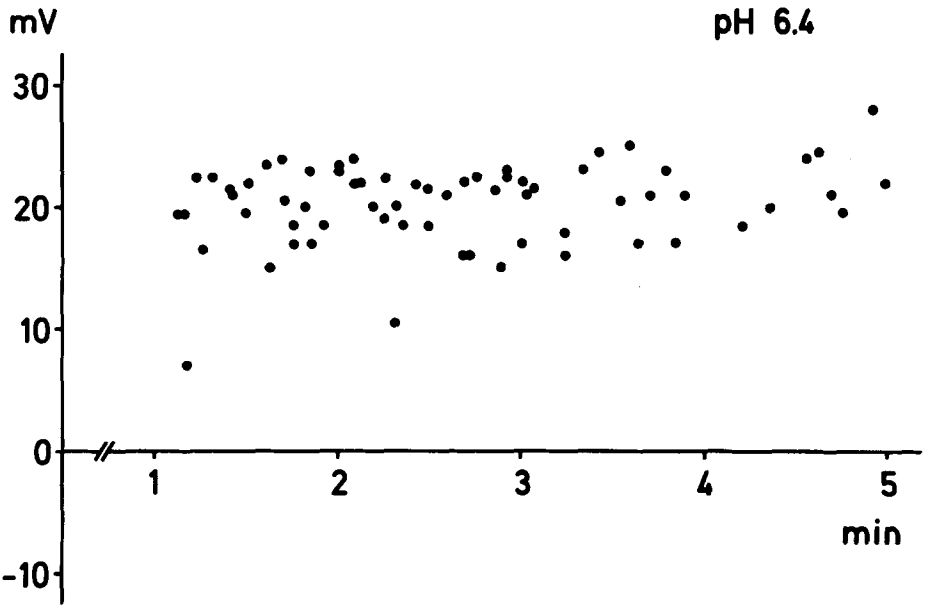

Fig. 7. Individual membrane potential measurements in *Amphiuma* red cells as a function of time after suspension of the cells in Ringer's containing 10 mM chloride (110 mM PAH), pH 6.4. *Ordinate:* membrane potential in mV; *abscissa:* time in min. 17 °C. Cells were preincubated at pH 7.2 (normal Ringer's)

ratio of chloride for hydroxyl ions. Since the cell membrane has a very effective exchange system for monovalent ions, such a change in Cl/OH ratio would be expected to cause an electrically silent counter flux of these ions. The fluxes of chloride and hydroxyl ions as a function of pH in a suspension of cells in a buffered medium (2 mM) with an extracellular chloride concentration of 16 mM are shown in Fig. 9. As expected, lowering the pH results in a reduction of the exchange rate of chloride for hydroxyl ions across the cell membrane. It is important to note that the assumption that an exchange takes place is substantiated by the fact that the fluxes of the two ions are approximately equal in magnitude and opposite in direction. Furthermore, the absolute magnitude of these fluxes indicates that only minor changes in equilibrium potentials for these ions take place during the period necessary for potential measurements (<5 min).

To determine whether intracellular production of HCO_3 from atmospheric CO_2 plays a role in determining the membrane potential, measurements were performed on cells suspended in a 10-mM chloride Ringer's containing 0.9 mM of the carboanhydrase inhibitor, acetazolamide. The

