Volume Regulation by *Amphiuma* Red Blood Cells

*The Membrane Potential and Its Implications Regarding the Nature of the Ion-Flux Pathways*

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**ABSTRACT** After osmotic perturbation, the red blood cells of *Amphiuma* exhibited a volume-regulatory response that returned cell volume back to or toward control values. After osmotic swelling, cell-volume regulation (regulatory volume decrease; RVD) resulted from net cellular loss of K, Cl, and osmotically obliged H₂O. In contrast, the volume-regulatory response to osmotic shrinkage (regulatory volume increase; RVI) was characterized by net cellular uptake of Na, Cl, and H₂O. The net K and Na fluxes characteristic of RVD and RVI are increased by 1–2 orders of magnitude above those observed in studies of volume-static control cells. The cell membrane potential of volume-regulating and volume-static cells was measured by impalement with glass microelectrodes. The information gained from the electrical and ion-flux studies led to the conclusion that the ion fluxes responsible for cell-volume regulation proceed via electrically silent pathways. Furthermore, it was observed that Na fluxes during RVI were profoundly sensitive to medium [HCO₃] and that during RVI the medium becomes more acid, whereas alkaline shifts in the suspension medium accompany RVD. The experimental observations are explained by a model featuring obligatorily coupled alkali metal–H and Cl-HCO₃ exchangers. The anion- and cation-exchange pathways are separate and distinct yet functionally coupled via the net flux of H. As a result of the operation of such pathways, net alkali metal, Cl, and H₂O fluxes proceed in the same direction, whereas H and HCO₃ fluxes are cyclic. Data also are presented that suggest that the ion-flux pathways responsible for cell-volume regulation are not activated by changes in cell volume per se but by some event associated with osmotic perturbation, such as changes in intracellular pH.

**INTRODUCTION**

Cell-volume regulation after osmotic perturbation is a non-steady-state response that tends to restore cell volume to normal control levels. Whereas in the invertebrates volume regulation is the result of changes in cell amino acid and, therefore, H₂O content (Gilles and Schoffeniels, 1969; Schoffeniels and...
Gilles, 1970), volume regulation by vertebrate cells is the result of net inorganic ion flux and osmotically obliged H2O flow. Those cells for which quantitative ion- and water-flux data are available include the red blood cells of the duck (Kregenow, 1971 a and b; Schmidt and McManus, 1977), flounder (Fugelli, 1967; Cala, 1977), dog (Parker, 1973 a and b), frog (Weissenberg and Katz, 1975), and human (Poznansky and Solomon, 1972). There are also data available for rabbit proximal tubule (Dellasega and Grantham, 1973; Grantham et al., 1974), mouse lymphoblasts (Roti Roti and Rothstein, 1973), and Ehrlich ascites tumor cells (Hendil and Hoffman, 1974). With the exception of the dog red blood cell, all of the above are high-K, low-Na cells, which regulate volume primarily by net inorganic ion fluxes that proceed via dissipative pathways. Typically, the volume-regulatory response to swelling is characterized by net cellular loss of K, Cl, and H2O. The response to shrinkage is variable in that some systems regulate cell volume by gaining Na, Cl, and H2O whereas others appear to require elevated external K and regulate volume by gaining Na, K, Cl, and H2O. In all of the systems thus far studied, inhibition of the Na-K pump with ouabain is without effect upon the rate and magnitude of volume regulation.

Although volume regulation is a fundamental cellular process, it is at present poorly understood. One of the major gaps in our understanding is that the nature (i.e., whether it is conductive or electrically silent) of the volume-regulatory ion fluxes is unknown. Evaluation of the flux pathways is complicated by the absence of electrical information, without which a precise determination of the relevant driving forces is impossible. Finally, the event or events associated with osmotic perturbation and responsible for activation of the volume-regulatory flux pathways remain obscure. Thus, a knowledge of the membrane voltage would clearly help in identifying the nature of the ion-flux pathways responsible for volume regulation. Furthermore, once the nature of the pathways is understood, reasonable attempts to understand the details of their activation could be initiated. It is generally assumed that the red blood cell membrane conductance to Cl (a passively distributed species) is sufficiently high that the cell membrane potential (V_m) equals the chloride equilibrium potential. This may not be true during volume regulation because net cation fluxes increase by 1–2 orders of magnitude, and it is no longer reasonable to assume that the membrane conductance is dominated by chloride. This is of particular relevance with respect to the present studies of Amphiuma red cells, because the steady-state ratio of Cl to K permeability is ~5 (Hoffman and Laris, 1974). Thus, the present study of volume regulation by Amphiuma red blood cells employs electrophysiological techniques to obtain direct measurements of V_m and thus avoid uncertainties associated with calculations based upon assumptions of equilibrium. Because of the size (5 × 10^3 μm^2) of the Amphiuma red blood cell, it is possible to directly measure V_m by impalement with glass microelectrodes (Lassen, 1971; Lassen and Vestergaard-Bogind, 1976; Smith and Levinson, 1976; Stoner and Kregenow, 1976; Lassen et al., 1978).
This communication reports ion-flux and electrical data obtained by studying the volume-regulatory response of *Amphiuma* red blood cells. Comparisons of data obtained by studying volume-regulating and volume-static cells in the absence and presence of the K ionophore valinomycin suggest the hypothesis that volume-regulatory ion fluxes are electrically silent. Tests of the hypothesis involving selective inhibition and stimulation of the various flux pathways are performed and evaluated by both electrical and chemical methods, and the hypothesis is accepted. The experimental observations are summarized in a model that would have net Na and K fluxes characteristic of the volume-regulatory response obligatorily coupled to H, whereas net Cl fluxes proceed in exchange for HCO₃. In addition, data are presented that suggest that H serves not only as a substrate for, but as an activator of, the alkali metal–proton exchange pathways.

Preliminary reports of some of the findings of this study were presented to the Red Blood Cell Club, Dallas, 1979, and have been published (Cala, 1980).

