A Single-Cell Technique for the Measurement of Membrane Potential, Membrane Conductance, and the Efflux of Rapidly Penetrating Solutes in *Amphiuma* Erythrocytes

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ABSTRACT We describe a single-cell technique for measuring membrane potential, membrane resistance, and the efflux of rapidly penetrating solutes such as Cl and H_2O. Erythrocytes from *Amphiuma means* were aspirated into a Sylgard (Dow Corning Corp.)-coated capillary. The aspirated cell separated a solution within the capillary from a solution in the bath. Each of these two solutions was contiguous with ~5% of the total membrane surface. Microelectrodes placed concentrically within the capillary permit the measurement of intracellular voltage, specific membrane resistance, and the electrical seal between the two solutions. The intracellular voltage averaged −17.7 mV (pH 7.6) and changed as either intra- or extracellular chloride was varied. The average specific membrane resistance measured by passing current across the exposed membrane surface was 110 ohm-cm^2. ^{36}Cl and tritiated H_2O fluxes ($0.84 \pm 0.05 \times 10^{-6}$ M·cm^{-2}·min^{-1} and $6.4 \pm 1.5 \times 10^{-3}$ M·cm^{-2}· min^{-1}, respectively) were determined by noting the rate at which isotope leaves the cell and crosses the membrane exposed to the bath. Our measured values for the flux of ^{36}Cl and tritiated H_2O approximate reported values for free-floating cells. ^{36}Cl efflux, in addition, is inhibited by 4-acetamido-4′-isothiocyano-stilbene 2,2′-disulfonic acid (SITS) and furosemide, known inhibitors of the anion exchange mechanism responsible for the rapid anion fluxes of red blood cells. One can also demonstrate directly that >89% of ^{36}Cl efflux is "electrically silent" by analyzing the flux in the presence of an imposed transcellular voltage.

INTRODUCTION

Although direct measurement of the intracellular voltage and membrane resistance have been common in many cell types, measuring these parameters directly in erythrocytes has proved difficult.

The initial attempts to measure the intracellular voltage in human red cells with microelectrodes (Lassen and Sten-Knudsen, 1968; Jay and Burton, 1969)

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gave values that were considered to be in good agreement with those calculated from the equilibrium potential for chloride.[1] Lassen (1977) has recently argued that these studies are subject to an inherent error related to a possible leak around the microelectrode tip. It is because this error is smaller when a larger cell is used that the giant erythrocytes of *Amphiuma means* have become the reference cell for electrical measurements.

The first measurements of intracellular voltage in *Amphiuma* erythrocytes by Chang (1965) gave a value of -5.75 ± 0.66 mV. These measurements disagree with those made subsequently and may be subject to the same uncertainties as the human red cell measurements. Hoffman and Lassen (1971), Lassen (1972), and Ronne and Lassen (1977) were the first to systematically vary the equilibrium distribution of chloride and simultaneously measure membrane potential in *Amphiuma* red cells. These workers, using a piezoelectric device to impale the cells, were able to analyze the initial 2-μs voltage deflection before it decayed 1–2 ms later. Their measured values were proportional to the calculated values for the chloride equilibrium potential and provided the first evidence that their microelectrode measurements were indeed recording biologically relevant intracellular potentials. Smith and Levinson (1975) have obtained more stable recordings by fixing *Amphiuma* red cells on a La^{+3}-coated glass surface before impaling them. Hoffman and Laris (1974) measured the potential indirectly using fluorescent dyes and also showed that the membrane potential changes in the expected direction in response to variations in the chloride concentration ratio.

Attempts to measure the membrane resistance in red cells have been both varied and imaginative but have produced diverse results. Initital studies used mammalian red cells. Fricke (1925) and Fricke and Morse (1925) passed an alternating current at frequencies as high as 4.5 MHz through packed suspensions of erythrocytes. The response at different frequencies permitted an estimate of membrane capacitance, conductance, and thickness. With simplifying assumptions, specific membrane resistance was estimated to be >10 ohm-cm^2 (Fricke and Curtis, 1935; Schwan, 1957). Johnson and Woodbury (1964) passed current through a human red cell held in the end of a capillary, a procedure similar to that used in this study. It is probable, however, that the values of specific membrane resistance (6–20 ohm-cm^2) found by these investigators are too low. In their study, cells were suspended

[1] Numerous studies suggest that Cl is distributed passively in the human erythrocyte. Changes in pH produce predictable variations in the ratio between the intra- and extracellular chloride concentration, as would be expected if the distribution of chloride were passive (Harris & Maizels, 1952). That this ratio agrees closely with the inverse of the concentration ratio for hydrogen ions (Warburg, 1922; Van Slyke et al., 1923; Harris and Maizels, 1952; Funder and Wieth, 1966) has also been taken to indicate that chloride distribution is only responsive to passive forces. In the human red cell, chloride permeability apparently is large relative to that of other permeant ions. Hunter's (Hunter, 1971; see also Hoffman and Laris [1974] and Knauf et al. [1977]) estimate of anion conductance from cation flux measurements in valinomycin-treated cells indicates that P_{Cl} is 100 times larger than the upper limit of either P_{Na} or P_K. These findings, taken together, have led to the conclusion that the steady-state membrane potential in the human erythrocyte is given by the Nernst equation for the chloride equilibrium potential.

in isotonic sucrose with the expectation that this procedure would prevent current leakage around the cell by producing a sucrose gap between the capillary wall and cell surface. Unfortunately, the large increase in KCl permeability of human red cells suspended in low ionic strength media (LaCelle and Rothstein, 1966) probably introduced intracellular electrolytes to the sucrose pathway and made it conductive. Part of the current, therefore, probably passed around the cell, and not through it as intended.

Using *Amphiuma* red cells, Lassen and co-workers have estimated total membrane resistance with several techniques. In their study of membrane potential Hoffman and Lassen (1971) and Lassen (1972) passed diphasic current pulses through the microelectrode after the method of Lettvin et al. (1958) and reported a specific membrane resistance of 1,000–2,000 ohm-cm^2. Rathlev and Lassen (1977) have reported the specific membrane resistance to be as high as 2.8×10^5 ohm-cm^2. This value was obtained by passing a current through a cell held at its ends by separate pipettes at a time when the cell surface between the two pipettes was bathed in an oil droplet. It was hoped that submersing the cell in the oil droplet would produce an "oil gap" much like that used in the study of excitable tissues.

There is very little direct experimental evidence that relates these bioelectric parameters and the transmembrane movement of ions such as chloride in the red cell. In this study we present a new experimental approach whereby membrane potential, membrane resistance, and the radioisotopic flux of ^{36}Cl and tritiated H_2O can be measured. Because the investigator can control the electrical and chemical driving forces across the cell, the methodology has the potential of permitting a more direct assessment of the mechanisms of ion movement in the red cell than was previously possible. These findings have been reported in abstract form (Stoner and Kregenow, 1976).

METHODS

We obtained Congo eels (*Amphiuma means*) from Mogul-Ed, Oshkosh, Wis. Animals were maintained at room temperature (20–25°C) in aquaria containing either pond water or dechlorinated tap water and were allowed to stabilize in this environment for at least one week before serving as blood donors.