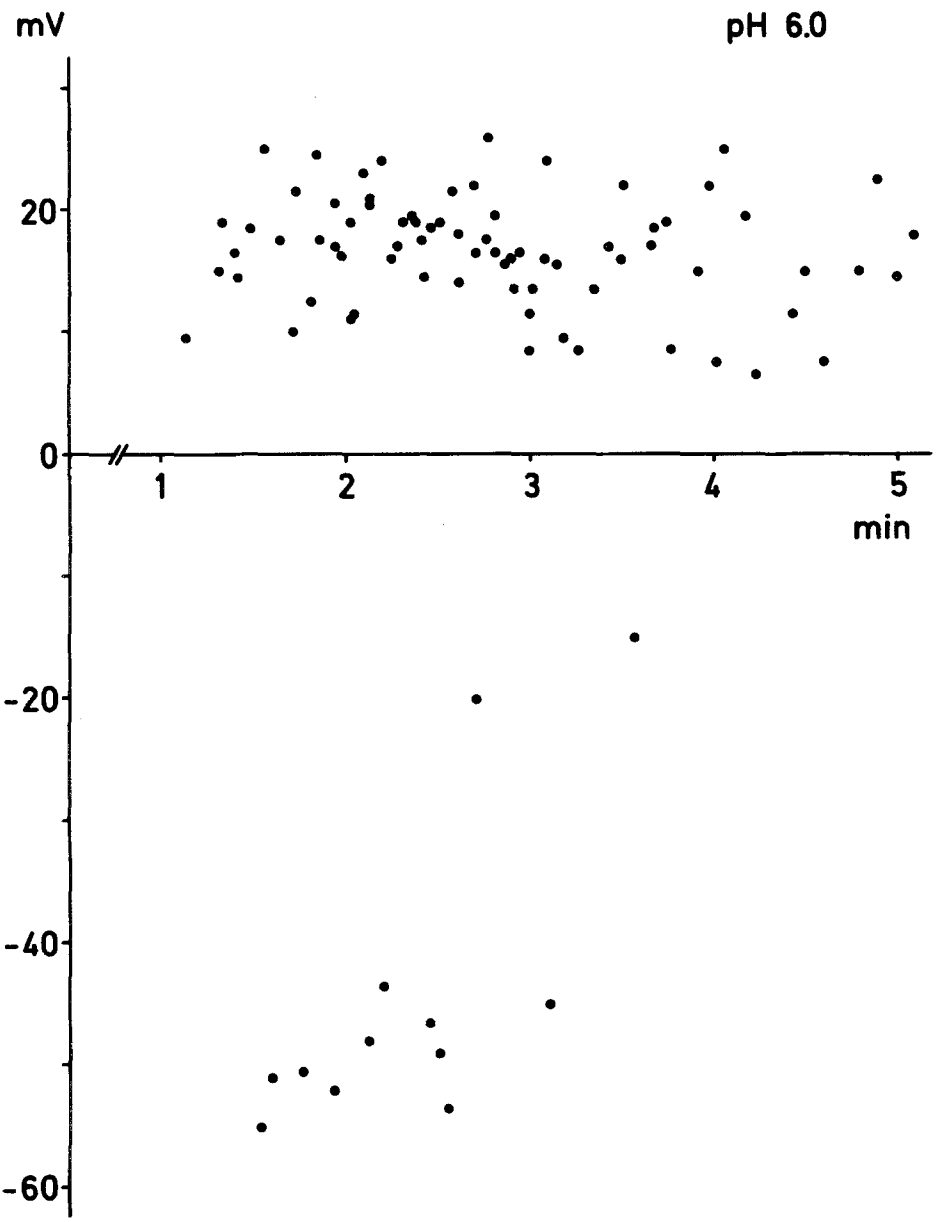

Fig. 8. Individual membrane potential measurements in *Amphiuma* red cells as a function of time after suspension of the cells in Ringer's containing 10 mM chloride (110 mM PAH), pH 6.0. Cells were preincubated at pH 7.2 (normal Ringer's)

results are shown in Fig. 4 and demonstrate that the membrane potentials with or without acetazolamide were identical at all pH values investigated.

