Further Studies of the Volume-regulatory Response of *Amphiuma* Red Cells in Hypertonic Media

*Evidence for Amiloride-sensitive Na/H Exchange*

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ABSTRACT When *Amphiuma* red cells are shrunken in hypertonic media, they return toward their original volume by gaining Na through an amiloride-sensitive pathway. As cells recover their volume during this volume-regulatory increase (VRI) response, acid is extruded into the medium. Medium acidification is correlated with cell Na uptake. Both medium acidification and cell Na uptake are blocked by 10⁻³ M amiloride or by replacing medium Na with K or choline. Perturbations that increase cell Na uptake (such as increasing medium osmolality) also increase medium acidification. As the medium becomes more acidic, the cells become more alkaline. These changes in cell and medium pH are increased if pH equilibration across the cell membrane is prevented by inhibiting the anion exchanger with SITS (4-acetamido-4'-isothiocyano-2,2'-stilbene disulfonic acid). The quantity of acid extruded by SITS-treated cells is the same as the quantity of Na gained, which strongly suggests 1:1 exchange of Na for H. Cell enlargement in SITS-treated cells results from the exchange of osmotically active Na ions for H ions that are not osmotically active when combined with cellular buffers. Previous evidence indicates that the normal VRI response involves an increase in the cellular content of Cl as well as Na. We show that SITS completely blocks net Cl uptake, which suggests that Cl enters via the anion exchanger. SITS also slows Na entry, presumably as a result of the above-mentioned increase in cell pH caused by SITS. We suggest that the initial event in the VRI response is net Na uptake via a Na/H exchanger, and that net Cl uptake results from secondary Cl/HCO₃ exchange via the anion exchanger.

INTRODUCTION

*Amphiuma* and duck red cells are two of the nucleated red cells known to regulate their volume in anisotonic media. After initially shrinking in hypertonic media...
or swelling in hypotonic media, these cells return toward their original size with continued incubation. These adaptive changes in size result from ouabain-insensitive, volume-regulatory ion transport processes, which, once activated, alter the internal salt content and bring about shifts in cell water (reviewed in Kregenow, 1981; Cala, 1983; Siebens, 1985).

The transport process activated when shrunken *Amphiuma* red cells gain NaCl and enlarge in hypertonic media is of particular interest. As reported in the preceding paper (Siebens and Kregenow, 1985), Na uptake takes place through an amiloride-sensitive pathway that shares several features with the Na uptake process at the mucosal border of some epithelia. These similarities have led to the suggestion that the Na transporter may be similar or identical in both *Amphiuma* red cells and these epithelia (Kregenow, 1981; Siebens and Kregenow, 1985).

In duck red cells, on the other hand, the analogous transport process activated by hypertonic media differs in several important ways from that in *Amphiuma* red cells. It is blocked by furosemide (Kregenow et al., 1976), but not by amiloride (Schmidt and McManus, 1977). In addition, the uptake process involves three electrolytes, two cations (Na and K) and an anion (Cl or Br). All three ions must be present in the bathing medium for enlargement to take place: a sufficient decrease in the medium concentration of any one prevents the simultaneous uptake of all three (Kregenow et al., 1976; Schmidt and McManus, 1977; Kregenow, 1978; Kregenow and Caryk, 1979; Haas and McManus, 1982).

The results of experiments designed to explore why Cl was limiting in the duck red cell response indicated that Cl and Na and K were transported together in what appears to be an electroneutral translocation step (Kregenow and Caryk, 1979). Two approaches proved useful in these duck red cell experiments. In the first, we replaced the Cl on both sides of the membrane with another anion. In the second, we obtained simultaneous measurements of Na, K, and Cl when the red cell anion exchanger was blocked with the disulfonic stilbene derivative SITS. By blocking Cl movements associated with the anion exchanger, SITS permitted the measurement of the portion of the Cl flux that was coupled to the volume-regulatory Na and K fluxes. In this paper, we have applied these same two approaches to the study of the *Amphiuma* red cell VRI response. These studies show, in contrast to the results in duck red cells, that the amiloride-sensitive transporter in *Amphiuma* red cells acts as a Na/H antiport (or a Na/OH symport or some other equivalent pathway).

Our interest in these studies was stimulated by the results of microelectrode measurements by Cala (1980a, b) and ourselves (unpublished), which indicate that the *Amphiuma* red cell membrane potential does not change substantially during the volume-regulatory increase (VRI) response despite large cation flux changes. These findings suggest that ion movements in the *Amphiuma* red cell VRI response are electrically silent. Preliminary evidence that Na/H exchange is the probable mechanism of Na uptake during the *Amphiuma* red cell VRI response was published simultaneously by ourselves (Siebens and Kregenow, 1980) and by Cala (1980a).
METHODS

Media

The standard isotonic medium, medium A₁, differed slightly in composition from medium A of the preceding paper (Siebens and Kregenow, 1985). It contained 19 mM NaHCO₃, 3 mM KCl, 1.8 mM Na₂HPO₄, 0.3 mM NaH₂PO₄, 0.6 mM MgSO₄, 0.5 mM CaCl₂, 5.6 mM glucose, and, on the average, 92 mM NaCl. The pH of this solution was maintained at 7.7 by gassing with 98% O₂/2% CO₂. As with medium A (see previous paper), the osmolality of medium A₁, as well as all other isotonic solutions, was adjusted to within 4 mosmol of the osmolality of the animal's plasma (range 204–225 mosmol) by varying the NaCl concentration.