Erthrocytes were collected by one of two methods, depending upon the quantity of blood needed. Small amounts (50 μl) could be obtained by surgically removing the distal one centimeter of the animal's tail and permitting a drop of blood to mix with 10 ml of saline. When larger quantities of blood (10–20 ml) were needed for experiments that involved the analysis of cellular ionic and water content, they were collected by cardiac puncture. In this procedure, the animals were doubly pithed before opening the thorax and exposing the heart. Heparin served as the anticoagulant.

The standard solution was bicarbonate-free but otherwise mimicked the osmolality and the major ionic content of *Amphiuma* plasma (Table I). Table I also presents the composition of the other stock solution used in the study in which *p*-aminohippurate (PAH) replaced Cl as the major anion. Notice that to make this solution isosmotic with the standard solution it was necessary to have the sum of the Na^+ and PAH content exceed the Na^+ plus Cl^- content of the standard solution. Various bath

chloride concentrations were achieved by mixing appropriate amounts of these two stock solutions. Solutions were titrated to the desired pH with either HCl or NaOH.

Macroanalysis

After the erythrocytes were separated by gentle centrifugation at 2,000 *g* and 20–25°C, the plasma and buffy coat were removed by suction. Cells to be modified by changing only the medium pH were washed in two stages—first with standard solution (pH, 7.5) and then again with standard solution at the appropriate experimental pH. Cells modified by changing both the pH and Cl content (PAH replacement) of the media were washed in three stages—first with the standard solution, then with an acidic standard solution at pH 6.5, and finally with media at pH 6.5 in which 15, 30, or 50 meq of PAH replaced Cl. Each stage of washing lasted 15–20 min and consisted of two rinses, each in 100 vol of the appropriate solution. Cells were separated between rinses by gentle centrifugation at 2,000 *g* (room temperature). Washing with either acidic or PAH-containing solutions produced the expected change in cellular Cl content (the result of an anion shift), while the Na and K content remained unchanged. We also found the cells to have nearly the same Na, K, Cl, and H_2O content 30 min after the final wash as at the end of the final wash.

TABLE I

Constituent	Chloride saline concentration	PAH saline concentration
	mM	*mM*
Na	110	121
K	4.0	4.0
Ca	1.8	1.8
Mg	0.6	0.6
Cl	108	1.8
SO_4	0.6	0.6
PO_4	2.0	2.0
PAH	—	121
Glucose	5.5	5.5
Albumin	1 g/l	1 g/l

Therefore, this procedure provided adequate time for the cells to re-equilibrate. The one possible exception occurred after introducing cells to a medium with 50 mM PAH and pH of 6.5. In this instance, cells contained 3 meq less Cl 30 min after the end of the last wash. This difference, however, was not statistically significant.

Duplicate aliquots of cells (hematocrit, 0.5%) that were to be analyzed for electrolyte, water content, or radioactivity were centrifuged in specially fabricated centrifuge tubes at 30,000 *g* and 20–25°C for 1 min. These centrifuge tubes had an opening at the base that could be occluded with a plastic plug and were tapered to form a cylindrical core (3 mm in diameter). They were capable of holding 150 μl of packed cells. After removing the supernate and top layer of packed cells, the remaining cell population (>96% of the total) was expressed through the opening at the base of the tube. The cells from one of the duplicate tubes were analyzed either for specific gravity or percent cell water (wt/wt) (Riddick et al., 1971). Those from the other tube were resuspended and hemolyzed. Aliquots were then removed for determination of Na, K, Cl, and Hgb content or the radioactivity associated with ^{14}C inulin, ^{36}Cl, or [^{14}C]PAH. Identical determinations, except for the measurement of Hgb content, were also performed on aliquots of the supernate.

The procedures for determining Na, K, Cl, and Hgb content have been described (Riddick et al., 1971). Samples containing radioactivity were freed of protein by perchloric acid (PCA), placed in scintillation vials containing Aquasol (New England Nuclear, Boston, Mass.), and radioassayed by liquid scintillation photometry.

From each animal cell population we obtained a well-mixed volumetric sample of packed cells that had incubated in the standard isotonic solution before centrifugation. This sample served as a reference. The Hgb value from this sample permits one to express the Na, K, and Cl content for all samples from the same cell population in terms of the identical number of cells (millimoles per liter$_{ONC}$) (Riddick et al., 1971). This is possible because a direct relationship exists between Hgb content and number of cells.

Despite changes in cell size, one can also calculate the concentration of Cl (mmoles per liter of cell H_2O) for any sample by using the following equation:

$$\text{mmol/liter cell } H_2O = \frac{(\text{millimol/liter}_{ONC})\left(\dfrac{W_{Du}/W_{Wu} \times \dfrac{P_u}{P_r}}{W_{Dr}/W_{Wr}}\right)}{\text{fractional cell } H_2O \text{ on a volume basis}}. \quad (1)$$

In this equation the values for chloride content in millimoles per liter$_{ONC}$, wet weight, W_{Wu}, dry weight, W_{Du}, specific gravity, P_u, and the fractional cell H_2O on a volume basis are those of the unknown cell sample and the values for net weight, W_{Wr}, dry weight, W_{Dr}, and specific gravity, P_r, are those of a reference isotonic cell sample. The value for the fraction of cell volume that can be accounted for by measurements of cell water content (fractional cell H_2O on a volume basis) was obtained from the experimentally determined percent cell water wt/wt and specific gravity as described by Riddick et al. (1971).

The chloride equilibrium was calculated from the Nernst equation:

$$V_m = 58 \log \frac{[Cl]_i}{[Cl]_o}, \quad (2)$$

in which $[Cl]_i$ represents the intracellular Cl concentration (mmoles per liter H_2O) and $[Cl]_o$ the extracellular chloride concentration. In several experiments, the Cl ratio $[Cl]_i/[Cl]_o$ was also determined with ^{36}Cl and found not to be significantly different from the chemically determined ratio.

We measured PAH uptake by including [^{14}C]PAH (ICN, International Chemical and Nuclear Pharmaceuticals, Irvine, Calif., 11.5 mCi/mmol) in the PAH wash solutions and taking samples initially, at the end of the wash, and 20 min later. Knowing the specific activity of PAH in the supernate and the number of counts per minute of ^{14}C in an aliquot of cells, one can calculate the rate of uptake (Kregenow, 1971).[2]

Techniques for Studying Single Red Blood Cells

The equipment and techniques used for studying single red blood cells were modified from those developed for perfusion of isolated renal tubules (Burg et al., 1966; Burg, 1972). Only pertinent modifications are presented here.

[2] We could not distinguish the radioactivity associated with [^{14}C]PAH uptake into cells during the experimental period (20 min) from the radioactivity associated with trapped plasma. Nevertheless, uptake measurements for longer periods did demonstrate PAH to be slightly permeable rather than impermeable and to enter cells with an influx value of ~1 mmol/liter of cells/h.