The use of PAH as a substitute for chloride is based on the assumption that this molecule is impermeant. This assumption was confirmed in

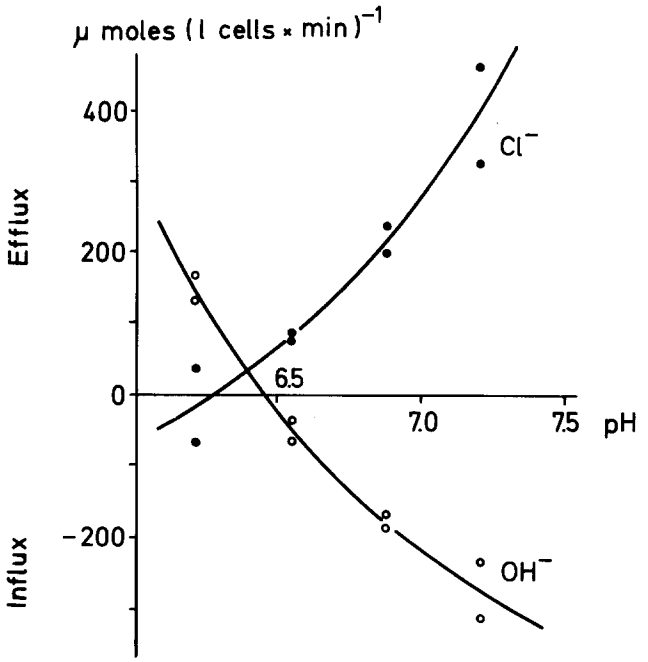

Fig. 9. Net fluxes of chloride and "hydroxyl" ions (hydroxyl, hydrogen, and bicarbonate ions) as a function of extracellular pH at an extracellular chloride concentration of 16 mM (104 mM PAH). *Ordinate:* Flux in μmoles (liter cells × min)$^{-1}$. *Abscissa:* extracellular pH. Cytocrit: 20–25%. 17 °C. Points represent separate experiments. Cells were preincubated at pH 7.2 (normal Ringer's)

experiments using ^{14}C labelled PAH (not shown). Further confirmation was obtained by substituting chloride with MOPS (morpholino-propane sulphonate) which is also believed to be impermeant. Substitution with MOPS gave results which were identical to those obtained with substitution by PAH.

Discussion

Calculation of the Transference Number for Chloride under Partial Electrochemical Equilibrium

Assuming that the ionic currents across the *Amphiuma* red cell membrane are independent of each other and neglecting any contribution from electrogenic pumps, the conductance for the jth ion can be defined as:

$$G_j = \frac{I_j}{(E_j - V_m)}$$

where I_j is the transmembrane current carried by the jth ion, E_j is the equilibrium potential for that ion, and V_m is the membrane potential. Thus the total membrane current can be expressed as:

$$I_T = \sum_j [G_j(E_j - V_m)]. \tag{1}$$

If one further assumes that the total current is carried by Na, K, Cl, OH, and HCO_3 ions, and thus the contribution from other ions, such as Ca, H, and HPO_4, is negligible, then the explicit expression for the total current becomes:

$$\begin{aligned} I_T = {} & G_K(E_K - V_m) + G_{Na}(E_{Na} - V_m) + G_{Cl}(E_{Cl} - V_m) \\ & + G_{OH}(E_{OH} - V_m) + G_{HCO_3}(E_{HCO_3} - V_m). \end{aligned} \tag{2}$$

If the total membrane current is zero, Eq. (2) can be rearranged to:

$$\begin{aligned} V_m &= \frac{E_K G_K + E_{Na} G_{Na} + E_{Cl} G_{Cl} + E_{OH} G_{OH} + E_{HCO_3} G_{HCO_3}}{G_K + G_{Na} + G_{Cl} + G_{OH} + G_{HCO_3}} \\ &= \sum_j E_j G_j / \sum_j G_j. \end{aligned} \tag{3}$$

The expression for membrane potential can also be written in terms of the transference numbers of the individual ions. The fractional conductance or transference number can be written as:

$$T_j = \left[\frac{G_j}{\sum_j G_j}\right]. \tag{4}$$

Substituting in Eq. (3) results in:

$$V_m = E_K T_K + E_{Na} T_{Na} + E_{Cl} T_{Cl} + E_{OH} T_{OH} + E_{HCO_3} T_{HCO_3}. \tag{5}$$