The five solutions used in the anion substitution experiments differed from medium A₁ in that KCl was replaced with KHCO₃, CaCl₂ was omitted, and NaHCO₃ was replaced with 15 mM Tris HEPES; if the solution was Cl-free, NaBr, NaI, NaNO₃, or NaSCN isosmotically replaced NaCl. Hypertonic versions of these solutions (350 mosmol) contained additional NaCl, NaBr, NaI, NaNO₃, or NaSCN.

In experiments in which we monitored extracellular pH and permitted the pH to change, we used minimally buffered media. These media were modifications (see figure legends) of medium I, the minimally buffered isotonic solution. The composition of medium I was: 2 mM KCl, 1 mM KHCO₃, 0.6 mM MgSO₄, 0.5 mM CaCl₂, 5.6 mM glucose, and, on the average, 110 mM NaCl. With the exception of the pH 8.1 solution in Fig. 2, the initial pH of these solutions was set at 7.7 by the addition of <1 mM Na₂HPO₄. In experiments in which extracellular pH was monitored but the pH was kept constant, medium II or some modification of it was used. Medium II was the same as medium I except that it was buffered by 15 mM Tris Cl (Tris Cl having replaced an equivalent amount of NaCl). Hypertonic versions of medium I and medium II (350 mosmol) contained additional NaCl. To vary the Na concentration of medium II or hypertonic medium II, we isosmotically replaced some or all of the NaCl with choline Cl.

In experiments in which extrusion of acid from the cells was quantified, a manual pH stat was used to maintain the pH of modifications of medium II at pH 7.7. The pH stat released small quantities of NaOH, which resulted in an increase in medium Na of at most 1 mM.

Experimental Procedures

The procedures described in the previous study for preparing cells were followed whenever possible. Amphiuma red cells were washed three times with medium A₁ and preincubated in medium A₁ (hematocrit 10%) for 1–2 h. The cells were then washed three times with an isotonic solution that was similar in composition to the experimental medium (for example, cells to be incubated in the hypertonic Br medium were washed with the isotonic Br medium). Experimental flasks were either stoppered or left open. All incubations were performed at 23–25°C.

Cells were treated with SITS by incubating them continuously with 0.12 mM SITS for a period starting before and lasting through the experimental period. Cells were first exposed to SITS for 0.5 h while they were preincubated in the bicarbonate-buffered medium A₁. They were then washed two to three times with a chilled isotonic wash solution containing SITS before being introduced to a SITS-containing experimental solution. SITS was dissolved in the wash and experimental media shortly before use, but was added as a solid to the suspension of preincubating cells and quickly dissolved by swirling the flask. Throughout the procedure, solutions containing SITS were either kept
in the dark or exposed to yellow light. This regimen, as will be shown, blocked \( \text{Cl} \) movements without appreciably affecting \( \text{Na} \) and \( \text{K} \) movements. SITS was used instead of DIDS \( (4,4'-\text{diisothiocyanato-2,2'-stilbene disulfonic acid}) \) because we could not find a suitable DIDS treatment regimen that did not result in a sizable increase in the \( \text{Na} \) permeability of control cells.

To monitor extracellular \( \text{pH} \) during the experimental period, we recorded the \( \text{pH} \) continuously on an Aminco (American Instrument Co., Silver Spring, MD) recorder using Radiometer (Westlake, OH) glass \( \text{pH} \) electrodes connected to a Radiometer \( \text{pH} \) meter. Washed cells were added to 3 cc of experimental medium, which resulted in a hematocrit of 3–5%. The suspension was gently stirred with a small stirring bar. Within a few seconds, the \( \text{pH} \) electrode was submerged in the cell suspension and the \( \text{pH} \) was monitored. A manually controlled switching device permitted more than one experimental vial to be monitored simultaneously. \( ^{24}\text{Na} \) or \( ^{22}\text{Na} \) was added immediately after the cells to measure \( ^{24}\text{Na} \) or \( ^{22}\text{Na} \) uptake. At the conclusion of the experimental period, the entire radioactive cell suspension minus an aliquot for hemoglobin determinations was measured volumetrically and then centrifuged. The \( ^{24}\text{Na} \) or \( ^{22}\text{Na} \) content of the packed cells was then measured to calculate the tracer \( \text{Na} \) uptake (Siebens and Kregenow, 1985). The \( [^{14}\text{C}]\) inulin method was used to correct the centrifuged cell sample for trapped extracellular \( ^{24}\text{Na} \) (Siebens and Kregenow, 1985). The quantity of cells present in the flask was calculated from the hemoglobin determination and the volume of the initial cell suspension.