Our electrical measurements required that we hold a single cell snugly in a glass holding-capillary so that the cell itself separated two fluid compartments. The procedure and electrode arrangements which proved most successful are diagrammed in Fig. 1. The tip of the outer holding-capillary was shaped to provide a parallel section of glass to grip the cell. The inner surface of this parallel section was coated

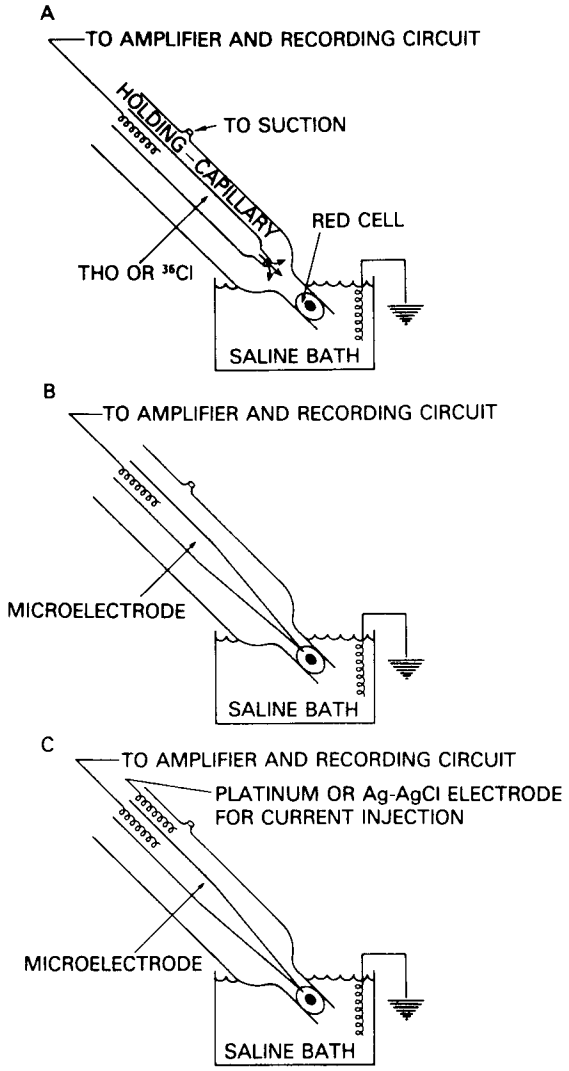

FIGURE 1. Schematic presentation of the pipette and electrode configurations used in this study. See text for a description of *A*, *B*, and *C*.

with Sylgard 184 (Dow Corning Corp., Ann Arbor, Mich.) to prevent contact between the glass and cell surface. The Sylgard, after being mixed with a catalyst, was applied as a liquid and polymerized by heat. It was necessary to aspirate air through the capillary during the polymerization process to apply the Sylgard uniformly onto the inner glass surface. Once the Sylgard had polymerized, cells from the bath could then

be drawn into the holding-capillary with gentle suction and held securely in place. Under these conditions, the cell physically separated the solution within the holding-capillary from that in the bath.

A second inner concentric glass capillary could be placed inside the cell holding-capillary. Two types of inner capillaries were used. *A* of Fig. 1 shows the inner capillary as a pipette with a tip diameter of 10 μm or less, and positioned so that the tip is adjacent to the inner surface of the aspirated cell. This inner capillary served two functions. First, when filled with isotonic saline and connected to an agar bridge, it acted as an electrode, which, along with the ground electrode in the bath, permitted one to record the voltage across the entire cell. Second, this inner pipette functioned as a superfusion capillary, allowing one to introduce and maintain solutions of different ionic or radioisotope composition at the inner membrane surface.

B of Fig. 1 shows the inner capillary as a Ling-Gerard microelectrode centered so that it has impaled a cell as the cell is aspirated into the holding-capillary. Impalement permits one to inject current into the cell and to measure the voltage difference across the outer membrane between cytosol and bath. Ling-Gerard microelectrodes were pulled on a commercial puller and subsequently filled (by boiling) with 3 M KCl. Electrodes were selected for input resistance of 20 to 100 MΩ and discarded if tip potentials exceeded 10 mv.

C of Fig. 1 shows a third arrangement in which a platinum or Ag-AgCl electrode has been placed inside the holding-capillary. This electrode is exterior to and spacially separate from a Ling-Gerard microelectrode, which is shown as having penetrated the cell. We used this additional electrode to pass current extracellularly at a time when the placement of the Ling-Gerard microelectrode permitted one to record the voltage drop across the outer membrane.

Intracellular or transcellular voltages were recorded between calomel cells connected to the bath and microelectrode by agar salt bridges. Calomel cells were connected either to a Transidyne General MP 106 (Transidyne General Corp., Ann Arbor, Mich.) or Burr-Brown model 3621K (Burr-Brown Research Corp., Tucson, Ariz.) preamplifier. The techniques for passing monophasic direct current pulses have been published elsewhere (Helman et al., 1971). We obtained permanent recordings of voltages on a Hewlett-Packard model 7402A oscillographic recorder (Hewlett-Packard Co., Palo Alto, Calif.)

Measurement of Membrane Resistance

The various electrode arrangements described in Fig. 1 permit one to pass current and measure input resistances in several ways. These input resistances, though obtained differently, can all be used to estimate the specific resistance of the cell membrane (assumed to be the primary resistive element in the cell). Using the arrangement described in Fig. 1 *B*, one can pass a small current into the cell after having recorded a stable intracellular voltage and measure the voltage deflection between cytosol and bath. The input resistance associated with this voltage deflection yields the specific resistance across the outer exposed membrane according to the following equation:

$$\text{specific resistance (ohm-cm}^2) = A \times R, \tag{3}$$

where R is the input resistance and A the membrane surface area (assumed to be a hemisphere with a diameter equivalent to the diameter of the holding-capillary).

Alternatively, by using the arrangement described in Fig. 1 *A*, one can pass small currents across the entire cell and record the resultant voltage deflections between the bath and the solution in the holding-capillary. This gives the input resistance of the

entire system, R_t. This value is equivalent to the sum of the input resistances across both exposed membranes at either end of the cell, if, as we estimate (see Results), current leakage around the cell through the Sylgard seal is minimal. Provided both exposed surfaces have the same specific resistance, one can calculate the specific resistance of the exposed membrane by using the following equation:

$$\text{specific resistance (ohm-cm}^2) = \frac{A_1 \times A_2}{A_1 + A_2} \cdot R_t, \tag{4}$$

where A_1 and A_2 are the surface areas of the outer and inner membranes, respectively.

Finally, using the arrangement described in Fig. 1 *C*, it is possible to pass current either extracellularly through the external platinum or Ag-AgCl electrode or intracellularly through the Ling-Gerard microelectrode and record, in both instances, the voltage drop across the outer membrane between the Ling-Gerard microelectrode and gound electrode. This gives the input resistance across the outer membrane when two different current sources are used and permits an assessment of how much current leaks around the cell with any given set of pipettes.