If all but one of the equilibrium potentials are maintained constant and the membrane potential measured as the remaining equilibrium potential is altered, the results should reflect the transport number for the corresponding ion. The results shown in Fig. (2) represent an experiment in which the equilibrium potential for chloride was altered by changing the extracellular Cl concentration, while the intracellular Cl concentration was assumed to remain at the normal physiological value. Chloride in the extracellular phase is substituted for by para-amino-hippuric acid, which is assumed to be impermeable; that is, PAH carries no current across the membrane. The appearance of the curve indicates a rather complex interplay between several factors including the fact that because of the change in membrane

potential, OH and HCO_3 ions are no longer in equilibrium and E_{OH} and E_{HCO_3} vary during the experiment. In order to circumvent this, the results shown in Figs. 3–6 can be evaluated to determine the conditions under which OH and HCO_3 do not contribute to the total membrane current. Each of the curves in Figs. 3–6 contains a single point at which the intracellular hydroxyl ions at a given extracellular Cl concentration are in electrochemical equilibrium with extracellular OH ions. That is:

$$[E_{OH} - V_m] = 0.$$

These points are represented in Fig. 10 by the intersection of the superimposed straight line with the jointly plotted curves from Figs. 3–6. This line is plotted from the calculated equilibrium potential for hydroxyl ions (E_{OH}) as a function of extracellular pH under the assumption that the intracellular hydroxyl ion concentration is equal to that in cells equilibrated in normal Ringer's at pH 7.2 and having a measured membrane potential of -12.5 mV. Furthermore, it can readily be shown that at constant partial

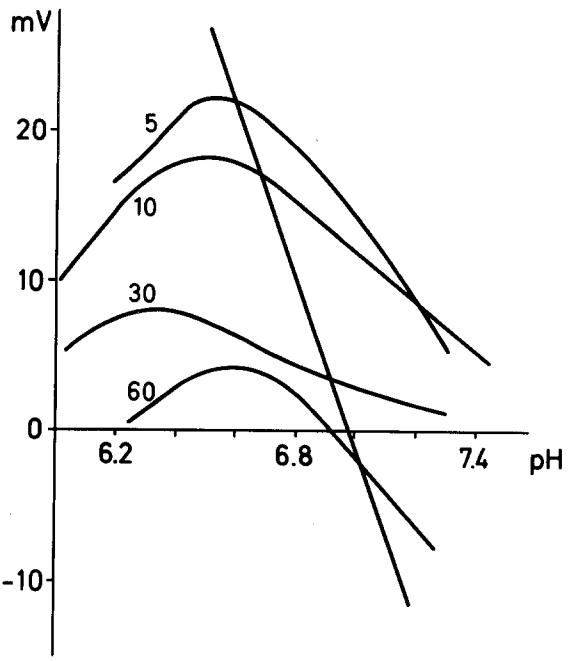

Fig. 10. Membrane potentials of *Amphiuma* red cells as a function of extracellular pH at various extracellular chloride concentrations. The curves are retracings of those shown in Figs. 3–6. The superimposed straight line represents the equilibrium potential for hydroxyl ions as function of extracellular pH assuming constant intracellular pH equal to that of cells equilibrated at pH 7.2. *Ordinate:* potentials in mV; *abscissa:* extracellular pH

pressure of CO_2 the conditions which provide equilibrium for OH ions will also do so for HCO_3 ions. Since all the Ringer's solutions, including suspensions of red cells, were equilibrated with atmospheric CO_2, we can assume that the term $(E_{HCO_3} - V_m)$ is also equal to zero for these intercept points.

It should be noted that, on the basis of the data in Fig. 9, E_{OH} does not stay constant due to the net flux of OH even at the pH values corresponding to the points of intercept in Fig. 10. A mean total influx of 250 μM per liter cells within 5 min can be calculated to give a change in E_{OH} of less than one mV (buffer capacity of *Amphiuma* red cells taken to be similar to that of human red cells; *see* Siggaard-Andersen, 1974). In the following we have neglected this small change in E_{OH}.