The procedure for measuring cell \( \text{pH} \) after disrupting the cells by freezing and thawing three times was similar to that used by Funder and Wieth (1966) in their study of human red cells. The centrifuged cell pellet was frozen with a slurry of dry ice and alcohol and thawed with a warm water bath. After the third thaw, the disrupted cell pellet was quickly brought to room temperature and the \( \text{pH} \) was measured by submerging the entire tip of a Radiometer \( \text{pH} \) electrode in the residue of disrupted cells. The procedure lasted 1 h and, as determined by light microscopy, disrupted 100% of the cells.

The procedures for measuring hemoglobin, \( \text{Na} \), \( \text{K} \), and \( \text{Cl} \) content, \( ^{24}\text{Na} \) or \( ^{22}\text{Na} \) uptake, cell water, cell specific gravity, and trapped extracellular \( \text{Na} \) using \( [^{14}\text{C}]\) inulin were described in the preceding paper (Siebens and Kregenow, 1985).

**Materials**

\( \text{Na} \) SITS was obtained from Aldrich Chemical Co., Cleveland, OH. The sources of \( ^{22}\text{Na}, ^{24}\text{Na}, [^{14}\text{C}]\) inulin, ouabain, amiloride, and choline \( \text{Cl} \) were noted in the preceding paper (Siebens and Kregenow, 1985).

**RESULTS**

In the preceding paper (Siebens and Kregenow, 1985), we presented evidence that volume recovery by osmotically shrunken *Amphiuma* red cells results from a net \( \text{Na} \) gain that can be blocked by amiloride. In the course of these studies, measurements of the medium \( \text{pH} \) indicated that a transmembrane shift in hydrogen or hydroxyl ions also develops during the response. Fig. 1 demonstrates this medium \( \text{pH} \) change. To readily detect \( \text{pH} \) changes, cells were incubated in the nearly buffer-free medium 1, either isotonic or hypertonic. Medium acidity increases dramatically in the hypertonic medium containing cells undergoing the VRI response. The time course of medium acidification is the same as the time course of \( \text{Na} \) uptake, including an initial "delay period" (Siebens and Kregenow, 1980, 1985; Cala, 1980b). Once cells stop enlarging and approach their original
volume (39 min in this experiment), the rate at which the medium accumulates hydrogen ions subsides and matches that of control cells. The rapid fall in medium pH is only observed if cells gain Na and H₂O and enlarge. Accordingly, there is no significant acidification above control levels if cells incubated in hypertonic media are treated with 10⁻³ M amiloride (bottom of Fig. 1). The response of cells in isotonic media with or without amiloride is shown as a control.

Fig. 2 provides additional evidence that the acidification process is an integral part of the VRI response of Amphiuma red cells. Experimental perturbations that

Figure 1. Time course of medium pH changes during the VRI response: the effect of amiloride. Cells from a single animal were divided into two equal portions. One portion was treated with SITS as described in the Methods (results are shown in Fig. 4), while the other was processed using a similar regimen but with SITS-free solutions. After the third wash, the cells of each portion were subdivided into four equal fractions; each fraction was then added to an experimental solution that was either isotonic or hypertonic (350 mosmol) medium I. All experimental media contained 10⁻⁴ M ouabain and either no amiloride or 10⁻³ M amiloride. We monitored extracellular pH as a function of time and also measured ⁴²Na uptake and the total number of cells in each flask (see Methods). The initial pH was 7.7. The results of the latter two measurements (not shown) confirmed that (a) the cells in each flask exhibited the expected Na transport response, and (b) all flasks contained the same number of cells.

calculate or change the rate of Na uptake (Siebens and Kregenow, 1985) have a similar effect on the rate at which the change in medium pH takes place. Curve 1 of Fig. 2A serves as a reference and shows the response of cells incubated in the usual hypertonic medium I. In the response shown in curve 2, we first preincubated cells in the usual hypertonic solution with 10⁻³ M amiloride for 25 min and then washed away the amiloride. Pretreatment with amiloride under these conditions caused the delay period to disappear: the maximal rate of acidification (Fig. 2A)
and Na uptake (Siebens and Kregenow, 1985; unpublished) was observed as soon as amiloride was removed. In curve 3, the extracellular pH was raised from 7.7 to 8.1 by adding NaOH, while in curve 4 the tonicity of the hypertonic bathing solution was increased by 50 mosmol. Both of these perturbations shortened the delay period and increased the maximal rate at which the acidification developed.

