Volume Regulation by Flounder Red Blood Cells in Anisotonic Media

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ABSTRACT   The nucleated high K, low Na red blood cells of the winter flounder demonstrated a volume regulatory response subsequent to osmotic swelling or shrinkage. During volume regulation the net water flow was secondary to net inorganic cation flux. Volume regulation after osmotic swelling is referred to as regulatory volume decrease (RVD) and was characterized by net K and water loss. Since the electrochemical gradient for K is directed out of the cell there is no need to invoke active processes to explain RVD. When osmotically shrunken, the flounder erythrocyte demonstrated a regulatory volume increase (RVI) back toward control cell volume. The water movements characteristic of RVI were a consequence of net cellular NaCl and KCl uptake with Na accounting for 75% of the increase in intracellular cation content. Since the Na electrochemical gradient is directed into the cell, net Na uptake was the result of Na flux via dissipative pathways. The addition of 10^{-4} M ouabain to suspensions of flounder erythrocytes was without effect upon net water movements during volume regulation. The presence of ouabain did however lead to a decreased ratio of intracellular K:Na. Analysis of net Na and K fluxes in the presence and absence of ouabain led to the conclusion that Na and K fluxes via both conservative and dissipative pathways are increased in response to osmotic swelling or shrinkage. In addition, the Na and K flux rate through both pump and leak pathways decreased in a parallel fashion as cell volume was regulated. Taken as a whole, the Na and K movements through the flounder erythrocyte membrane demonstrated a functional dependence during volume regulation.

INTRODUCTION

The phenomenon of cellular volume regulation is characterized by the adjustment of cell volume back toward original, steady-state volume after osmotic perturbation. The volume regulatory response has been described by a number of workers studying a variety of different vertebrate cell types (1-10). Volume regulation by vertebrate cells is the result of net inorganic cation flux and osmotically obliged water flow. One of the early investigations was performed by Fugelli using red blood cells of the European flounder *Pleuronectes flesus* (2). This author demonstrated that while volume regulation did occur in response to osmotic swelling, only one-eighth of the fluid transported could be linked to changes in the cellular content of ninhydrin-positive substances. The volume

regulatory response of duck erythrocytes was studied by Kregenow (4, 5). These cells were shown to rely almost solely upon net inorganic ion fluxes as a means of effecting net water flow during volume regulation. When swollen, the duck red cell decreases volume as a result of net KCl and water loss. In response to osmotic shrinkage the duck erythrocyte increases volume by gaining NaCl, KCl, and osmotically obliged water. Net K uptake accounts for 75% of the total cation gained by the duck erythrocyte during regulatory volume increase (RVI). Volume regulation by shrunken duck red cells was observed only under conditions of elevated external K.

The volume regulatory response of mouse leukemic cells (L5178Y) was studied by Roti Roti and Rothstein (10). The cells were observed to demonstrate both regulatory volume decrease (RVD) and RVI. As was the case with the duck erythrocyte, RVD was characterized by net loss of KCl and osmotically obliged water. When osmotically shrunken by transfer from control (325 mosM) to hypertonic media the cells did not demonstrate a volume regulatory response. If, however, after RVD in hypotonic medium (150 mosM) the cells were osmotically shrunk by resuspension in control (325 mosM) medium the cell volume was regulated back toward control values as a result of net KCl and osmotically obliged water uptake. Upon the basis of an analysis of net and unidirectional fluxes Roti Roti and Rothstein concluded that the ion fluxes responsible for water flow after osmotic swelling are the result of increased membrane permeability to K. After osmotic shrinkage the cells are thought to regulate their volume as a result of K uptake mediated by the pump. In contrast, unidirectional flux studies performed upon human red cells (8) suggest a net Na and K loss after swelling and net uptake of both Na and K after shrinkage. Since the unidirectional flux data predict net contragradient Na and K fluxes during RVD and RVI, repectively, it would seem that volume regulation by human erythrocytes is dependent upon metabolic energy (8).

The red blood cells of the winter flounder (*Pseudopleuronectes americanus*) were chosen for the present study. This study is intended to illustrate the dynamic changes in the Na and K transport pathways during cell volume regulation. Data will be presented demonstrating that, as in the systems described above, the red blood cells of the winter flounder regulate volume by adjusting intracellular Na and/or K content. The data will show that after osmotic swelling the flounder red cell loses K, presumably anion, and osmotically obliged water. Since these cells are of high K (130 mM/liter cell water), low Na (24 mM/liter cell water) type, the net K loss associated with RVD is passive. When flounder red cells are osmotically shrunk, they recover volume as a result of net Na, K, Cl, and water uptake. Na is the major cation accumulated, accounting for 75% of the total cation gained. Since Na moves into the cell down its electrochemical gradient during RVI, the net ion flux leading to net water flow appears to be passive. The net gain in intracellular K during RVI is inexplicable by passive means and probably represents cation rather than volume regulation.