Measurement of ^{36}Cl and Tritiated H_2O Fluxes Across the Cell

We measured the ^{36}Cl or tritiated H_2O (THO) flux across the outer membrane of a cell that had been positioned in a capillary and allowed to equilibrate with a radioactive solution bathing its inner membrane surface.[3, 4] The efflux was determined at room temperature by noting the rate at which isotope entered the bath. The radioactive solution used to load the cell was introduced at the inside membrane surface through a superfusion pipette like that described above and in Fig. 1 *A*. Fluid in this capillary could be changed at will (Burg, 1972). Once we began superfusing with a radioactive solution,the superfusion rate remained rapid and constant until the end of the flux measurement. It was mandatory that a cell be secured before radioactive solutions were introduced to the superfusion capillary, since failure to do so resulted in substantial radioactive contamination of the bath chamber. Contamination of this nature was troublesome because the observed counts during a flux measurement were small (two to four times background). We allowed the superfused ^{36}Cl solution or tritiated water 5 min to equilibrate with the cell before beginning the flux measurements. After the equilibration period, the bath was removed and the chamber thoroughly rinsed with nonradioactive saline. We determined the flux by noting the rate at which isotope entered the bath during the next 20 min interval. At the end of 20 min the bath contents (0.5 ml) and a 2.5-ml wash of nonradioactive saline were removed for counting. This combined sample was placed in scintillation vials containing Aquasol or Aquascint (ICN, International Chemical and Nuclear Pharmaceuticals) and, along with blanks and standards, was radioassayed by liquid scintillation photometry.

^{36}Cl and THO fluxes were calculated with the following equation:

$$J\ (\text{mol/cm}^2 \cdot \text{min}^{-1}) = \frac{C_2}{S^* \cdot A_1}, \tag{5}$$

where S^* is the specific activity of ^{36}Cl or THO in the cell, A_1, the membrane surface

[3] $H^{36}Cl$ was purchased from ICN (sp. act., 10.4 Ci/g, titrated to pH 7.4 with NaOH). The resulting $Na^{36}Cl$ replaced some of the NaCl in formulating an isotonic solution. The final solution was kept frozen until used.

[4] Tritiated H_2O (THO) was purchased from ICN (sp. act., 1 Ci/g) and added to the standard solutions so that the final sp. act. was 1 mCi/g.

area exposed to the bath, and C_2, the counts entering the bath per minute. We calculated S^* by assuming that the cell was a single well-mixed compartment whose specific activity was intermediate between that of the superfusion solution at the inner membrane and the tracer-free bath solution at the outer membrane. The following equation therefore applies:

$$S^* = \frac{S_1}{2} \cdot \frac{A_2}{A_1}, \tag{6}$$

in which S_1 is the specific activity of the superfusion solution and A_1 and A_2 are the surface areas of outer and inner membranes, respectively. This calculation also assumes that both exposed membranes are homogeneous and that they provide the only rate-limiting barrier for exchange of Cl or tritiated water between the intra- and extracellular compartment.

Unless stated otherwise, the results represent experiments carried out at least three times with blood from different animals. Results are expressed as ± SEM (n = number of cells).

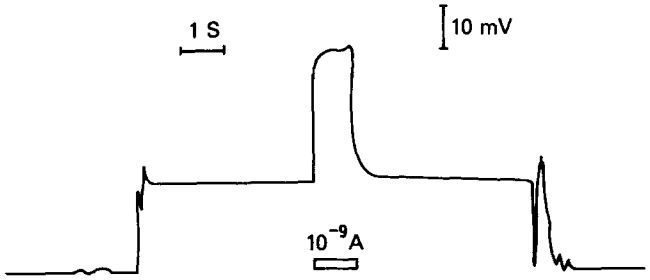

FIGURE 2. Representative tracing of an intracellular voltage measurement in an *Amphiuma* red cell. The bathing medium was the standard solution (pH, 8.0). The bar denotes the voltage deflection when an additional 10^{-9} A was injected into the cell via the microelectrode. Noise seen at the beginning and end of impalement is associated with membrane movement before penetration and after removal of cell. Although not a constant feature, noise of this nature was present in less than half of the tracings.

RESULTS

Membrane Potential

To determine the intracellular voltage in *Amphiuma* red cells, we impaled the cell with a Ling-Gerard microelectrode and recorded the voltage deflection upon penetration of the membrane (see Methods, Fig. 1 *B*). Fig. 2 shows a representative electrical recording. Note that the voltage changes abruptly upon penetration and remains essentially constant for seconds. In this experiment, we injected 10^{-9} A for a brief period after the microelectrode had penetrated the cell. The voltage deflection associated with this current injection is denoted by the bar. The cell was finally removed by applying positive pressure; this resulted in the voltage returning suddenly to its original baseline value.

Measurements were obtained on cells in which the chloride concentration ratio, $[Cl]_i/[Cl]_o$, was varied by one of two methods. In the first method, we kept the medium chloride concentration constant and changed the intracellular chloride concentration by varying the pH of the medium. In the second, cellular concentrations were constant and extracellular concentrations were varied by replacing medium chloride with the relatively impermeant anion

TABLE II

EFFECT OF THE CHLORIDE RATIO ON THE INTRACELLULAR VOLTAGE OF *AMPHIUMA* RED CELLS

Condition			
pH	Bath [Cl]	Intracellular voltage	Number of cells
	mM	*mV*	
6.5	110	−8.8±0.6	61
7.6	110	−17.7±0.8	55
8.0	110	−25.1±0.9	19
6.5	110	−9.7±0.7	51
6.5	78	−4.9±0.8	60
6.5	58	−2.8±0.7	49

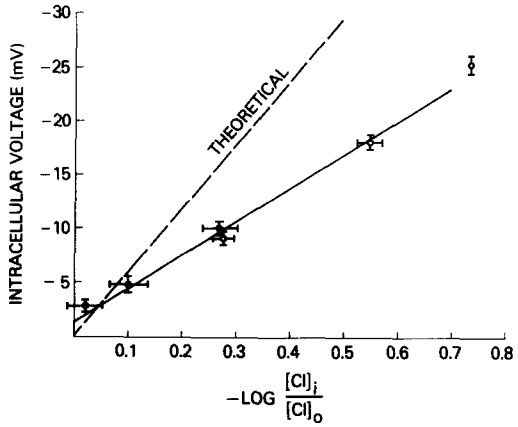

FIGURE 3. Plot of the intracellular voltage against the negative logarithm of the chloride concentration ratio. Chloride ratios were varied by one of two methods: altering the pH of the bathing solution (open symbols) or replacing part of the extracellular chloride with PAH (closed symbols). The solid line has been drawn by linear regression analysis. Bars denote standard errors of the mean.

PAH. (Cells had previously been equilibrated with an acid environment, pH, 6.5; see Methods.) The effects of those perturbations on the intracellular voltage are presented in Table II. Both methods of changing the chloride ratio produce changes in voltage that are in the direction predicted if the distribution of chloride is passive.

In Fig. 3, we have plotted the observed values for the membrane potential, obtained by varying the chloride ratio by both methods, as a function of the

negative logarithm of the measured chloride concentration ratio (rCl). As can be seen, the membrane potential changes in the expected direction in response to varying rCl. Voltage and the negative log of rCl can be described by a straight line whose slope is 33 mV per decade change in the chloride concentration ratio. Two points should be noted. First, the value is less than the theoretical value of 58 predicted by the Nernst equation. Second, the observed values of intracellular voltage when only the pH is varied (open circles) are identical to those already reported by Hoffman and Lassen (see review by Lassen, 1977) for *Amphiuma* red cells under similar conditions (see Fig. 8 in Discussion).