**Figure 2.** Effects of pH, osmolality, amiloride, and cation substitution on the VRI response. Preincubated cells from a single animal were divided into six equal fractions. Each fraction was then washed with either isotonic medium I or an isotonic medium similar to the experimental medium (e.g., K substituted for Na) before being divided in half and added to one of several hypertonic experimental solutions that contained either 10^-3 M amiloride or no amiloride. All the hypertonic solutions had an osmolality of 350 mosmol except the two 400-mosmol solutions. To obtain the LiCl, KCl, and choline Cl solutions, all of the NaCl in isotonic or hypertonic medium I was replaced with an isosmotic quantity of LiCl, KCl, or choline Cl, respectively. With the exception of the pH 8.1 solution, the initial pH was 7.7. Additional NaOH was added to medium I to obtain the pH 8.1 solution, while additional NaCl was added to the usual hypertonic medium I to obtain the 400-mosmol hypertonic solution. In the amiloride wash-off experiments, all of the cells from one of the original fractions were pretreated with 10^-3 M amiloride in hypertonic medium A (350 mosmol) for 25 min. These cells were then centrifuged and washed free of amiloride with 100 vol of chilled amiloride-free hypertonic medium I. Half of these cells were then incubated in hypertonic medium I containing 10^-3 M amiloride, while the other half were incubated in amiloride-free hypertonic medium I. Approximately 2 min elapsed between the beginning of the wash with amiloride-free hypertonic medium I and the introduction of cells to experimental media. In each experimental flask, we monitored extracellular pH as a function of time. As in Fig. 1, we also measured 22Na uptake and the total number of cells contained in each flask (results not shown).
in the same manner that they affected Na uptake (Siebens, 1983; Siebens and Kregenow, 1985). Finally, Fig. 2B shows that replacing the medium Na with Li had little effect on the pH response (compare curve 6 with curve 1 of Fig. 2A), while replacement of Na with K (curve 7) or choline (curve 8) blocked acidification. These results are consistent with the findings of the previous paper (Siebens and Kregenow, 1985) that Li can substitute for Na, but K and choline cannot. Note that in K and choline media, there is an amiloride-sensitive alkalization (compare curves 7, 8, and 9). This alkalization correlates with a small loss of cell Na that develops under these Na-free conditions (data not shown). Thus, net ion movements responsible for the acidification observed under high-Na conditions appear to be reversed when extracellular Na is removed.

It has long been recognized that Cl/HCO₃ exchange plays a critical role in pH equilibration in red blood cells (Jacobs and Stewart, 1942; Whittam, 1964; Hladky and Rink, 1977). In addition, the disulfonic stilbene derivative SITS has been shown to block anion exchange in red cells almost completely, blocking Cl self-exchange in human red cells by 99.9% (Knauf et al., 1977). In Fig. 3, we show experiments that verify the critical role of Cl/HCO₃ exchange in pH equilibration in Amphiuma red cells. In Fig. 3A, a small bolus of NaOH was introduced into an isotonic medium containing Amphiuma red cells at the point in time indicated by the arrow. This addition of NaOH caused the medium pH to rise sharply and then fall abruptly before reaching a new stable value more alkaline than the initial pH. The downward pH swing reflects the pH equilibration step across the membrane, when HCO₃ enters the cell in exchange for cell Cl. The HCO₃ then equilibrates with cell H, H₂O, H₂CO₃, and CO₂ (HCO₃ + H = H₂CO₃ = H₂O + CO₂). Because H also equilibrates with cellular buffers such as hemoglobin, the net effect of Cl/HCO₃ exchange is to allow cellular buffers to buffer medium pH changes. The actual rate at which pH equilibrates is more rapid than shown (half-time, ~0.2 s; Whittam, 1964): the recorded response is limited by the solution mixing time and delays inherent in the response of the electrode and recording apparatus. As in human red cells (Jacobs and Stewart, 1942), removing bicarbonate from the medium (B) slows the downward equilibration step appreciably. (It should be remembered that the solutions are not actually HCO₃-free since at this pH a small amount of medium HCO₃ is in equilibrium with dissolved CO₂ from air.) In C, anion exchange was slowed further by using the same HCO₃-depleted solutions but then replacing Cl on both sides of the membrane with SCN, an anion that the anion exchanger transports more slowly than Cl (Dalmark and Wieth, 1972; Dissing et al., 1982; Kregenow, F. M., unpublished observations). Finally, D shows that treating Amphiuma red cells with SITS prevented the downward pH shift and any apparent equilibration of hydrogen or hydroxyl ions across the membrane.

Fig. 4 shows the results of an experiment in which we examined the effect of SITS on the medium acidification observed during the VRI response. A comparison of the rate of medium acidification in Fig. 4 with that in Fig. 1 indicates

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1 Tracer efflux of SCN in SCN-loaded Amphiuma red cells is more than an order of magnitude smaller than tracer efflux of Cl in Cl-loaded cells at 4°C (unpublished observation).
that treatment of cells with SITS increased the rate of medium acidification severalfold. (Note that the scale of the ordinate is fivefold greater in Fig. 4 than in Fig. 1.) As with cells not treated with SITS (Fig. 1), medium acidification by SITS-treated cells in hypertonic media was blocked by amiloride (bottom of Fig. 4).

Experiments such as those shown in Figs. 1, 2, and 4 suggest that the Na gained during the VRI response might enter by exchanging for H, thereby causing medium acidification. The observation that SITS greatly increases medium acidification (Fig. 4) would then be explained as resulting from prevention of secondary equilibration of cell and medium pH by Cl/HCO₃ exchange. If the initial Na entry step is exchange of Na for H, one would predict that the intracellular pH would increase as the medium pH decreases because the cell is the source of the acid added to the medium. In addition, SITS would be expected
to increase cell alkalization as well as medium acidification by preventing pH equilibration. Because the buffer power of red cells is large, ~50 mmol/Lcell/pH unit (Whittam, 1964), millimolar quantities of Na entering for H would only be expected to change cell pH by a few hundredths of a pH unit.