**MATERIALS AND METHODS**

**General Procedures**

The procedures outlined for manipulating the cells and determining cellular Na, K, Cl, and water content have been described (Cala, 1977). Cells were obtained from healthy, adult *Amphiuma* by cardiac puncture. Blood was removed with a heparinized syringe and immediately washed in 40 vol of control Ringer's solution (1 R) of the following composition (mM): NaCl, 110; KCl, 3; MgCl₂, 1; CaCl₂, 1; imidazole, 20; glucose, 10. This medium was gassed with water-saturated room air and buffered at pH 7.65 at 23°C. The medium also contained 10⁵ U/l penicillin and 0.25 g/l streptomycin sulphate. The cells were then suspended at a hematocrit of <10% and incubated in the medium described above for 3 h to ensure that they had reached steady state with respect to ion and H₂O content before experimental treatment. At the beginning of an experiment, the cells were centrifuged from suspension in the control medium and at time zero resuspended at a hematocrit of 10% in the appropriate medium (T = 23°C). To all cells intended for ion and H₂O analyses, [³⁵Cl]polyethylene glycol was added to the experimental medium as an extracellular space marker. 375-μl samples were then removed at predetermined intervals (15, 30, or 60 min), placed in 400-μl polyethylene tubes, and centrifuged in a microcentrifuge (model 3200, Brinkmann Instruments, Inc., Westburg, N. J.) for 2 min at 12,000 g. After centrifugation the supernate was separated from the cell pellet and saved for analysis. The cell pellet (9-18 mg) was then weighed, heated in an oven at 80°C for 48 h, cooled in a desiccator, reweighed to determine dry weight (4-10 mg), and extracted in 250 μl of glass-distilled H₂O. After a 3-d extraction, the extract was sampled, and ion and ¹⁴C analyses were performed.

**Cell Ion and H₂O Content**

The following aliquots of cell extract were analyzed: 100 μl for chloridometry (Buchler Instruments Div., Searle Diagnostics, Inc., Fort Lee, N. J.) 50 μl for Na and K flame photometry (model 343, Instrumentation Laboratory, Inc., Lexington, Mass.) and 40 μl for scintillation counting (Mark II liquid scintillation system, Searle Diagnostics, Inc., Des Plaines, Ill.). The data thus obtained and the wet and dry weights of the cell
pellets were used to calculate cellular ion and H₂O content corrected for extracellular contribution. The extracellular space correction typically was <5%.

Experimental Media

Unless otherwise specified, medium osmolarity was altered by changing [NaCl]. All media contained glucose, trace elements (Mg²⁺, Ca²⁺), and the antibiotics penicillin and streptomycin. 20 mM, imidazole was the usual buffer. When HCO₃-buffered medium was desired, the imidazole was deleted, 32 mM NaCl was replaced with 42 mM Na HCO₃, and the medium was gassed with a mixture of 95% O₂-5% CO₂ (final pH, 7.65 at 23°C). When ouabain or 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS; Calbiochem-Behring Corp., San Diego, Calif.) were added to cells, they were added before experimental treatment (during the preincubation period) because they required 30 min to 1 h to produce their maximal effect at 23°C. Experiments with DIDS were performed in a dimly lighted room, and the cell suspensions were kept from light except during sampling. DIDS was present throughout the preincubation and experimental period and was made fresh for each experiment.

Fluorescence Measurements

Fluorescence was measured with a Farrand MK-1 spectrophotometer (Farrand Optical Co., Inc., Valhalla N. Y.). The fluorescence levels were recorded on a Hewlett-Packard recorder (Hewlett-Packard Co., Palo Alto, Calif.). The protocols employed were those described by Hoffman and Laris (1974). The dye employed in these studies was Di-S-Cs(5) obtained from Dr. Alan Waggoner of Amherst College, Amherst, Mass. Cells were suspended at a hematocrit of 0.33% in medium containing 1.5 × 10⁻⁶ M Di-S-Cs(5).

Electrical Measurement

Measurements of the Amphiuma red blood cell membrane potential were made with glass microelectrodes filled with 2 M KCl. All electrodes were beveled at an angle of 22° with a beveler (model 1200, W-P Instruments, Inc., New Haven, Conn.). I found that beveling the electrodes greatly facilitated successful cell impalement. The tip diameter of these electrodes typically was between 0.3 and 0.5 μm, and resistance was 15–50 MΩ. The electrode was in contact with a silver–silver chloride wire connected to a microelectrode amplifier (G model 5, Getting, Los Altos, Calif.). The cells were observed at × 200 with an inverted microscope (model CK phase-contrast, Olympus Optical Co., Tokyo, Japan) mounted on a vibration isolation table (model 1201, Kinetic Systems, Waltham, Mass.). The cells were allowed to settle to the bottom of a plastic petri dish containing 10–25 ml of fluid. Upon settling the cells stuck to the dish, obviating the need for a means of holding them stationary. The electrode initially was advanced at angles of between 30° and 50° with a rather coarse micromanipulator (Stoelting Co., Chicago, Ill.). Fine movements and impalement were achieved with a hydraulic microdrive (David Kopf Instruments, Tujunga, Calif.) with stepping motor. Electrical data were recorded on a strip chart recorder (model 7702B, Hewlett-Packard). One channel of the recorder was used to measure the amplifier output in real time, providing a record of the stable potential as well as permitting resistance measurements. The other channel of the recorder was used to record data indirectly in that it recorded fast transients <16 ms. The fast transients were first recorded with a storage oscilloscope with an analogue to digital converter (OS4000, Gould Inc. Cleveland, Ohio) with automatic playback. Thus, it was possible to record voltage excursions occurring in the millisecond range and play them back
over minutes. Consequently, the response time of the recorder was not a factor in obtaining faithful traces of the electrical events.