Plotting the membrane potential of the intercept points in Fig. 10 vs. the associated $\ln(Cl)_o$ results in a curve approaching a straight line as shown in Fig. 11. With the above simplifications the expression for the total membrane current I_T, given by Eq. (2) reduces to:

$$I_T = G_K(E_K - V_m) + G_{Na}(E_{Na} - V_m) + G_{Cl}(E_{Cl} - V_m). \quad (6)$$

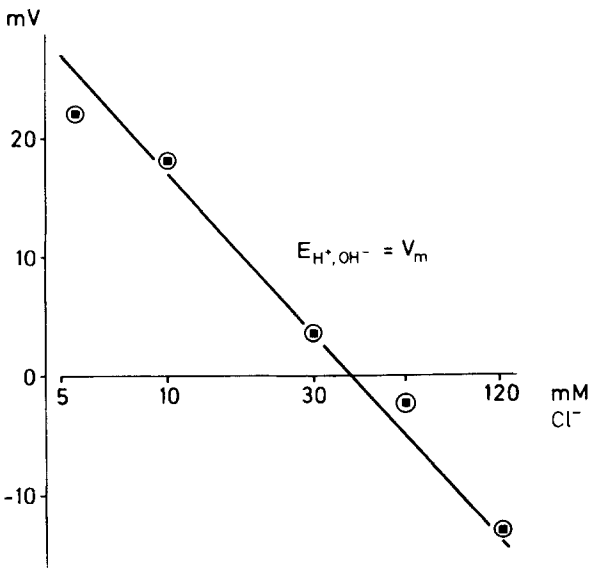

Fig. 11. Membrane potential of *Amphiuma* red cells as a function of extracellular chloride concentration. Values for membrane potentials for various extracellular chloride concentrations taken from the intercepts of the "OH^- equilibrium line" and the curves shown in Fig. 10. For discussion, *see text*

As shown in Fig. 7, the measured membrane potentials remained constant for a period of at least 10 min. Since the potentials shown in Fig. 11 were all measured within 5 min after cells were transferred to the appropriate PAH Ringer's, the total membrane current is taken to be zero. Thus Eq. (6) on rearranging gives:

$$V_m = \frac{E_K G_K + E_{Na} G_{Na} + E_{Cl} G_{Cl}}{G_K + G_{Na} + G_{Cl}}. \tag{7}$$

At partial electrochemical equilibrium the transference number T_j' for the jth ion, in parallel with Eq. (4), is given as:

$$T_j' = \left[\frac{G_j}{\sum G_{j'}}\right] \tag{8}$$

where j' is now explicitly limited to Na, K, and Cl ions. For zero total current Eq. (7) becomes:

$$V_m = E_K T_K' + E_{Na} T_{Na}' + E_{Cl} T_{Cl}'. \tag{9}$$

T_j' shall be referred to as the partial transference number and is defined as the fractional conductance at zero transmembrane current and electrochemical equilibrium with respect to hydroxyl and bicarbonate ions. Although Eqs. (9) and (5) are similar in appearance, there is an important difference between the normally used (fundamental) transference number and the partial transference number defined above. Whereas the total membrane conductance can be calculated from a single conductance G_j and the corresponding fundamental transference number T_j such a calculation is not possible using a single conductance and the corresponding partial transference number.