The effects of SITS on amiloride-sensitive changes in intracellular ions and water were examined in cells undergoing the VRI response for 30 min. The extracellular pH was kept constant by using a well-buffered medium and a low (3–5%) hematocrit (see Methods). The results are shown in Table I. The initial value of cell pH, ~6.95, was the same in osmotically shrunken cells and in isotonic controls (see the legend to Table I). Column 1 indicates that cell pH became more alkaline during the VRI response and that SITS substantially increased cell alkalization. This is particularly noteworthy in that SITS-treated cells gained less Na than did untreated cells (column 2). The magnitude of the increase in cell pH for a given Na gain was more than twice as great in SITS-treated cells as in untreated cells. Perhaps the most striking result in Table I is that SITS almost completely inhibited net Cl uptake during the VRI response (column 4). Thus, the Cl normally gained during the VRI response appears to enter via the anion exchanger. Experiments with $^{36}$Cl indicate that $^{36}$Cl efflux from SITS-treated cells undergoing the VRI response was unaffected by amiloride (data not shown). These $^{36}$Cl findings and the findings in columns 2 and 4 in Table I suggest that Na and Cl move through different transporters during the

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Time course of medium pH changes during the VRI response of SITS-treated cells: the effect of amiloride. The protocol of this experiment was described in the legend of Fig. 1; cells from the same animal were used in the experiments of both figures. The only modification of the protocol of Fig. 1 was that cells in this experiment were exposed to $1.2 \times 10^{-4}$ M SITS both before and during the experimental period.
VRI response. Column 5 of Table I indicates that the cells underwent a net increase in cell Na + K + Cl content and therefore gained H₂O (column 6) in both the presence and absence of SITS. Volume-regulatory fluid movements are therefore not blocked by SITS and are not dependent on a net increase in cell Cl content. On the other hand, by blocking Cl uptake (column 4) and partially inhibiting Na uptake (column 2), SITS decreased by about half the amount of Na + K + Cl and H₂O gained in 30 min. Possible explanations for the inhibition of Na uptake by SITS will be described in the Discussion. Column 7 indicates that the amount of H₂O gained for a given increase in cell Na + K + Cl content

| SITS (1.2 × 10⁻⁴ M) | Increase in cell pH/30 min | Electrolyte change (mmol/Lcell/30 min) | Gain in cell H₂O (ml/Lcell/30 min) | Ratio of gain in cell H₂O to gain in Na + K + Cl |
|----------------------|----------------------------|----------------------------------------|-----------------------------------|-------------------------------------------------|
| +                    | 0.30±0.02                  | 38.8±3.2                                | 117±6                             | 2.10±0.16                                       |
| -                    | 0.18±0.02                  | 38.2±3.1                                | 117±6                             | 2.10±0.16                                       |

Cells from each animal were separated into two fractions. One fraction was treated with SITS (see Methods); the other was left untreated. In these experiments, bicarbonate-free isotonic medium II (KCl having replaced KHCO₃) served as wash medium, while the usual bicarbonate-containing hypertonic medium II served as the experimental medium. All experimental solutions contained 10⁻⁴ M ouabain. 1.2 × 10⁻⁴ M SITS was included in the wash and experimental media that were to be used with SITS-treated cells. After the third wash, each fraction was again divided in half and one of the two halves was incubated in experimental media containing 10⁻⁵ M amiloride. The other fraction was incubated in amiloride-free experimental media. Each experiment was performed in triplicate and the results were averaged. The changes in the various parameters were determined by subtracting the average values for cells at zero time from the values at 30 min and then subtracting the 30-min change in amiloride-treated cells from the corresponding change in cells not treated with amiloride. The pH of cells treated with amiloride did not change during the experimental period. The pH of untreated cells in hypertonic media (6.94 ± 0.01) at zero time was the same as that of isotonic control cells (6.94 ± 0.01, N = 5). Similarly, the pH of SITS-treated cells in hypertonic media (6.98 ± 0.02) at zero time was the same as that of isotonic control cells treated with SITS (6.98 ± 0.01, N = 5). In the table, all differences caused by SITS are statistically significant (P < 0.01), except that of column 7. To calculate the increase in cell H₂O on a volume basis (column 7), we used the amiloride-sensitive gain in percent cell H₂O (W/W) and the appropriate cell density determinations as described in the preceding paper (Siebens and Kregenow, 1985).
during the initial 30 min of the VRI response. Acid extrusion was measured with a pH stat technique (see Methods). It was found that SITS decreased Cl uptake by 12 ± 2 mmol/L onc/30 min and increased acid extrusion (i.e., decreased HCO₃⁻ loss) by 11 ± 2 mmol/L onc/30 min. These results are entirely consistent with secondary pH equilibration involving a 1:1 exchange of Cl for HCO₃⁻.