Resistance Measurements

Fig. 4 shows that when we passed current across the cell positioned in a holding-capillary, by injecting current through an electrode like that described in Fig. 1 *A*, the voltage response is linear. Thus, the entire system acts as a constant resistor over the range of currents used in this study ($1–10 \times 10^{-9}$ A).

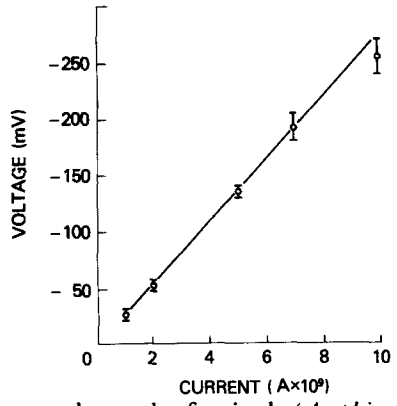

FIGURE 4. A current-voltage plot for single (*Amphiuma*) red blood cells. Current pulses, varying from 1×10^{-9} to 10^{-8} A were passed across a cell held in a pipette. Symbols represent the mean of six cells. Bars denote standard errors of the mean.

Since in these experiments injected current may pass not only through the cell but also through a shunt pathway (between the membrane and Sylgard coating), it is necessary to describe the entire system with two parallel resistors, R_S and $(R_{M_1} + R_{M_2})$. The following equation, therefore, applies:

$$\frac{1}{R_t} = \frac{1}{R_{M_1} + R_{M_2}} + \frac{1}{R_S}, \tag{7}$$

where R_t, as determined above, is the input resistance across the entire cell, R_{M_1} and R_{M_2} are the input resistances of the exposed membrane surface at the outer and inner ends of the cell, and R_S is the resistance of the shunt pathway. Besides R_t, one can also measure the input resistance across the outer membrane surface, R_{M_1}, in a separate experiment (see Methods, Fig. 1 *B*).

Table III presents the pooled results of a series of experiments in which separate measurements of R_t and R_{M_1} were made on different cells. Values are given both as input resistance and conductance. Assuming the specific resistance of both membranes to be equal, R_{M_2} (in MΩ) can be calculated from the measured value of R_{M_1} and the ratio of the calculated surface areas, A_1 and A_2, according to the following equation:

$$R_{M_2} = \frac{R_{M_1} \cdot A_1}{A_2}. \tag{8}$$

Knowing R_t, R_{M_1}, and R_{M_2}, we then calculated R_s from Eq. 7. The mean calculated value for R_s was large relative to that of R_{M_1} and R_{M_2} and is, therefore, consistent with the hypothesis that most of the current passed through and not around the cell. The reader is reminded that since R_{M_1} and R_{M_2} are input resistances, a difference in these values does not indicate

TABLE III

INPUT RESISTANCE AND CONDUCTANCE VALUES FOR THE MEMBRANE AND SHUNT PATHWAYS

	Whole cell	Outer membrane	Inner membrane*	Shunt*
Input resistance, MΩ	39.6±2.7 (16)	24.6±3.1 (18)	15.3	5,268
Conductance, $\times 10^{-2}\ \Omega^{-1}$	2.53±0.18 (16)	4.07±0.26 (18)	6.54	—

* Values calculated from Eqs. 7 and 8. Ratio of inner to outer membrane surface areas was 1.61. Data were pooled from two separate experiments. Number of cells measured is given in parentheses.

membrane damage but reflects instead a difference in the surface areas of the two membranes. Some uncertainty about the absolute value for R_s remains, however, both because it was necessary in these experiments to obtain data from two groups of cells rather than one and because the standard errors of the means are large.

Another way to test whether significant current passes around the cell is to vary the diameter of the capillary. Enlarging the diameter will reduce the resistance of both the shunt and membrane pathways, but the effect on the shunt resistance will be greater. A cell need not elongate as much to enter a capillary with a large diameter as to enter a small one. Thus, a cell introduced to a capillary whose diameter is twice that of a smaller one will form a cylinder one-fourth as long. This has the effect of reducing the length of the shunt pathway and consequently the shunt resistance by a factor of four. At the same time, the larger capillary, by virtue of its larger circumference alone, will have one-half the shunt resistance of the smaller capillary (assuming the shunt pathway has the same thickness in both pipettes.) Therefore, as one doubles the diameter, the combined effect of changes in length and circumference should decrease the shunt resistance by a factor of eight.

On the other hand, doubling the diameter will decrease the input resistance of the membrane only by a factor of four—a consequence of quadrupling the exposed membrane surface area. (The true membrane input resistance should only be affected by changes in the exposed surface area.)

The specific membrane resistance values computed by using Eqs. 3 and 4, possibly included components originating from both shunt and membrane resistances rather than membrane resistance alone. If all of the current flows through the membrane, the *actual* specific membrane resistance should be independent of any changes in surface area produced by doubling the diameter. (By definition specific membrane resistance is independent of surface

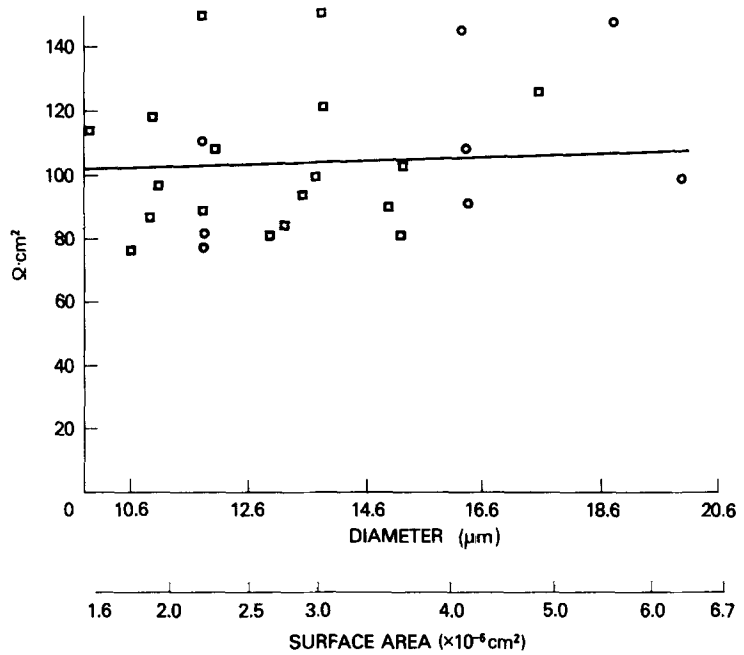

FIGURE 5. A plot of the calculated specific membrane resistance against the diameter of the pipette used to hold the cell. Each value represents the mean of 5–20 determinations performed on different cells with a single holding-capillary. Values were obtained by passing current through the entire cell (open squares) or by injecting current into cells via a microelectrode (open circles).

area.) In contrast, if all the current flows through the shunt pathway, the *calculated* specific membrane resistance will decrease to one-half of its initial value as the diameter doubles. This is a consequence of the shunt input resistance changing twice as much as the membrane input resistance (8 vs. 4). Fig. 5 shows the calculated specific resistance in ohm-cm^2 as a function of capillary diameter and total exposed membrane surface area. We measured the input resistance across the entire cell (open squares), using the electrode arrangement described in Fig. 1 *A* and across the outer membrane (open circles), using the arrangement described in Fig. 1 *B*. Specific membrane

resistance was calculated by using Eqs. 4 and 3, respectively. Holding-capillary diameters ranged from 9.8 μm, the point at the far left, to 19.6 μm, the point at the far right. The smallest capillary enclosed a cell whose exposed surface was 1.5×10^{-6} cm^2; whereas the largest capillary contained a cell with an exposed surface area of 6.0×10^{-6} cm^2. Specific resistance is independent of capillary diameter over the nearly fourfold increase in exposed surface area, an observation consistent with the postulate that most of the electrical conductance passes throught the membrane.