The direct measurement of the *Amphiuma* red cell membrane potential is complicated by the fact that it is difficult to obtain an electrically tight seal between glass microelectrode and membrane. Thus, one is forced in most cases to measure a peak value. Yet, as will be shown, these peak values agree well with theory. The validity of using the peak values of rapidly changing potentials also gains support from studies by Stoner and Kregenow (1975) and Smith and Levinson (1976). These authors have been able to obtain more stable measurements that decay in seconds or minutes rather than milliseconds. To the extent that studies have been duplicated, the values reported herein agree with those of these authors as well as those inferred from studies with voltage-sensitive dyes (Hoffman and Laris, 1974). For a more complete discussion of the methodology, its difficulties, and validity, see Lassen (1977) and Lassen and Rasmussen (1977).

**RESULTS**

*Volume-regulatory Ion Fluxes*

The data presented in Figs. 1 and 2 depict changes in ion and water content of *Amphiuma* red blood cells after osmotic swelling and shrinkage, respectively. After osmotic swelling (Fig. 1) by transfer of cells from control medium (1 R; 245 mosM) to hypoosmotic medium (0.75 R; 183 mosM) at time zero \((t = 0)\), the cells exhibit regulatory volume decrease (RVD), losing K, Cl, and osmotically obliged \(H_2O\). After osmotic shrinkage (Fig. 2) by transfer of cells from 1 R to hyperosmotic Ringer's solution (1.3 R; 319 mosM), the cells undergo regulatory-volume increase (RVI), gaining \(H_2O\) in response to net cellular uptake of Na and Cl. The net K and Na fluxes characteristic of the volume-regulatory responses are in the direction of their respective electrochemical gradients (dissipative fluxes), and the rates and magnitudes of these fluxes are insensitive to ouabain. As first reported by Siebens and Kregenow (1978) and confirmed (data not shown) in the present studies, net Na fluxes associated with RVI are completely inhibited as a result of osmotic shrinkage in the presence of the diuretic drug amiloride \((10^{-3} \text{ M})\), (Merck, Sharp & Dohme, West Point, Pa.). Because normal steady-state Na flux is amiloride insensitive, it appears that the Na-flux pathway responsible for RVI is activated by some event(s) associated with osmotic shrinkage and that the pathway is different from the classical leak (Tosteson and Hoffman, 1960). With the exception of data relating to amiloride sensitivity, which has not been tested in most cases, the volume-regulatory responses of *Amphiuma* red cell are similar to those described for other systems (Kregenow, 1971a and b; Roti Roti and Rothstein, 1973; Dellasega and Grantham, 1973; Weissenberg and Katz, 1975) and remarkably similar to volume regulation as exhibited by flounder erythrocytes (Cala, 1977).

*Validation of Electrical Methods*

After it was established that *Amphiuma* red blood cells exhibit a volume-regulatory response, attempts were initiated to obtain direct measurements of \(V_m\) by impaling cells with glass microelectrodes. Two typical microelectrode
Figure 1. RVD after cell swelling. These data were obtained from *Amphiuma* red blood cells that were transferred from control medium (245 mosM) to hypotonic medium (183 mosM) at *t* = 0. Samples were removed at the indicated times and prepared as described in Materials and Methods. Note that the units on the ordinate in this and all other graphs depicting cell ion and water content express amount. Concentration can be obtained by dividing the values for cell ion content by the corresponding cell water content values.
recordings are presented in Fig. 3. These data were obtained from *Amphiuma* red blood cells whose K conductance ($G_K$) had been increased by exposure to the K ionophore Valinomycin (Val; (Calbiochem-Behring Corp.)). Trace $A$ was obtained in medium in which $[K]_o = 3$ mM, and trace $B$ was obtained at
[K]₀ = 20 mM. The relative magnitudes of the peak measured values of \( V_m \), at [K]₀ = 3 and 20 mM are consistent with the expected behavior of the \( V_m \) of a membrane with a \( G_K \) that is a significant fraction of the total membrane conductance. Yet, as shown in Fig. 3 A and B, the recorded \( V_m \) is not stable at the maximum value but falls over milliseconds to a lower, stable potential. Thus, in the present studies, as it was in studies of Amphiuma red cells described by others (Lassen, 1971; Lassen, 1977; Lassen et al., 1978), it was necessary to measure a rapidly changing potential, choosing the peak value as the actual value of \( V_m \) before impalement. Though the conditions of measurement are less than ideal, the data obtained are qualitatively if not quantitatively correct (see Figs. 3 and 4 and Tables I and II).

The inability to obtain stable measurement of \( V_m \) by conventional means is probably due to the combined effects of mechanical instability and an unusually high membrane resistance.

![Figure 3](image)

**Figure 3.** Measured excursion in transmembrane voltage obtained upon impaling Val (2.5 \( \mu M \))-treated Amphiuma red cells suspended in medium at external [K] equal to 3 or 20 mM. Upon cell penetration there is a sharp drop in potential followed by a less rapid decay to more positive values. Note that although peak values are those that would be expected of the \( V_r \) of a K-selective cell (as a function of external K), the quasi-stable values are not.