Fig. 5 depicts the results of experiments in which amiloride-sensitive Na and H movements were measured at various external Na concentrations. Equilibra-
Fig. 6 shows the results of experiments designed to examine whether the Amphiuma response has a specific Cl requirement. $^{24}\text{Na}$ uptake was measured in cells incubated in media in which all Cl was replaced with Br, I, SCN, or NO$_3$. As shown in Fig. 6, replacement of Cl with Br or NO$_3$ did not affect $^{24}\text{Na}$ uptake, while replacement with I or SCN appeared to partially inhibit Na uptake. With all anions tested, large Na movements were observed that could be inhibited by amiloride. Thus, the Amphiuma red cell VRI response does not require Cl.
DISCUSSION

The results indicate that the mechanism of Na entry in the *Amphiuma* red cell VRI response is amiloride-sensitive Na/H exchange (or its equivalent, such as Na/OH cotransport). Before the results supporting the concept of Na/H exchange in these cells are summarized, we present a model in Fig. 7 that depicts the ion transport pathways important in the *Amphiuma* red cell VRI response. These include the putative Na/H exchanger, the anion exchanger, and the Na/K pump. The Na/K pump is included because it keeps the intracellular Na low so that Na entry is energetically feasible. The lack of a direct effect of the Na/K pump on cell volume regulation in *Amphiuma* red cells was described in the previous paper (Siebens and Kregenow, 1985) and has also been described elsewhere (Cala, 1980b). Indeed, the Na/K pump was inhibited with ouabain in all of the experiments of the present study.

**Evidence for Na/H Exchange Followed by Cl/HCO₃ Exchange**

The following findings presented in the Results support the concept of amiloride-sensitive Na/H exchange in the VRI response. (a) Na (or Li) entry is associated with medium acidification (Figs. 1 and 2) and cell alkalization (Table I). (b) Amiloride (10⁻⁵ M) completely inhibits the pH changes associated with shrinkage-stimulated Na movements (Figs. 1 and 4). (c) If SITS is used to prevent equilibration of Cl and HCO₃ (and therefore H and OH) across the membrane, medium acidification (Fig. 4) and cell alkalization (Table I) are increased, and Na uptake equals acid extrusion (Fig. 5). The finding that Na uptake and acid extrusion are equal in SITS-treated cells over a wide range of extracellular Na concentrations (Fig. 5) is strong evidence that the stoichiometry of Na/H ex-
change is 1:1. Cala (1980b) has also presented evidence that the medium acidifies during the *Amphiuma* red cell VRI response, and that Na uptake and acid extrusion are equal when the anion exchanger in blocked. (d) The substitution of Br, I, SCN, or NO₃ for Cl does not substantially inhibit amiloride-sensitive Na uptake (Fig. 6), which indicates that the volume-regulatory Na gain does not require Cl. (e) Microelectrode measurements of the membrane potential, $V_m$, indicate that ion movements during the VRI response are electroneutral (Cala, 1980; Kregenow, F. M., unpublished data). As shown in the preceding paper (Siebens and Kregenow, 1985), values of $V_m$ estimated from Cl concentration ratios agree with the microelectrode values.

It should be pointed out that electrical coupling between Na and H via conductive Na and H channels is unlikely in these cells. Indirect evidence from microelectrode measurements of $V_m$ indicates that the H conductance of the membrane is too low to account for the Na movements observed during the VRI response (Cala, 1980b). Similarly, voltage-sensitive dye studies of the VRI responses of both dog red cells (Parker and Castranova, 1984) and lymphocytes (Grinstein et al., 1983) support the view that Na uptake results from electroneutral amiloride-sensitive Na/H exchange.

The results shown in Table I indicate that the Cl that normally enters during the VRI response (see also Table II in Siebens and Kregenow, 1985) enters via the anion exchanger. Net Cl uptake is almost completely blocked by inhibition of anion exchange with SITS. The conclusion that Cl normally enters via 1:1 exchange for HCO₃ (or OH) is strongly suggested by the findings that (a) changes in medium and cell pH observed in the normal VRI response are greatly exaggerated by SITS, and (b) SITS treatment results in a decrease in Cl uptake and an increase in acid extrusion (presumably because of decreased HCO₃ exit) that are equal in magnitude.

Returning to the model of Fig. 7, we will attempt to explain the findings of the present study. We will first consider the case in which anion exchange is prevented by SITS. First, Na enters in exchange for H. Removal of H from the cell then results in a disequilibrium in the cellular buffer systems, such as HCO₃ (i.e., $H + HCO_3 \equiv H_2CO_3 \equiv H_2O + CO_2$), and proteins, such as hemoglobin (i.e., $H + Hb \equiv HHb$). Because the intracellular buffer power is large, millimolar quantities of H can be released from the cellular buffers while maintaining $[H]$, at $\approx 10^{-7}$ M. This means that the osmotic content of the cell is increased by an amount equivalent to the Na gain, inasmuch as the decrease in the osmotic content caused by H loss is immeasurable. Thus, the cell enlarges by Na/H exchange alone, the source of almost all of the H being the intracellular buffers.