The following experiment, which was an extension of the preceding study, provides even more convincing evidence that the specific membrane resistance is independent of capillary diameter. A holding-capillary was so constructed (i.d., 8 μm) that it provided sufficient space to aspirate only one-half of a cell before it was impaled by the microelectrode. The remaining portion of the cell became spherical and had on the average a surface area of $\sim 20 \times 10^{-6}$ cm^2 exposed to the bath. The average calculated specific resistance for 10 such cells was 169 ± 22 ohm-cm^2, which is not statistically different from the resistances reported in Fig. 5.

TABLE IV

Experiment	pH	Intracellular voltage	ΔV_i 10^{-9} A injected intracellularly	ΔV_o 10^{-9} A injected extracellularly	$\Delta V_i - \Delta V_o$
		mV	*mV*	*mV*	*mV*
1	7.6	−14.2	−32.4 (9)	−36.3 (9)	+3.9
2	7.6	−12.0	−30.3 (6)	−25.6 (6)	−4.7
3	6.5	−3.8	−48.6 (8)	−46.1 (8)	−2.5
4	6.5	−5.2	−15.0 (10)	−11.8 (10)	−3.2
X±SEM (n)			−31.6±7.9 (4)	−32.5±8.4 (4)	−1.6±2.2 (4)

We performed a third type of experiment to assess the magnitude of the shunt pathway. The electrode arrangement described in Fig. 1 *C* was used. In this approach, we recorded the voltage drop across the outer membrane of the same cell, while injecting current from two different sources, one extracellular, the other intracellular.

If all the current injected extracellularly passes through the cell, the measured voltage drop across the outer membrane should be identical to that measured while injecting current intracellularly. The extent to which these two values differ is an indication of the amount of current that failed to enter the cell and passed instead around it. Note that in any given cell we measured the voltage drop across the same membrane surface each time; thus, the two values are directly comparable and independent of surface area considerations. Table IV presents the results of four experiments; the pH of the bathing medium and the intracellular voltage are shown in columns 2 and 3, respectively. Column 4 presents values for the voltage drop across the outer membrane after injecting current intracellularly; column 5 shows comparable values after injecting current extracellulary. Column 6 presents the difference between the two voltage deflections (column 4 minus column 5). This difference, as discussed above, is an indication of the amount of current that goes

around the cell. The observed difference is not significantly different from zero. These experiments suggest that little or no current leaks around the cell.

^{36}Cl Fluxes

Most of the tracer ^{36}Cl movement across the red cell membrane takes place via a mechanism that obligatorily exchanges an extra- and intracellular anion in a one to one fashion (Fortes, 1977). The transport process is saturable (Cass and Dalmark, 1973; Gunn et al, 1973; Dalmark, 1975 and 1976; Brahm, 1977) and, as such, probably depends upon a membrane component(s) in finite concentration. It can be inhibited by furosemide (Deuticke, 1970; Brazy and Gunn, 1976) and more specifically by 4-acetamido-4′-isothiocyano-stilbene 2,2′-disulfonic acid (SITS) (Passow and Schnell, 1969; Knauf and Rothstein, 1971). Early experiments with ionophores (Chappel and Crofts, 1966; Harris and Pressman, 1967; Henderson et al., 1969) suggested that most

TABLE V

EFFECT OF VOLTAGE ON CHLORIDE FLUX ACROSS SINGLE *AMPHIUMA* RED CELLS

Cell	Control J_{Cl}	Imposed voltage	Experimental J_{Cl}	Change in J_{Cl}
	$\times 10^{-6} M \cdot cm^{-2} \cdot min^{-1}$	mV	$\times 10^{-6} M \cdot cm^{-2} \cdot min^{-1}$	
1	0.98	195	1.50	0.52
2	0.71	187	1.45	0.74
3	0.44	273	1.19	0.75
4	0.91	279	1.41	0.50
5	0.86	241	1.77	0.91
6	0.82	229	1.38	0.56
7	1.15	209	1.39	0.24
8	0.83	217	1.51	0.68
$\bar{X}$	0.84±0.07	229	1.45±0.06	0.61±0.07

of the exchange did not generate a current and was, therefore, electrically silent. Two studies, in particular, confirmed this view and indicated that >99.9% of the tracer was probably electrically neutral. The first was Hunter's (1971) estimate of total anion conductance using valinomycin-treated human erythrocytes and a constant field approach. The second was Lassen's (Hoffman and Lassen, 1971; Lassen, 1972) measurement of total conductance in *Amphiuma* red cells. Although there is a consensus that most of the tracer exchange is electrically neutral, direct evidence and precise quantitation are lacking.

This transport process provides another means of assessing whether our methodology is measuring properties of the cell membrane or a nondiscriminate shunt around the cell. By measuring the ^{36}Cl flux across the outer membrane in the presence and absence of any electrical gradient, we should be able to determine directly whether the flux is unresponsive to an electrical gradient. The second column of Table V shows that the ^{36}Cl efflux across the outer membrane of control cells, at 23°C, averaged $0.84 \pm 0.07 \times 10^{-6}$ M·cm^{-2}·min^{-1}. This value corresponds to an equivalent resistance of 19 ohm-

cm^2. It compares acceptably, considering the many assumptions inherent in the flux calculation, with a calculated equivalent resistance of 12.2 and 9.9 ohm-cm^2 obtained from tracer efflux studies on suspensions of *Amphiuma* red cells by Brahm[5] and Knauf,[6] respectively. Brahm's measurement was direct and obtained at 23°C using a rapid flow tube, whereas Knauf's values were calculated from measurements made at 0°C.