This paper will present data demonstrating that the volume regulatory response of flounder red blood cells is a direct result of changes in permeability to Na and K. The behavior of the Na-K pump during the volume regulatory

response and its contributions will be discussed. In addition, evidence will be presented which suggests that during the volume regulatory response, transmembrane Na and K movements are, at least functionally, linked.

MATERIALS AND METHODS

General Protocol

Blood was drawn from adult winter flounder which had been captured and maintained in seawater at 10°C for no longer than 1 wk. Blood was taken from the caudal vein into a heparinized syringe and immediately centrifuged, and the plasma and buffy coat were removed by aspiration. The cells were then washed four times in 30 vol of solution A (Table I). Since the analysis of flounder plasma for total osmolarity gave a mean value of 340 mosM with a range of 150 mosM, it was considered necessary to preincubate cells overnight in solution A to assure that a steady state with respect to cell volume and ion content was reached before experimental treatment. All experiments were carried out at

TABLE I

EXPERIMENTAL MEDIA

Solution	A	B
	mM	mM
NaCl	170	100
KCl	3	3
CaCl$_2$	0.75	0.75
MgCl$_2$	1	1
Dextrose	5	5
Imidazole	3	3
penicillin (U)	10^6/liter	10^6/liter
Streptomycin sulfate	0.25 g/liter	0.25 g/liter
pH	7.95	7.95

All ringers were gassed with air saturated with H$_2$O at 5°C before use.

pH 7.95 and 10°C. After preincubation the cell suspension was split into experimental and control groups. The suspensions were then centrifuged, the supernate was removed by aspiration, and the resultant cell pellets were resuspended in known volumes of the desired solutions (Tables I and II). Samples were taken at known intervals by removal of 250-μl or 500-μl portions, which were transferred to preweighed siliconized Pyrex culture tubes (6 × 60 mm). The samples were centrifuged for 3 min at 20,000 g in a Clay Adams autocrit centrifuge (model 0571, head CT-2915, Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N. J.). The resultant cell pellets were immediately separated from the supernate by aspiration and both were stored for analysis. Before analytical treatment, the cell pellets were dried to constant weight (24-48 h) in the culture tubes at 80°C. The dried pellets were then extracted in 250 μl of 15 mM LiNO$_3$ (internal standard for flame photometry).

Chemical Analysis for H$_2$O, Na, K, and Cl

All cells taken for analysis of Na, K, Cl, or H$_2$O were suspended at a hematocrit of 8% in media containing tracer quantities of [^{14}C]polyethylene glycol([^{14}C]PEG) mol wt 4,000, which served as an extracellular space marker. This marker was chosen as a result of studies by Schmidt-Nielsen et al., (11). While there was some indication of marker

metabolism when inulin was used, [^{14}C]PEG appeared to function as a good marker of extracellular space. The extracellular marker was used in all experiments since standard corrections lead to errors because of random variability as well as an inverse relationship between cell volume and extracellularly trapped fluid. The relatively high hematocrit was chosen in order to provide a sufficient number of cells for chemical analysis. It should also be noted that ^{14}C associated with PEG was the only isotope present. A 40-μl sample of the cell extract was taken for liquid scintillation counting of [^{14}C]PEG as was a 10-μl sample of the experimental bathing medium. ^{14}C cpm/μl of extracellular fluid were determined and used to correct for the extracellular contribution to cell water and ion content. The ^{14}C associated with PEG was counted in a Packard Tri-Carb Liquid Scintillation Counter model 3002 (Packard Instrument Co., Inc., Downers Grove, Ill.). The scintillation cocktail consisted of 800 ml Toluene, 200 ml ethanol, 0.3 g 1,4-bis[2-(5-phenyloxazolyl)]benzene (POP), 7 g 2,5-diphenyloxazole (PPO), and 5 g carbosil. From the same pellet extract used for scintillation counting, a 100-μl portion was removed for chemical determination of Na and K. The analysis was performed with an Instrumenta-

TABLE II
EXPERIMENTAL PROTOCOL

1, Withdraw blood from the caudal vein into a heparinized syringe.

2, Wash cells four times in 30 vol each wash, with solution A at 10°C.

3, Preincubate cells overnight in solution A at a hematocrit of <1%.

   Maximum inhibition of active Na-K transport by flounder red blood cells at 10°C by 10^{-4} M ouabain requires 30 min of exposure. Therefore cells to be used in experiments employing ouabain were exposed to 10^{-4} M ouabain for the last 30 min of the preincubation period.

4, Separate cells from perincubation medium by centrifugation followed by aspiration and split cells into experimental and control groups.

5, Resuspend cells in appropriate experimental and control media at a hematocrit of ≈8%.

   Experimental cells are placed in solution B while control cells are suspended in solution A. Both experimental and control suspensions contain [^{14}C]PEG as an extracellular space marker.

6, Sample with time.

   Immediately separate cells from supernate by centrifugation followed by aspiration.