In the case in which the anion exchanger is not blocked, the disequilibrium in the HCO₃ buffer system caused by movement of H from the cell to the medium results in a decrease in HCO₃ outside the cell and an increase in HCO₃ inside the cell, so that the ratio $[HCO_3]/[HCO_3]_o$ is no longer the same as $[Cl]/[Cl]_o$. Cl and HCO₃ then re-equilibrate, almost entirely via the anion exchanger, until $[HCO_3]/[HCO_3]_o = [Cl]/[Cl]_o$. This re-equilibration of Cl and HCO₃ during volume recovery was discussed in the Discussion of the preceding paper and has recently been reviewed (Siebens, 1985). The key points are that the net Cl uptake will be smaller than the net Na uptake because of cellular buffering of part of
the Na/H exchange-induced pH change, and that the ratios $[\text{HCO}_3^-]/[\text{HCO}_3^-]_0$, $[\text{Cl}^-]/[\text{Cl}^-]_0$, and $[\text{H}^+]/[\text{H}^+]_0$ will all move toward unity, i.e., the cell will alkalize (Table I).

Another point briefly mentioned in the preceding paper (Siebens and Kregenow, 1985) is that similar effects on anion ratios and pH changes are expected during volume regulation by red cells regardless of the initial step for net cation uptake. For example, like the *Amphiuma* red cell VRI response, the duck red cell VRI response involves medium acidification and a shift of the Cl ratio toward unity (Kregenow, 1971, 1977). However, the initial step in the duck response is Na/K/Cl cotransport (Kregenow, 1981; Haas and McManus, 1982). In the duck response, part of the Cl that enters by Na/K/Cl cotransport then moves out through the anion exchanger in exchange for medium HCO$_3^-$ during anion re-equilibration. This contrasts with the *Amphiuma* red cell VRI response, in which Cl moves in through the anion exchanger. Thus, inhibition of anion exchange in the duck response increases net Cl uptake (by decreasing Cl loss) and prevents medium acidification (by preventing cellular HCO$_3^-$ uptake), i.e., the opposite of the findings of the present study. If anion exchange is not blocked, the final anion and H distributions in duck red cells and *Amphiuma* red cells following the VRI responses are expected to be similar. Thus, the changes in anion and H distributions observed during the normal *Amphiuma* and duck red cell VRI responses result from the redistribution of anions and H subsequent to volume-regulatory cation uptake (reviewed in Kregenow, 1981; Siebens, 1985).

Recall from Table I that SITS not only prevents net Cl uptake, but also decreases Na uptake. The mechanism of this inhibition of Na uptake is uncertain. A likely explanation is that the increase in cell pH caused by SITS (Table I) decreases the driving force for Na/H exchange relative to the driving force in cells not treated with SITS. The observed 0.12 pH unit difference in cell pH caused by SITS (Table I) represents a 32% decrease in $[\text{H}^+]$. In addition, the pH and Na gradients in the unstirred layer immediately adjacent to the membrane are undoubtedly smaller than those estimated from measurements of [Na] and [H] in the bulk medium and cytoplasm. The effect of SITS on the pH at the intracellular transport site may therefore be underestimated in Table I. Another mechanism whereby an increase in pH may inhibit Na/H exchange is interaction of H with a regulatory, or modifier, site. The rate of acid extrusion (Roos and Boron, 1981), specifically Na/H exchange (Aronson et al., 1982), is thought to be decreased by increases in cell pH in other systems, presumably via the interaction of H with a modifier site. The data from cells in pH 8.1 media (curve 3, Fig. 2) indicate that if an intracellular modifier site is present in these cells, it is not the dominant factor regulating Na/H exchange. When medium pH is increased from 7.7 to 8.1, cell pH will also increase by equilibration (Whittam, 1964). If a modifier site similar to that mentioned above were the key controlling factor, an increase in cell pH would decrease transport. However, an increase was observed, which indicates that other factors dominate any possible inhibitory effects of pH. Finally, SITS may directly inhibit Na uptake. This seems unlikely, inasmuch as SITS does not appear to inhibit Na/H exchange in other systems (Kinsella and Aronson, 1980; Boron and Boulpaep, 1983).

The partial inhibition of Na uptake by SCN and possibly I (Fig. 6) is not readily
explained in terms of the model of Fig. 7. The rate at which these anions are transported by the anion exchanger (Dalmark and Wieth, 1972; Dissing et al., 1982) is more than sufficient to equilibrate pH over the time periods used in the present study, so that a SITS-like effect is unlikely. Cala (1983) has also reported inhibition of the Amphiuma red cell VRI response with SCN, while Parker (1983a) has observed that SCN almost completely inhibits volume-induced Na/H exchange in dog red cells. In contrast to these findings, SCN does not appear to inhibit Na/H exchange in rabbit microvillus membrane vesicles (Kinsella and Aronson, 1980). NO3 has also been reported to be inhibitory in both dog (Parker, 1983a) and Amphiuma red cells (Cala, 1983), although we found no inhibition. The reasons for these differences in results remain to be determined. Recent evidence in glutaraldehyde-treated dog red cells suggests that SCN acts by inhibiting the activation of shrinkage-induced Na/H exchange rather than by inhibiting the transporter itself (Parker, 1984). It is possible that inhibition of activation also accounts for the inhibitory effect of SCN shown in Fig. 6.