We then imposed an electrical gradient (~230 mV holding pipette negative) across the cell using the electrode arrangement described in Fig. 1 *A* and remeasured the flux. For the moment, let us assume that all of the ^{36}Cl movement does not enter the cell but passes around it through a shunt pathway. For a diffusive pathway of this nature, the Ussing equation (Ussing, 1949) describes how the ratio of the two unidirectional fluxes across the cell will change as a result of the newly imposed voltage. To ascertain the change in either individual flux one must apply the following equation, whose derivation utilizes the Ussing relationship (Linderholm, 1952; Schultz and Zalusky, 1964):

$$J_{i\to 0} = {}^{0}J_{i\to 0}\,(zFE_{i\to 0}/RT)/(\exp[zFE_{i\to 0}/RT] - 1). \quad (9)$$

In this equation $J_{i\to o}$ is the flux from the holding capillary to the bath, ${}^{0}J_{i\to 0}$ is a vectorally similar flux but measured at a time when the voltage across the cell is zero, and $E_{i\to 0}$ is the imposed voltage. z, F, R, and T have their usual meaning. Since ${}_{0}J_{i\to 0}$, the flux at zero voltage, is 0.84×10^{-6} M·cm^{-2}·min^{-1}, and $E_{i\to 0}$ equals 229 mV, Eq. 9 predicts a value of 6.40×10^{-6} M·cm^{-2}·min^{-1} sor $J_{i\to 0}$. Therefore, the predicted increase in the unidirectional flux is 5.56×10^{-6} M·cm^{-2}·min^{-1} ($6.40 - 0.84 \times 10^{-6}$ M·cm^{-2}·min^{-1}, and $E_{i\to 0}$ equals 229 mV, Eq. 9 predicts a value of 6.40×10^{-6} M·cm^{-2}·min^{-1} for $J_{i\to 0}$. Therefore, the predicted increase in the unidirectional flux is 5.56×10^{-6} M·cm^{-2}·min^{-1} ($6.40 - 0.84 \times 10^{-6}$ M·cm^{-2}·min^{-1}). In contrast, column 5 of Table V (column 4 minus column 2) shows that the flux increased on the average 0.61×10^{-6} M·cm^{-2}·min^{-1}. Since this an exchange mechanism whose characteristics resemble those described above, we performed the following two experiments. Fig. 6 shows that replacing 88% of the Cl in the solution exposed to the outer membrane with a relatively impermeant anion, PAH, inhibits the chloride flux 40%. The removal of Cl from the *trans* side of the membrane should alter a ^{36}Cl flux originating from the *cis* side if transport involves an obligatorily coupled exchange process. Since PAH replacement alters the membrane potential, it might also reduce a ^{36}Cl efflux which was diffusive. We therefore examined the effects of two known inhibitors of the anion exchanger. Fig. 7 shows that furosemide (10^{-3} M) and SITS (5×10^{-4} M) applied to the outside of the outer membrane inhibit the control flux 35 and 90% respectively.

THO Fluxes

We determined THO effluxes by the same procedure used to measure control ^{36}Cl effluxes. Our measured value of $6.4 \pm 1.5 \times 10^{-3}$ M·cm^{-2}· min^{-1} ($n = 6$)

[5] Personal Communication.
[6] Personal Communication.

corresponds to a permeability of $2.0 \pm 5 \times 10^{-3}$ cm·s^{-1}. This value should be compared with that which Brahm and Wieth (1977) found for *Amphiuma* red cells at 25°C, using a rapid flow tube technique ($0.7–0.8 \times 10^{-3}$ cm·s^{-1}). Values for the diffusional permeability of tritiated water in mammalian red cell membranes are higher ($4.1–5.1 \times 10^{-3}$ cm·s^{-1}) (Paganelli and Solomon, 1957; Villegas et al., 1958).

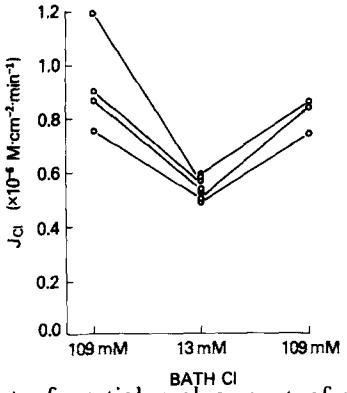

FIGURE 6. The effect of partial replacement of medium chloride with the impermeant anion, PAH, on ^{36}Cl efflux. We used seven cells in this study. On four of the cells the initial measurements were performed when the bathing solution in the bath contained 109 mM Cl; the measurements were then repeated after replacing this solution with one containing 13 mM Cl. The sequence was reversed with the other three cells; the initial bathing solution contained 13 mM Cl. Isoosmotic quantities of Na PAH replaced 96 mmol of NaCl in the Cl-depleted medium. The pH of both bathing media was 7.6.

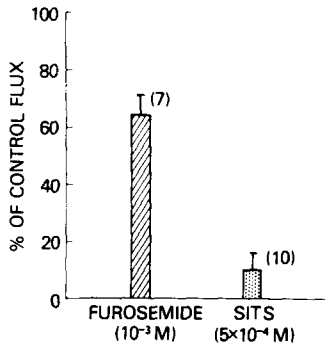

FIGURE 7. The inhibitory effects of furosemide and SITS on the ^{36}Cl efflux of single red blood cells. The crosshatched and stippled bars represent the percent of the control flux remaining after application of the drug. Numbers in parentheses represent standard errors of the mean.

DISCUSSION

The major findings in this work are, first, a technique that provides a direct means for measuring membrane potential and resistance in the *Amphiuma* erythrocyte and, second, the modification of this technique to study the

transport of fast moving substances such as Cl and water. It is necessary to have knowledge about membrane potential and conductance to understand thoroughly how electrolyte transport occurs in red cells. We provide here a direct means for studying these parameters. Importantly, our study of ^{36}Cl fluxes is the first to investigate how a voltage gradient applied directly to the red cell affects the transport of a charged substance, in this case chloride.

Our measurements of membrane potential, Cl, and THO effluxes are in reasonable agreement with both direct and indirect measurements of other investigators. In addition, all the changes in the ^{36}Cl efflux produced by an imposed voltage gradient and such recognized inhibitors as furosemide and SITS are consistent with previously published observations. Taken together,

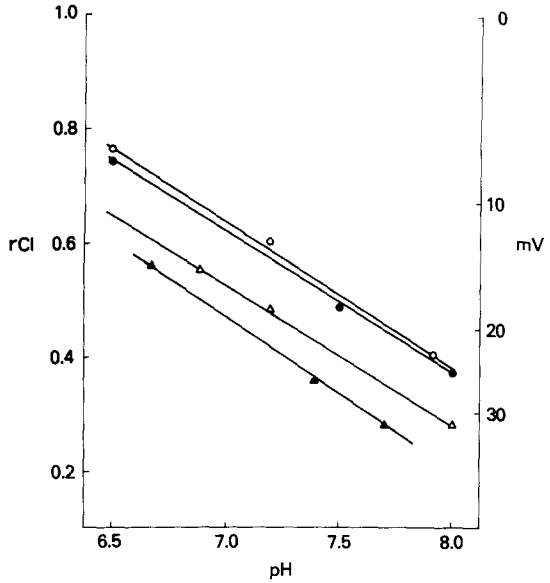

FIGURE 8. A plot of the chloride concentration ratio, $rCl = [Cl]_o/[Cl]_i$ (triangles), and the measured intracellular voltage (circles) against the pH of the bathing medium. Open symbols represent the data from this study and closed symbols the data of Lassen (1977).

these studies support the postulate that we are studying properties of a normal red blood cell membrane whose function has not been altered by the technique. They also indicate that we are not studying the properties of a nondiscriminating shunt pathway.