Since the sum of transference numbers equals one, we have in the case of partial transference numbers:

$$T_K' + T_{Na}' + T_{Cl}' = 1. \tag{10}$$

Eqs. (9) and (10) contain three unknowns, namely the transference numbers. Determination of one of these would then permit calculation of the other two. The straight line shown in Fig. 11 makes such a determination possible. Brown, Walker and Sutton (1970) and Christoffersen (1973) have shown that if a plot of V_m vs. $\ln(C_j)_o$, determined under conditions where all equilibrium potentials other than E_j are held constant, results in a straight

line, then

$$\frac{dV_m}{d\ln[C_j]_o} = \frac{F}{RT} T_j. \tag{11}$$

Eq. (11), which is derived from Eq. (5), is also valid in the case of partial equilibrium (e.g., $E_{OH} = V_m$ as in Eq. (9)). Since it is reasonable to assume that E_{Na} and E_K remain constant during the measurement of V_m, then T'_{Cl} can be determined from the slope of the line shown in Fig. 11 and is 0.48. Using this value in connection with Eqs. (9) and (10) gives the following values for T'_K and T'_{Na}:

$$T'_K = 0.27$$

$$T'_{Na} = 0.25.$$

The calculation involves the implicit assumption that the chloride concentration gradient remains constant during the experimental determination of V_m.

Calculation of the Chloride Conductance

From Eq. (8) it can be seen that:

$$\frac{T'_K}{T'_{Cl}} = \frac{G_K}{G_{Cl}}. \tag{12}$$

Thus a value for G_{Cl} can be obtained from the values for T'_K and T'_{Cl} and an experimental determination of the K conductance. In order to avoid a constant field assumption as well as problems associated with calculations based on K exchange, the K conductance was calculated on the basis of net flux rather than tracer flux determinations. The K flux was measured in ouabain treated cells incubated in 15 mM Cl/105 mM PAH-Ringer's at a pH at which OH and HCO_3 ions were in equilibrium. Since the net flux was moderate and quite constant for a period of at least 20 min, and the membrane potential during this period is known, G_K can be calculated from the single ion current:

$$I_K = [E_K - V_m]\, G_K = F \cdot J_K. \tag{13}$$

The resulting value for G_K is $2.0 \times 10^{-7}\ \Omega^{-1}$ per cm^2. This value is for all practical purposes identical with that obtained for ouabain treated cells in

normal Ringer's (pH 7.2), thus substantiating the assumption that G_K is neither a function of the PAH/Cl ratio nor the extracellular pH. Inserting the value for G_K in Eq. (12) gives a chloride conductance of $3.9 \times 10^{-7}\,\Omega^{-1}$ per cm^2.

Estimate of a Maximum Value for the Sum of the Hydroxyl and Bicarbonate Conductances

The influence of OH and HCO_3 ions on the membrane potential is partially reflected in the difference between the curves shown in Figs. 2 and 10. In Fig. 2 the membrane potential is given as a function of E_{Cl} with E_{Na} and E_K constant. With decreasing values of the extracellular chloride concentrations, the values of $[E_{OH} - V_m]$ and $[E_{HCO_3} - V_m]$ however, continuously increase. This results in a constant increase in the currents from the two ion species. At the same time, degradation of the chloride concentration gradient by way of the anion exchange mechanism becomes increasingly important (*see* Fig. 9).

Eq. (3), which contains the contribution from OH and HCO_3 ions, can be rewritten in the following form:

$$V_m = \frac{E_{Na} G_{Na} + E_K G_K + E_{Cl} G_{Cl} + E_a G_a}{G_{Na} + G_K + G_{Cl} + G_a} \tag{14}$$

where

$$G_a = G_{OH} + G_{HCO_3} \quad \text{and} \quad E_a = E_{HCO_3} = E_{OH}$$

From Eq. (8) we get the explicit expression:

$$T'_{Cl} = \frac{G_{Cl}}{G_K + G_{Na} + G_{Cl}} = 0.48$$

which when solved for G_{Na} gives a value of $2.2 \times 10^{-7}\,\Omega^{-1}$ per cm^2. This value together with the values for G_K, G_{Cl}, E_K, E_{Na}, and the measured value for V_m at pH 7.2 and an extracellular Cl concentration of, for example, 30 mM can now be used to solve Eq. (14) for G_a. The result is $0.6 \times 10^{-7}\,\Omega^{-1}$ per cm^2.

Since an important assumption is that the chloride ion concentration gradient has not been degraded by way of the anion exchange mechanism, this is a maximum value.