It has been proposed that the mechanism responsible for triggering the VRI response is an assumed initial decrease in cell pH brought about by osmotic shrinkage (Cala, 1980b). We found that cell shrinkage does not result in an initial pH change (see the legend to Table 1), which suggests that the trigger mechanism is not a change in cell pH. A similar absence of an initial pH change has been observed in the lymphocyte VRI response (Grinstein et al., 1983).

Recall from Fig. 2 that increasing medium pH increases medium acidification. Increasing medium pH is also expected to cause the following three effects (Jacobs and Stewart, 1947; Whittam, 1964): (a) an increase in cell pH (caused by pH equilibration); (b) an increase in the transmembrane pH gradient (because the cell pH change will be less than the medium pH change); (c) a decrease in cell volume (caused by the Hamburger shift). If the rate of Na/H exchange in these cells is regulated by a modifier site similar to that described in renal microvillus vesicles (Aronson et al., 1982), increasing pH should decrease Na/H exchange and medium acidification. On the other hand, increasing the outwardly directed H gradient and decreasing the cell volume are both expected to stimulate Na/H exchange. The relative contributions of the pH gradient and the cell volume to the observed increase in acid extrusion remain to be determined. Similarly, the effects of medium and cell pH per se remain to be examined in these cells. It should be noted that our data are not consistent with the finding of Cala (1980b) that increasing medium pH (to pH 8.65 under experimental conditions different from those of the present study) decreases Na uptake during the VRI response. Further studies will be required to explain this difference in results.

A word of caution is perhaps appropriate with respect to measurements of intracellular pH in nucleated red cells. Unlike in human red cells, in which H and OH have been shown to be distributed passively (Funder and Wieth, 1966), studies of DMO (5,5-dimethyloxazolidine-2,4-diene) and ammonia distributions in chicken red cells indicate that the nucleus is more acidic than the cytoplasm (Bone et al., 1976). Other organelles, such as a few mitochondria, are also present in nucleated red cells (Kregenow, 1977). Thus, the cell pH values of the present
study must be considered approximations of the cytoplasmic pH values. However, the reported changes in cell pH are almost certainly in qualitative agreement with changes in cytoplasmic pH.

It should be mentioned that ion exchange results in energy transduction as part of the energy in the electrochemical gradient of the “driver” ion is transferred to the electrochemical gradient of the other ion. Thus, the energy that drives net Cl uptake during the VRI response is derived from the initial Na gradient established by the ATP-driven Na/K pump. The cascade of reactions is therefore Na/K exchange, Na/H exchange, equilibration of H with HCO₃ and OH, and, finally, exchange of HCO₃ for Cl.

**Na/H Exchange in Other Systems**

Evidence consistent with shrinkage-induced Na/H exchange has been presented for frog red cells (Palfrey and Greengard, 1981), dog red cells (Parker, 1983a, b, 1984; Parker and Castranova, 1984), Necturus gallbladder (Ericson and Spring, 1982), and lymphocytes (Grinstein et al., 1983). The role of Na/H exchange in cell volume regulation has been discussed in several recent reviews (Kregenow, 1981; Spring and Ericson, 1982; Cala, 1983; Grinstein et al., 1984; Siebens, 1985). A role of Na/H exchange in pH regulation has been demonstrated in mouse muscle fibers (Aickin and Thomas, 1977), crayfish neurons (Moody, 1981), and salamander proximal renal tubules (Boron and Boulpaep, 1983). Na/H exchangers have been identified in the apical membranes of a number of epithelia, including small intestine (Murer et al., 1976), renal tubules (Murer et al., 1976; Kinsella and Aronson, 1980, 1981; Schwartz, 1981; Warnock et al., 1982; Boron and Boulpaep, 1983), and gallbladder (Weinman and Reuss, 1982; Ericson and Spring, 1982). Na/H exchange has also been observed during fertilization in marine eggs (Johnson et al., 1976). For a further discussion of Na/H exchange in eukaryotic cells, the reader is referred to a recent review by Benos (1982). Na/H exchange has also been observed in bacteria (Lanyi, 1979).

**Conclusion**

The results presented in this paper and the preceding paper, as well as the findings of Cala (1980a, b), indicate that the initial event in the *Amphiuma* red cell VRI response is amiloride-sensitive Na/H exchange. Re-equilibration of anions and H via the anion exchanger then results in a net uptake of Cl, but blockage of this re-equilibration with SITS does not prevent shrinkage-induced Na uptake. Both Cl/HCO₃ exchange and cellular buffering minimize Na/H exchange-induced changes in cell pH. This permits the cell to dramatically increase its osmotic content without substantially altering cell pH.

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