With regard to the former, any nonaxial movement of the electrode tip upon advancement will contribute to membrane damage. As a result of small cell size and the lack of electrical support from adjacent cells, such cell damage can seriously impair the ability to obtain stable measurements of \( V_m \). With regard to membrane resistance (\( r_m \)), the same low-resistance shunt around the electrode that prevents measurement of stable \( V_m \) also makes it impossible to obtain direct measurements of \( r_m \) by current injection (typically, the \( r_m \) measured upon stabilization of \( V_m \) is on the order of 1-2,000 \( \Omega \) cm²). Yet, it is possible, by indirect means, to obtain estimates of \( r_m \) that, while underestimated, are within an order of magnitude of the actual value. One such attempt involves the use of the protonionophore 3,3',4,4'-tetrachlorosylcylanilide (TCS) (1 \( \mu M \)), which increases \( G_H \) enough that the Val-K current is no longer sufficient to produce membrane hyperpolarization (i.e., \( G_T^{\text{TCS}} \gg G_K^{\text{at}} \)). Because under normal conditions the Amphiuma red cell \( V_m \) is equal to the H equilibrium potential (\( E_H \)), the large \( G_H \) induced by TCS essentially clamps \( V_m \) at its normal "resting" value. Thus, it is possible to add Val to cells previously exposed to TCS and measure the Val-induced K flux uninfluenced by changes in \( V_m \). Under such conditions, K fluxes from 30 to 50 mmol/kg dry cell solids (des) × h are obtained. Since there are 4.7 × 10¹¹ cells/kg des at 5 × 10⁶ \( \mu m^2 \)/cell, calculated values for
$G_{\text{K}}^{\text{Val}}$ are on the order of $5.8 \times 10^{-6}$ $\Omega^{-1}$ cm$^{-2}$. Given that in the presence of Val, $V_m \neq E_K$, yet $V_m$ is sensitive to changes in $[K]_o$, then $G_{\text{K}}^{\text{Val}}$ must be close to (within an order of magnitude) $G_m$. Thus, membrane resistivity ($R_m$) is on the order of $2 \times 10^3$ $\Omega$ cm$^2$ and membrane resistance ($r_m$) must be near $10^{10}$ $\Omega$. The values for $R_m$ and $r_m$ are very large relative to those values obtained from cells typically used in electrophysiological studies ($R_m$ on the order of $10^3$ $\Omega$ cm$^2$). Thus, rather than some peculiarity of the *Amphiuma* red cell membrane-electrode glass interaction, the inability to obtain a tight electrical seal may simply be a reflection of high $R_m$. That is, if the interface between the membrane and the electrode tip is a low resistance relative to $R_m$, then current will flow through this low-resistance shunt and not across the membrane. Therefore, the resistance between the electrode and the *Amphiuma* red cell membrane (leakage resistance) may be no lower than that in systems typically used for electrophysiological studies, yet this pathway may constitute a low-resistance shunt by virtue of the extremely high values of the *Amphiuma* red cell $R_m$. In support of this hypothesis is the observation that in the presence of TCS, $V_m$ is stable over seconds and $R_m$ is on the order of $1.6 \times 10^3$ $\Omega$ cm$^2$ (see also Lassen et al. [1978]).

Before employing electrical techniques in studies of the cell-volume regulatory response, I performed confirmatory studies to demonstrate the validity

| Condition | mV/10-fold change in medium concentration |
|-----------|----------------------------------------|
| $\Delta [K]_o + 2.5$ µM Val | 40-50 |
| $\Delta [H]_o$ | 10-14 |
| $\Delta [Cl]_o$ | 30-40 |

* The maximum slope was obtained graphically at the steepest point of the curve, relating $V_m$ to medium concentration.

‡ In the absence of Val $V_m$ was insensitive to alteration in medium $[K]$, and $[K]_o$ was altered at the expense of $[Na]_o$.

§ Changes in $[Cl]_o$ were achieved by replacement with $p$-aminobipurate.

of the measurements. Manipulations chosen to produce predictable (based upon sound theory) effects upon $V_m$ were performed, and the results are presented in Table I. These data are presented as the maximum slope in millivolts per 10-fold change in the concentration of an extracellular ionic species to which $V_m$ was expected to respond. The first horizontal row presents data obtained in experiments in which Val was present in the medium. Val, by increasing the membrane conductance to K ($G_K$), should cause $V_m$ to approach the K equilibrium potential ($E_K = -90$ mV). In the extreme case in which $G_K^{\text{Val}}$ is equal to the total membrane conductance, the $V_m$ should equal $E_K$ and vary by 59 mV/10-fold change in $[K]_o$. The observation that at high external K concentrations, when contributions of K to total membrane current are the greatest, $V_m$ varies by 40 mV/10-fold $\Delta[K]_o$ (see also Fig. 4) is consistent with known effects of Val. Furthermore, that the addition of Val to *Amphiuma* red cells produces a membrane hyperpolarization of some 20 mV (see also Table II) agrees with data obtained by others studying Val-treated *Amphiuma* red blood cells (Hoffman and Laris, 1974; Smith and Levinson,
1976; Lassen, 1977) and thus supports the electrophysiological technique employed in the present studies. Since the erythrocyte membrane potential is a Donnan potential, the magnitude of which depends upon the magnitude of the net negative charge on intracellular impermeant anions, then titration of intracellular anion with H should lead to positive shifts in $E_m$. The data in the second horizontal row of Table I show that $V_m$ again changes by 14 mV/

$$V_m = F \log [K]^0$$

**Figure 4.** The membrane potential of Val (2.5 μM)-treated *Amphiuma* red blood cells as a function of external [K⁺]. The [K⁺] was altered by replacing Na⁺ with K⁺ such that the total medium concentration of Na⁺ + K⁺ was constant. With the exception of changes in Na⁺ and K⁺, the media were identical to the control medium. Cell impalement was as described in Materials and Methods.

10-fold $ΔH$, in good agreement with data reported by others (Hoffman and Laris, 1974; Stoner and Kregenow, 1976; Lassen, 1977; Lassen et al., 1978). In *Amphiuma* red cells, as is true of other red cells, $G_{Cl}$ is a significant fraction of $G_m$ in media of physiological composition. Consequently, changes in $[Cl]^0$ should give rise to changes in $V_m$. That this is the case can be seen in the third row of Table I, which shows that $V_m$ changes by 30-40 mV/10-fold change in
Thus, in all cases studied, measured values of $V_m$ agree with theoretical predictions and previous observations.