The Effect of pH on the Conductances

The presence of maxima in the region of pH 6.4 in the average curves shown in Figs. 3 to 6 presents a special problem. On the basis of the absolute values of the various equilibrium potentials, four explanations can be considered. First, G_K increases as the external pH decreases. Secondly, G_{Na} decreases with decreasing extracellular pH. Thirdly, G_{Cl} decreases in the same range of pH but possibly with a stronger dependence on pH (since the weight of E_{Cl} is less than that of E_K within the range of membrane potentials in question). Finally, a combination of these conductance changes can take place simultaneously. With regard to the first possibility, the fact that a small percentage of cells suspended at low pH in normal as well as PAH Ringer's were strongly hyperpolarized may be significant. However, as mentioned above, K net effluxes at low extracellular Cl concentrations and low pH were within normal range. Thus, although a possible G_K dependence on pH cannot be ruled out, its role would appear to be insignificant.

With regard to a possible decrease in G_{Na} with decreasing pH, although the determinations of net Na fluxes are not as accurate as the results from corresponding determinations of K fluxes, there is no indication of a substantial decrease in G_{Na} with decreasing extracellular pH.

Considering the third possibility, we previously proposed (Vestergaard-Bogind & Lassen, 1974) that the conductance of chloride might take place by way of a slip in the otherwise obligatory anion exchange mechanism. If this is so, then the significant decline in self-exchange of chloride with decreasing pH in the range 7.8 to 5.5 as reported by Gunn *et al.* (1973) may be accompanied by a comparable decrease in chloride conductance. Since the pH dependence of the exchange mechanism is more pronounced around 6.6 than 7.1, the decrease in G_{Cl} should be most apparent at the lower end of the pH range. Therefore the value of the membrane potential at 5 mM extracellular chloride and pH 6.6 may represent an actual deflection of the line shown in Fig. 11. If a decreasing chloride conductance with reduction in extracellular pH is the primary explanation for the maxima seen in Figs. 3–6, then this dependence suggests that besides the ground permeability of the lipid bilayer, chloride conductance takes place one way or another through the anion exchange unit. This idea is supported by the finding that phloretin, which blocks chloride self-exchange in human red cells by about 99.5% (Wieth *et al.*, 1973), blocks chloride conductance almost completely in *Amphiuma* red cells (Vestergaard-Bogind & Lassen, 1974). In addition, Fortes and Hoffman (1973) reported that both persantin and ANS

inhibit chloride self-exchange and chloride conductance in human red cells (estimated from experiments with valinomycin) to the same degree. In a recent study of the inhibition of anion transport in human red cells by stilbene derivatives, Knauf, Fuhrman, Rothstein and Rothstein (1977) conclude that a common element may be involved in both net and exchange anion transport.

Influence of the Anion Exchange Mechanism

The net in- and effluxes of Cl and OH ions shown in Fig. 9 represent one of the problems involved in this type of experiments. Under conditions represented by the intercepts between the membrane potential curves and the equilibrium potential line for hydroxyl ions (Fig. 10), no current should be carried by hydroxyl and bicarbonate ions. Nevertheless, as expected and verified in Fig. 9, a significant exchange of intracellular chloride for extracellular hydroxyl and bicarbonate ions takes place at the intercept values taken from Fig. 10. For practical reasons the experiments described in this figure were performed at an extracellular chloride concentration of 16 mM and a pH value of 6.8, corresponding to an intercept value of about +12 mV in membrane potential. Since the measurement of fluxes was based on the technique of extracellular recording of changes of pH in a phase of known buffer capacity, the influx of hydroxyl ions actually represents the sum of proton effluxes and hydroxyl and bicarbonate ion influxes. With the accuracy obtainable in such experiments, it can be seen from these curves that the difference between in- and effluxes, i.e., the current carrying net flux, is always a chloride efflux as should be expected.