Certain aspects of our findings, however, are unexpected and require further comment. If chloride distributes passively, and the membrane permeability to chloride is much greater than that of other ionic species, chloride will distribute according to a Donnan equilibrium. Under these conditions the difference between intra- and extracellular chloride concentration produces a diffusion potential that both defines the membrane potential and satisfies the Nernst equation. Even if the permeability to other ions is significant, provided

chloride distributes passively and the cell has reached a steady state, the Nernst equation for Cl will provide the magnitude of the membrane potential. Thus, if either of the above circumstances applies, one should observe a 58-mV change for each decade change in the chloride concentration ratio. Fig. 3 shows, however, that our measured slope is 33, not 58. It is necessary, therefore, to consider whether this discrepancy stems from a shortcoming in the techniques used to measure the membrane potential or from a failure of our cells to satisfy the previously mentioned prerequisite conditions.

To consider the former possibility, we have compared our measured values of intracellular potential and chloride ratio (open circles of Fig. 3) with those of Hoffman and Lassen (see review by Lassen, 1977). Fig. 8 shows both sets of values as a function of the pH of the bathing medium. Intracellular voltage measurements from the two studies are essentially identical. The chloride ratios from the two studies also have nearly parallel lines. There is, however, a small difference in the measured value of rCl at all pH values in the two studies. The reason for this disparity is unclear; differences in medium composition and the quantity of cell water are possibilities. It is noteworthy that when the voltage values in either study are plotted as a function of the negative logarithm of the chloride ratio neither generates a line with the theoretical slope of 58. Equivalent findings in both studies, in which the techniques for measuring potential are so dissimilar, make it unlikely that the failure to achieve an ideal slope results from a consistent error in the way voltages were measured.[7] In addition, the stability of our intracellular potential measurements with time (see Fig. 2) and our evidence of an electrical seal between the site of impalement and the opposite side of the membrane across which we measure voltage argue against the possibility that leakage around the electrode is an important consideration in our studies.

In the next few paragraphs, we consider some of the factors which might explain why our cells fail to demonstrate an ideal Nernst relationship. Since both our study and that of Hoffman and Lassen (Lassen, 1977) demonstrate equivalent findings, these comments could apply equally well to either study. Similar findings in both studies, one of which was performed on free-floating cells, and the other on cells held in a capillary, indicates that holding the cell within the capillary has not brought about changes sufficient to affect the membrane potential measurement. Both studies, however, could be subject to artifacts induced by the electrode itself. That the intracellular voltage is constant with time (Fig. 2) argues against this possibility in our study.

The Nernst equation requires that the ratio of chloride concentrations and the ratio of chloride activity coefficients be known. Our calculations assume that intracellular Cl is distributed uniformly in the cell water and that intra- and extracellular Cl activity coefficients are identical. There is uncertainty, therefore, about the actual intracellular chloride activity values. Thus, it may

[7] Lassen (1977) has also reported positive voltages for *Amphiuma* red cells suspended in a medium in which much of the Cl has been replaced with PAH. We did not try to reproduce these findings because we had established in preliminary experiments that only PAH replacement solutions that had substantially higher Cl concentrations satisfied our steady-state requirements.

be that we fail to achieve the theoretical slope because changes in intracellular Cl activity occur that are different from our calculated changes. This question must await reliable intracellular Cl activity measurements.

With regard to this possibility, Hoffman[8] (see also Lassen [1972]) has suggested that the nucleus excludes chloride and has offered this explanation for the difference between measured and calculated membrane potential values. Nuclear exclusion of chloride, if it occurred uniformly over the entire range of pH values and PAH concentrations used in our study, would change all calculated chloride ratio values nearly similarly and would not appreciably alter the observed experimental slope in Fig. 3. For this hypothesis to explain our experimental findings entirely, the amount of chloride excluded from the nucleus must vary with pH and PAH concentrations. This is because the size of the nuclear compartment (20–30% of total cell volume) requires that for the slope to be 58, Cl must be absent from the nucleus at pH 8.0 but present at concentrations that approach or exceed those found in the cytosol at pH 6.5. There is no data available on this point, so Hoffman's hypothesis remains a possibility.

Other possible explanations for the failure to observe an ideal Nernst relationship exist. P_{Cl} may be so low that the permeabilities of other ions such as Na and K may also affect voltage. Lassen's calculation of P_{Cl} from indirect measurements supports this premise (Lassen et al., 1978), as does his most recent estimate of total membrane conductance (Rathlev and Lassen, 1977).

In our study, direct conductance measurements obtained by passing current place total electrical conductance approximately three orders of magnitude lower than the estimates of Rathlev and Lassen (1977). To explain our conductance measurements, the permeability(ies) of some ionic species must be large. It is also possible that under our experimental conditions ions other than Cl contribute to the observed voltage. Callahan and Hoffman (1976) have provided evidence that suggests that OH or H conductance can sometimes contribute appreciably to total conductance in human red cells.

Another possible explanation is that, even though bulk analysis indicates that the cells have achieved a steady state, the steady-state condition may be only apparent. Electrolyte concentrations could be changing slightly during the experimental period but at a magnitude too small to be perceived by our techniques.

An Analysis of Conductance Measurements

The two direct measurements of specific membrane conductance in *Amphiuma* red cells by Lassen and co-workers are at variance with our value. Even their higher value of $0.5–1.0 \times 10^{-3}$ ohm^{-1}cm^{-2} (Hoffman and Lassen, 1971) would require that, in our study, 90% of the current that passes across the cell never enters it. Though we know of no experiment that can demonstrate the complete absence of a shunt pathway, we have presented three lines of evidence that support our postulate that most of the current permeates the membrane. First, an erythrocyte held in a Sylgard-lined capillary has the

[8] Unpublished observation.

properties expected of two resistors in series. Second, the calculated specific membrane resistance, like the true specific membrane resistance, is independent of the exposed membrane surface area. Finally, identical currents passed extracellularly across the entire cell or intracellularly via the microelectrode produce the same voltage deflection across the outer membrane. All three approaches indicate that our measured resistances are membranous in origin.

Our measurements, however, are subject to the criticism that aspirating the cell into the holding capillary may result in a membrane alteration that raises conductance. That our intracellular potentials, tritiated H_2O and ^{36}Cl effluxes agree with data generated by other laboratories using different techniques, all of which conform to patterns generally found in other erythrocytes, argues against a major change in membrane properties.

Rathlev and Lassen (1977), using the oil gap method (Lassen, 1977), have reported a conductance value as low as 3×10^{-6} ohm^{-1} cm^{-2}. It is conceivable that this measurement may include the resistance of a thin layer of oil that has covered the conducting elements of the membrane. This possibility remains tenable because of the following: first, the exterior surface of the red cell membrane tends to seek a hydrophobic environment (Mudd and Mudd, 1931); thus oil would have a tendency to spread over the entire red cell surface; and, second, the resistivity of silicone oil (20/200), 1×10^{14} ohm-cm, is such that even a very thin layer could account for this high resistance measurement.

It is unclear, therefore, why our conductance values and those of Lassen differ. An explanation must resolve how the two research groups can have such divergent conductance measurements yet still obtain identical intracellular potentials. It may be useful to remember that, in our study, measurements of membrane potential and conductance differ in that conductance measurements are obtained by imposing a voltage gradient across the membrane. Thus, our conductivity measurements may be subject to a voltage-induced permeability change.

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