Table II depicts membrane potential data obtained from osmotically perturbed and volume-static (control) cells in the absence and presence of Val. That there is no difference in the $V_m$ of the cells in the absence of Val, regardless of osmotic considerations, is seen by the first value in each pair. This finding was unexpected in light of the fact that in the presence of Val, unidirectional K flux across the *Amphiuma* red blood cell increases by only a factor of 3–4, yet produces rather large hyperpolarizations of the membrane. In contrast, during volume regulation, net cation fluxes increased from 10 to 100 times, yet produced no change in $V_m$. The possible explanations are as follows: (a) During volume regulation the cation flux is via electrically silent pathways, or (b) during volume regulation the membrane conductance to cations and anions increase in parallel, such that the ratio of anion to cation conductance remains constant, and thus the membrane potential remains unchanged. The latter possibility seems unlikely because in the presence of Val $V_m = -40$, regardless of cell volume status (Table II). If indeed both $G_{anion}$ and $G_{cation}$ increase during volume regulation, then $G_{K}^{Val}$ would be a smaller fraction of $G_m$ of volume-regulating cells, and Val would therefore be expected to be less effective in charging the membranes of the volume-regulating cells. That the membranes of volume-regulating and volume-static cells are hyperpolarized to the same extent in the presence of Val is a strong argument in support of the hypothesis that the large increases in cation fluxes, characteristic of volume-regulating cells, are via electrically silent pathways. Finally, it is important to note that the data presented in Table II were also

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1 Hoffman, J. F. Personal communication.
confirmed using the voltage-sensitive dye Di-S-C3(5) (Sims et al., 1974; Kregenow, 1977). Although no attempt was made to calibrate the dye, the fluorescence levels were the same for control and volume-regulating cells. Furthermore, the addition of Val caused the fluorescence of all groups, regardless of volume status, to decrease to the same level. The correspondence of the data obtained by direct impalement and from the voltage-sensitive dye is further evidence of the validity of the electrical measurements.

To further test the hypothesis that cation fluxes during volume regulation are electrically silent, cells were preincubated in 1 R plus DIDS (Knauf et al., 1977) for 1 h and subsequently suspended in hyposmotic medium. Because DIDS is a potent inhibitor of anion transport by red cells (Knauf et al., 1977) and if the K associated with RVD fluxes via a current-carrying pathway, then the membrane should hyperpolarize during RVD in the presence of DIDS. Direct measurement of $V_m$ during RVD in the presence of DIDS gave $V_m = 24 \pm 2$ mV ($n = 60$), whereas $V_m$ values for control volume-static or DIDS-free cells were $26 \pm 1.3$ ($n = 50$) and $24 \pm 1.9$ ($n = 80$), respectively. Fig. 5 depicts the ion and water content of cells treated as described above upon which flux studies were performed in parallel with electrical measurements. If net K loss during RVD is through a current-carrying pathway, then no net H2O loss and minimal net K loss would be expected under conditions where GCI is blocked by DIDS, because $V_m$ should move toward or to $E_K$, decreasing the net force acting to promote K loss. Although the data show no net H2O or Cl loss, there are large net Na and K fluxes associated with osmotic swelling in the presence of DIDS. Inasmuch as the membrane potential remains unchanged, it must be concluded that the exchange of K for Na is not coupled through the membrane potential but through some other event(s) associated with the treatment described above. The addition of $10^{-3}$ M amiloride to cells treated identically to those described in relation to Fig. 5 resulted in 100% inhibition of net Na uptake and 80% inhibition of net K loss relative to amiloride-free cells (data not shown). While K fluxes are insensitive to amiloride under all other circumstances studied, Na fluxes during RVI are exquisitely sensitive to amiloride. Thus, osmotic swelling in the presence of DIDS (absence of a functional anion exchange) results in activation of an Na-flux pathway, which, by virtue of its amiloride sensitivity, I believe to be that normally activated by osmotic shrinkage and responsible for RVI.

The Nature of Volume-regulatory Ion-Flux Pathways

The data in Table II and observations associated with Fig. 5 strongly suggest that the ion fluxes associated with volume regulation are electrically silent. Furthermore, the data in Fig. 5 suggest a coupling of Na and K during RVD in the absence of anion transport, and the amiloride sensitivity of the net Na and K fluxes indicates that the Na fluxes under such conditions move by the same pathway as that activated during RVI. On the basis of these data, I hypothesized that alkali metal–ion fluxes characteristic of volume regulation occur in exchange for H, whereas the chloride fluxes characteristic of the volume-regulatory response exchange for HCO3. Such a scheme is consistent
with observed ion fluxes during volume regulation in that net alkali metal-ion and chloride fluxes proceed in the same direction. The hypothesis is also consistent with the electrically silent nature of these fluxes, as well as with the activation of net Na uptake under conditions associated with the data in Fig.
5. That is, if alkali metal-ion flux occurs in exchange for protons, then the coupling between Na and K during RVD in DIDS (see Fig. 5) may be via protons. Yet, because the driving force for Na through such a pathway is normally directed into the cell, I further hypothesized that increased cell [H] as a result of the K-H exchange activates the Na-H pathway. The hypothesis that cellular pH changes activate the alkali metal-ion flux pathways responsible for volume regulation is consistent with predictions based on the Donnan theory in that cell pH should decrease due to osmotic shrinkage and increase as a result of swelling.

The Role of Protons in the Volume-regulatory Response

Experiments were performed to test the hypothesis that changes in cell pH activate ion fluxes responsible for cell-volume regulation (Table III). These data show that transfer of cells to more acid medium results in net Na uptake. Furthermore, when cells are osmotically swollen by transfer to hypoosmotic medium at pH 6.65, net Na uptake is activated, whereas net K loss is depressed relative to cells identically treated at pH 7.65. In contrast, osmotic shrinkage by transfer of cells from control medium at pH 7.65 to hypertonic medium at pH 8.65 results in net Na uptake that is <50% of that observed when cells are shrunk in medium at pH 7.65. If the stimulus for ion fluxes responsible for RVI is an acid shift in the cell interior occurring as a result of cell shrinkage, it should be possible to depress the volume-regulatory response to cell shrinking by minimizing acid shifts in intracellular pH. Toward this end, cells were shrunk in medium made hyperosmotic by the addition of sodium p-aminohippurate (middle curve in Fig. 6), [Cl] being unchanged relative to isoosmotic control medium. Shrinking cells in medium containing an impermeant anion should result in a smaller acid shift in the pH of the cell interior (pHi) than shrinking cells in medium made hyperosmotic by the addition of NaCl. Consistent with the hypothesis that acid shifts in pHi are responsible for activation of the Na-flux pathway during RVI, the data in Fig. 6 show that cells shrunk in medium made hyperosmotic by sodium p-aminohippurate

| Medium            | pH   | ΔNa<sup>+</sup> | ΔK<sup>+</sup>  |
|-------------------|------|----------------|----------------|
| Isotonic (245 mosM) | 7.65 | 5±2 (4)       | -6±3 (4)       |
| Isotonic (245 mosM) | 6.65 | 21±4 (4)      | -5±2 (4)       |
| Hypotonic (183 mosM) | 7.65 | 6±3 (4)       | -44±4 (4)      |
| Hypotonic (183 mosM) | 6.65 | 26±4 (4)      | -29±3 (4)      |
| Hypertonic (319 mosM) | 7.65 | 31±5 (4)      | -6±2 (4)       |
| Hypertonic (319 mosM) | 8.65 | 14±2 (4)      | —              |