At the intercept value (with respect to pH) there is a total efflux of about 100 μmoles of chloride per liter cells per minute. This corresponds to a total efflux of about 2% of the intracellular chloride within a period of 10 min. Thus, no appreciable degradation of the chloride concentration gradient takes place by way of the exchange mechanism during this period of time. This is in full agreement with the fact that the measured membrane potentials stayed constant for this period (*see* Fig. 7).

During potential measurements, atmospheric CO_2 which dissolves in the extracellular phase can permeate the membrane and, catalyzed by carboanhydrase, cause a constant production of H_2CO_3 which, in turn, dissociates into bicarbonate and protons. In order to evaluate the effect of this process, membrane potentials were measured in cells suspended in a 10 mM chloride, 110 mM PAH Ringer's containing 0.9 mM of the carboanhydrase inhibitor acetazolamide. The membrane potentials measured in the

presence of acetazolamide did not deviate significantly from those obtained in acetazolamide-free Ringer's (*see* Fig. 4). This indicates that under the experimental conditions employed, no current passes the membrane as net efflux of bicarbonate ions originating in atmospheric CO_2.

Comparisons with other Estimates of Chloride Conductance in Red Cell Membranes

Hunter (1971, 1977) and Tosteson *et al.* (1973), based on experiments in which valinomycin was used as a K-ionophore, estimated the chloride conductance for human and sheep red cells to be two orders of magnitude higher than the value for *Amphiuma* red cells reported here. It is important to note that the results reported by Hunter and Tosteson *et al.* were primarily obtained under experimental conditions involving high net chloride effluxes. The value of chloride conductance reported here was obtained on the basis of experiments characterized by very small net fluxes of chloride across the membrane.

Furthermore, Hunter (1971, 1977) based his estimate of G_{Cl} on the assumption that a constant field regime can be used in the treatment of the experimentally observed large net effluxes of KCl over the entire range of extracellular potassium concentration. In contrast, addition of valinomycin in optimal concentration (4×10^{-6} M) induces only small net effluxes of KCl in *Amphiuma* red cells. As judged from the degree of hyperpolarization, the K-ionophoric effect of valinomycin is maximum at this concentration, implying that the effect of valinomycin on *Amphiuma* red cells is far less drastic than on human and sheep red cells. In addition, we have shown that the variation in *Amphiuma* red cell membrane potential as a function of extracellular K concentration (Lassen *et al.*, 1975) indicates that the constant field regime may not be valid for valinomycin treated *Amphiuma* red cell membranes. Nevertheless, a rough estimate of the chloride conductance in the presence of the ionophore results in a value which agrees well with that obtained in the PAH substitution experiments. The difference in the effect of valinomycin on red cells from various species does not necessarily imply that there is a difference in the G_{Cl} of the unperturbed red cell membrane.

In a previous paper we (Lassen *et al.*, 1976) described a transient hyperpolarization in *Amphiuma* red cells induced by a sudden increase in the extracellular concentration of Ca. We also found that increased extracellular Ca results in a transient net efflux of KCl of considerable

magnitude, equivalent to a much higher value of G_{Cl} than that found in the experiments with PAH or valinomycin. Whether or not this difference is due to the fact that Ca induces a parallel increase in the conductances of both K and Cl remains to be elucidated.

Based on spectrophotofluorometric data using the dye diS-C3-(5), Callahan and Hoffman (1976) found that the membrane potential of human red cells changed with extracellular pH in a way "suggesting that the red cell membrane behaves as a hydroxyl electrode". Using the same dye, Hladky and Rink (1976) found that the hydroxyl ion/proton conductances of human red cells seemed to be extremely low. On the other hand, Tosteson *et al.* (1973), in their valinomycin experiments, found an appreciable G_{OH} in sheep cells. Our estimate of the sum of G_{OH} and G_{HCO_3} seems to be compatible with the estimate of Hladky and Rink (1976), since our sum conductance is based on concentrations of $10^{-7.5}$ M hydroxyl ions and about 1.5×10^{-4} M bicarbonate ions.

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