These data were obtained in the same way as those depicted in Figs. 1 and 2, the experimental difference being that media at a pH other than 7.65 were used. The data are presented as mean ± SEM, with the number of observations in parentheses.
(middle curve) gain less than one-third the Na taken up by cells shrunk in medium in which osmolarity increases are produced by the addition of NaCl (top curve). These data are in support of the hypothesis that acid shifts in pH serve as the signal for Na uptake during RVI.

If net alkali metal-ion flux is countercoupled to protons, then, to the extent that the rate of the turnover of the alkali metal ion-proton exchange exceeds that of the Cl-HCO₃ exchange, medium pH changes associated with volume regulation should occur. That is, during RVD the medium should become more alkaline as a result of cellular H uptake in exchange for K, whereas during RVI, cellular H extrusion in exchange for Na should lead to medium acidification. The results of such an experiment are presented in Fig. 7. These data show that the medium pH changes in a fashion consistent with the proposed alkali metal-proton exchanges and that the addition of amiloride to cells undergoing RVI prevents medium acidification. These data agree with the hypothesis that the net alkali metal-ion fluxes characteristic of cell-volume regulation occur in exchange for protons.

Support for the hypothesis that a K-H exchange is responsible for net K loss during RVD was obtained from studies of RVD in the presence and absence of Val. In five paired experiments net K loss during RVD in the
absence and presence of Val was $40 \pm 3$ and $52 \pm 4$ mmol/kg dcs in 90 min, respectively. Direct measurement of $V_m$ of Val-treated cells during RVD yielded a value of $45 \pm 3$ mV ($n = 80$). The corresponding value for Val-free cells was $24 \pm 2$ mV (50 cells). $E_K$ was 78 mV in both cases. Furthermore, the addition of Val to volume-static cells resulted in a net K loss of $12 \pm 3$ mmol/kg dcs in 90 min. Given that Val results in a membrane hyperpolarization of 21 mV, if net K loss during RVD were via a conductance pathway, the driving force favoring K loss during RVD and after the addition of Val would
be decreased by 40%, and thus net K loss during RVD would be expected to decrease. That net K flux does not decrease but actually increases by the amount observed in studies of Val-treated, volume-static cells argues for K loss via an electrically silent pathway. Similarly, Val-induced membrane hyperpolarizations are without effect upon net Na fluxes during RVI. That net alkali metal-ion fluxes associated with cell-volume regulation are unaltered by Val-induced changes in the membrane potential argues in favor of electrically silent transport.

The Role of HCO₃ in RVI
If net KCl loss during RVD and NaCl uptake during RVI are the result of alkali metal–proton exchanges working in parallel with the anion exchange, then there are four ions that would be expected to determine the kinetic behavior of net ion fluxes during volume regulation. Because the experiments thus far presented were performed in medium buffered with imidazole in room air, the normal [HCO₃] is only a fraction of 1 mmol/liter. Thus, of the four ions that must traverse the membrane during volume regulation, HCO₃ availability is most limited and thus probably is the rate-limiting substrate for the overall reaction. Accordingly, experiments similar to that depicted in Fig. 2 (RVI) were performed but in a medium gassed with 95% O₂–5% CO₂ buffered with HCO₃ at total [HCO₃] = 42 mM (pH 7.65). As can be seen upon comparison of the data presented in Fig. 8 with that in Fig. 2, the presence of 42 mM HCO₃ increased the initial rate of Na uptake from 50 to 140 mmol/kg des in 1 h. In contrast to the experiments illustrated in Fig. 2, in which the cells required about 2 h for the volume-regulatory response, cells in high-HCO₃ medium regulate volume in about 30 min. Clearly, the presence of high HCO₃ in the medium has a strong stimulatory effect upon the net Na movements associated with RVI.

The Correspondence between Net Na Uptake and H Loss during RVI
The most compelling evidence for the electrically silent nature of Na uptake during RVI and the role of protons in the overall response is presented in Fig. 9. These data were obtained from cells that were osmotically shrunk in the presence of DIDS after a 1-h preincubation in DIDS-containing isosmotic medium. The cells regulated volume, gaining Na (60 mmol/kg des in 90 min) and H₂O, yet there is no net anion flux. Furthermore, when the experiment was repeated, net Na flux and medium pH were measured, and the suspension was back-titrated from 7.49 to 7.65, it was observed that net Na uptake was 47 ± 2 mmol/kg des in 90 min while 40 ± 3 mmol H/kg des were added to the suspension medium. These observations support the hypothesis that net Na fluxes characteristic of RVI are electrically silent and proceed via a Na-H exchange.

DISCUSSION
The present communication is an attempt to understand cell-volume regulation by *Amphiuma* red blood cells based upon analyses of ion fluxes and associated electrical phenomena. Although the measurement of net ion flux is
Figure 8. RVI in bicarbonate-buffered medium. These data were obtained from cells treated as described in Fig. 2, except the data were obtained from cells shrunk in a HCO₃-buffered medium. The bicarbonate concentration was 42 mM, pH 7.65.

A well-established, straightforward technique, the measurement of electrical characteristics of small cells is somewhat more complicated. As has been reported in numerous papers and reviews of the subject (Lassen, 1971; Lassen and Vestergaard-Bogind, 1976; Lassen et al., 1978; Lassen and Rasmussen,
Figure 9. Net Na⁺ uptake by cells osmotically shrunk in DIDS-containing medium. The data were obtained from cells preincubated in DIDS (10⁻⁴ M) for 1 h before shrinkage in DIDS-containing medium. With the exception of the DIDS treatment, these cells were studied under the experimental conditions described in Fig. 2.

1977), the measured potentials are transient due to poor electrical sealing between the cell membrane and a glass microelectrode. Therefore, it is necessary to measure the peak value of the rapidly changing potential. Although this is less than ideal, the values of such peak potentials agree with theory (see Fig. 4 and Table I) as well as with potential values inferred from
voltage-sensitive dyes (Hoffman and Laris, 1974). On the basis of measured ion fluxes and associated potential changes I calculate that the membrane resistivity \( (R_m) \) is on the order of \( 10^{6} \Omega \text{ cm}^2 \) (see Validation of Electrical Methods above), whereas, the input resistance \( (r_m) \) is close to \( 10^{10} \Omega \text{ cm}^2 \) (see also Lassen et al. [1978] and Hoffman et al. [1979]). Thus, the inability to obtain a tight electrical seal is probably attributable to the value of \( r_m \) and not to an inordinately high value for the leakage resistance \( r_L \).

In support of this hypothesis is the observation that in the presence of a 1 \( \mu \text{M} \) protonionophore TCS \( R_m \) is decreased, and the membrane potential decays over minutes rather than milliseconds. That TCS produces large decreases in \( R_m \) can be inferred from the fact that in the presence of TCS, Val does not produce membrane hyperpolarization (i.e., \( G_H^{\text{TCS}} \gg G_K^{\text{Val}} \)). Furthermore, in the presence of TCS the measured value of \( R_m \) is \( 1.6 \times 10^3 \Omega \text{ cm}^2 \), a value typical of many cells used in electrophysiological studies. Thus, while it does not appear to be possible to impale *Amphiuma* red cells and record a stable potential by conventional means, the available electrical and flux information suggests that the peak potential can be a reliable index of \( V_m \). Given that measured values of \( V_m \) are less than ideal, the approach employed was to design flux experiments that would test hypotheses suggested by the electrical data, thus avoiding conclusions based solely upon measured values of \( V_m \).

Figs. 1 and 2 show that *Amphiuma* red blood cells regulate volume after swelling and shrinkage, respectively. These data also show that cell-volume regulation proceeds due to net alkali metal ion–chloride fluxes and osmotically obliged \( \text{H}_2\text{O} \) flow. Although these net fluxes are at least 10-fold greater than those observed in the steady-state (volume-static) cells, there is no change in cell membrane potential associated with the volume-regulatory response (Table II). However, Val-induced net K fluxes much smaller than those observed during volume regulation produce a 20–30-mV hyperpolarization of the *Amphiuma* red cell membrane. The Val-induced hyperpolarization occurs even during volume regulation (Table II), when, due to the large net ion fluxes (assuming that these fluxes are via current-carrying pathways) associated with RVI and RVD, it is expected that \( R_m^{\text{Val}} \) should be a smaller fraction of the membrane current and therefore less effective in altering \( V_m \). That during volume regulation Val produces the same membrane hyperpolarization as observed in studies of volume-static cells strongly suggests that the ion fluxes characteristic of the cell-volume-regulatory response are electrically silent. Further evidence for the electrically silent nature of the ion fluxes was obtained from the experiments presented in Figs. 5 and 9, which depict ion and water content of cells osmotically swollen and shrunk in the presence of DIDS. If the net Na and K fluxes associated with volume regulation were via conductive pathways, then activation of those pathways when the Cl flux pathway is inhibited by DIDS should lead to large membrane hyperpolarization during RVD, and depolarization during RVI. Yet, when such experiments were performed the membrane potential remained unchanged. If the net Na flux depicted in Fig. 9 were via a current-carrying pathway, given the surface area over which the flux occurred (\( 2.4 \times 10^7 \text{ cm}^2 \)) and a reason-
able value for the membrane capacitance (1 μF/cm²), a 245-V (an extraordinary value) change in $V_m$ is predicted. Yet, direct measurement of $V_m$ reveals no change. Inasmuch as the above value is about three orders of magnitude greater than that at which dielectric breakdown is expected, it is reasonable to conclude that net Na flux associated with RVI is electrically silent. Similarly, the electrically silent nature of ion fluxes during RVD is illustrated by flux and electrical measurements obtained during RVD in the presence of DIDS. Although such experiments are characterized by large net exchanges of cell K with medium Na (Fig. 5), coupling between K and Na is not via changes in $V_m$, because simultaneous measurements of $V_m$ (1–90 min postswell) revealed no change. Furthermore, the net movements of both K and Na are amiloride inhibitable. The data discussed thus far can be adequately explained by a model (Fig. 10) that has alkali metal ions exchanging with H in parallel.
with, and functionally coupled to, the Cl-HCO₃ exchange via H. Consistent with the proposed model is the observation that net Na uptake associated with RVI is exquisitely sensitive to HCO₃ concentration. As shown in Fig. 8 the maximum rate of Na uptake during RVI in HCO₃-buffered medium ([HCO₃]ₗ, 42 mM) is three times that observed in the imidazole-buffered medium. Studies of RVI in nominally CO₂-free medium result in a maximum rate of Na uptake on the order of 5 mmol/kg dcs × h (data not shown).

The electrical and ion-flux data support the proposed model and would be difficult to rationalize by any thermodynamically distinguishable model. In this regard, a model that is the thermodynamic equivalent of that described above is alkali metal-HCO₃ cotransport, with HCO₃ being recycled via the anion exchange. The two models are kinetically distinguishable on the basis of the HCO₃ sensitivity of net Na and K fluxes during RVD in the presence of DIDS (RVD plus DIDS) (see Fig. 5 and associated discussion). That is, it has been argued that the functional coupling between Na and K during RVD by DIDS-treated cells (Fig. 5) is due to the obligatory countercoupling of K and Na to H. If this is correct then net Na and K fluxes during RVD plus DIDS should be insensitive to the presence of HCO₃. If however, net alkali metal fluxes are cocoupled to HCO₃, then net Na and K fluxes during RVD plus DIDS should be inhibited by HCO₃ removal. Accordingly, cells were osmotically swollen in DIDS- and acetazolamide (Calbiochem-Behring Corp.) (5 × 10⁻⁴ M)-containing medium gassed with 95% O₂-5% N₂ (CO₂ free). The net Na and K fluxes in red cells treated as just described were indistinguishable from paired controls treated as those illustrated in Fig. 5 (RVD plus DIDS). Thus, the possibility that the alkali metal-ion fluxes responsible for volume regulation are cocoupled to HCO₃ is rejected in favor of the model presented in Fig. 10.

The data depicted in Fig. 5 show that osmotic swelling in the absence of the Cl-HCO₃ exchange results in activation of both the K-H and Na-H exchanges. Although an increase in cell H as a result of K efflux and H uptake would increase the driving force favoring Na entry, there is already a force of some 70 mV favoring cellular Na gain. Thus, I felt that increased cellular H may also exert a kinetic effect upon the amiloride-sensitive Na transport pathway. Because Donnan theory predicts acid shift in cellular pH due to swelling and alkaline shifts due to shrinkage, this possibility is attractive and consistent with experimental observations. The notion that changes in intracellular pH activate the net ion flux pathways responsible for volume regulation gains support from the data presented in Fig. 6 and Table III. The data in Fig. 6 show that by osmotically shrinking cells in medium in which [H]ₗ and [Cl]ₗ are unchanged relative to control medium and, therefore, minimizing decreases in pHᵢ, the rate and magnitude of net Na uptake associated with RVI are depressed relative to observations made on cells osmotically shrunk in medium in which [H]ₗ × [Cl]ₗ is increased relative to control medium. Similarly (as shown in Table III), osmotically shrinking cells in medium at pH 8.65 depresses net Na gain associated with RVI by 55% relative to cells identically treated at pH 7.65. The inhibitory effect of alkaline pH upon net
cellular Na uptake during RVI is not apparent, however, if [HCO₃⁻] is allowed to increase with pH, a fact attributable to the stimulatory effect of HCO₃⁻ upon net Na uptake during RVI. In this regard (data not presented in Table III), osmotic shrinkage in medium at pH 6.65 depresses net Na uptake relative to observations made in studies of cells shrinked at 7.65 or swollen at 6.65. That net Na uptake by cells undergoing RVD at 6.65 is greater than that observed during RVI at the same pH is explainable in terms of the model and the role of HCO₃⁻ in the overall response. That is, although acid shifts in pH may stimulate net Na uptake associated with RVI, if acidification is great enough to significantly decrease [HCO₃⁻], the rate of net cellular Na gain will be decreased. That net Na gain during RVD at pH 6.65 is greater than that observed during RVI at the same pH is consistent with the proposed model, because as a result of simultaneous operation of both the Na-H and K-H exchanges and (to the extent to which the turnover of the K-H exchange approaches that of the Na-H exchange) net Na flux will become independent of [HCO₃⁻]. Furthermore, that medium acidification increases net Na gain by cells in isotonic medium while depressing net K loss during RVD is in support of the proposal that changes in pH are important in activating the alkali metal ion-flux pathways responsible for volume regulation. The effect of pH upon ion flux is not thought to be a result of titration of a carrier because the pathways activated by ΔpH deactivate with time, even though pH is held constant. Moreover, activation of the alkali metal-ion fluxes appears to depend upon a change in pH and not on the absolute value of pH. That the Na-uptake pathway normally activated during RVI can be activated by swelling cells in acid medium argues against any direct effect of cell volume in the activation of the Na-flux pathway during RVI. Also of interest in this regard are the studies by Thomas (1977), who has shown that after acid shifts in the cell interior, the intracellular pH of snail neurons is regulated via an amiloride-sensitive Na-H exchange. Such a system for pH regulation in a red blood cell is clearly redundant, given the presence of the anion-exchange mechanism, but it would, regardless of where it is present, serve as both a pH- and a volume-regulating mechanism. That a H-activated, amiloride-sensitive Na-H exchange has elsewhere been demonstrated, lends credence to the hypothesis that changes in pH associated with osmotic perturbation serve as a signal for the alkali metal-H exchanges ultimately responsible for cell-volume regulation.

In summary, the data obtained by direct measurement of $V_m$ and ion fluxes are consistent with the hypothesis that ion fluxes responsible for cell-volume regulation are electrically silent. The effect of Val on the $V_m$ (Table II) of volume-regulating and volume-static cells is inconsistent with the relative magnitudes of the ion fluxes observed in the presence of Val and during volume regulation, if those associated with the latter occur via conductance pathways. Support for the electrically silent nature of volume-regulatory ion fluxes is obtained from experiments in which DIDS-treated, volume-regulating cells were studied. During RVD plus DIDS (Fig. 5) *Amphiuma* red blood cells exhibit large net K loss and Na gain with no change in $V_m$. During RVI plus
DIDS (Fig. 9) cells gain a large amount of Na, in the absence of compensatory fluxes of K or Cl, again with no change in \( V_m \). While this speaks to the electrically silent nature of the volume-regulatory ion fluxes, other studies identify H as the counterion for alkali metals, and HCO\(_3^-\) as that for Cl. The involvement of H is inferred from medium pH changes that occur during volume regulation (Fig. 7), and the correspondence between cellular Na gain and H extrusion during RVI in DIDS provides quantitative evidence. Furthermore, the profound stimulatory effect of HCO\(_3^-\) upon net Na fluxes during RVI (Fig. 8) would be difficult to rationalize by models thermodynamically distinguishable from that shown in Fig. 10. A further argument favoring the proposed model is that the counter ions for Na, K, and Cl cannot, if volume is regulated by changes in the number of intracellular osmotically active particles, exert an osmotic effect. The proposed model for coupled transport satisfies this criterion because it predicts net alkali metal–ion and chloride fluxes, with H\(_2\)CO\(_3\) cycled between cells and medium.\(^2\)

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