7, To study RVI repeat steps 4-6 suspending both experimental and control cells in solution A.

tion Laboratory Flame Photometer (model 343, Instrumentation Laboratory, Inc., Lexington, Mass.). The supernates were also analyzed and the values of Na and K thus determined were used to correct for extracellularly trapped ion in the cell ion determinations. Chloride analysis was performed on 50-μl samples of the pellet extract and on the supernate. The samples were analyzed by coulometric titration with silver ions by use of a Bucher-Cotlove chloridometer (no. 4-2000). The chloride values obtained by analysis of the cell pellet extract were corrected for the contribution of extracellularly trapped chloride. Cell water content was determined by difference between wet and dry weight corrected for trapped medium.

Membrane Potential (E_m)

The membrane potential was calculated by using the Nernst equation for chloride. It was assumed that chloride is distributed at electrochemical equilibrium (12) and thus $E_{Cl} = E_m$; i.e.:

$$E_m = E_{Cl} = RT/zF \ln [Cl]_i/[Cl]_o.$$

Electrochemical Potential Difference ($\Delta \bar{\mu}$)

The electrochemical potential difference for Na or K distributed across the flounder erythrocyte membrane was calculated as the difference between E_m and the equilibrium

potential for the ion in question, since:

$$\Delta \bar{\mu}_J = (RT \ln [J]^i + z_J F E^i) - (RT \ln [J]^0 + z_J F E^0)$$
$$= z_J F(E^i - E^0) - RT \ln [J]^0/[J]^i$$
$$= z_J F E_{m1} = z_J F E_J$$
$$= z_J F(E_m - E_J).$$

Where $\Delta \bar{\mu}$ is the electrochemical potential difference for ion J, the superscripts i and 0 refer to the inside and outside of the cell, respectively, and R, T, z, and F have their usual meanings. The quantity $Z_J F(E_m - E_J)$ expresses $\Delta \bar{\mu}$ in Joules/mole, while the difference $(E_m - E_J)$ is an expression of $\Delta \bar{\mu}_J$ in millivolts.

RESULTS

Content of Cell Water, Na, K, and Cl during RVD and RVI

Table III presents the ion and water content of volume static control cells preincubated and maintained in solution A (see Materials and Methods). While

TABLE III

CONTROL WATER AND ION CONTENT OF CELLS
SUSPENDED IN SOLUTION A

H_2O	Na	K	Cl
ml/kg dcs		*mmol/kg dcs*	
1,659.0*	38.2	210.0	129.0
±4.52	±0.284	±1.16	±0.52
(60)	(60)	(60)	(60)

* Mean ± SEM (*n*).

some sample to sample variability in control cell ion and water content was observed, there was no significant change during the 4-h sampling period. Fig. 1 is a presentation of the water content of experimental (solid circles) and control cells (open circles) as a function of time. The experimental cells in the first 120 min of the figure were transferred from solution A (325 mosM) to solution B (200 mosM) at time (T) = 0. After rapid osmotic swelling to 145% of control cell volume the cells begin to regulate their volume back toward initial values. The rate of water loss is initially rapid (≈ 400 ml/kg dry cell solids (dcs) × h) and decreases to <10% of the initial rate in 2 h. The degree to which the cells osmotically swell and regulate volume is seen as the difference between the water content of control and of experimental cells. Under the experimental conditions employed, cell volume regulation is not complete, cell volume being essentially stable at 124% ± 1.6% of control in 2 h. After osmotic swelling and RVD in solution B (200 mosM), resuspension in solution A (325 mosM) results in rapid osmotic shrinkage until cell water content is 88.5% ± 1.5% of controls (second 120 min of Fig. 1). The data show that RVI begins immediately after osmotic shrinkage and, as was the case during RVD, the rate at which volume is regulated is greatest at the beginning. In 120 min cell volume has increased to 97.3% ± 0.7% of control cell volume.

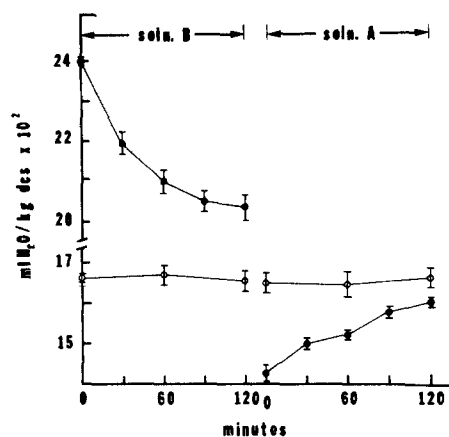The net ion fluxes associated with the water movement described above are

FIGURE 1. Regulatory changes in cell water content after cell transfer to hypotonic and hypertonic media. Cell water content (ml/kg dcs, ordinate) is plotted vs. time in minutes (abscissa). Experimental cells are depicted as closed circles, while control cells are shown as open circles. All data are expressed as mean ± SEM where $n = 11$. At T = 0 (first 120 min), cells which had been preincubated overnight in solution A were transferred to solution B (experimentals; closed circles) and fresh solution A (controls; open circles). At 120 min, the experimental cells were centrifuged from solution B and resuspended in solution A at T = 0 (second 120 min).
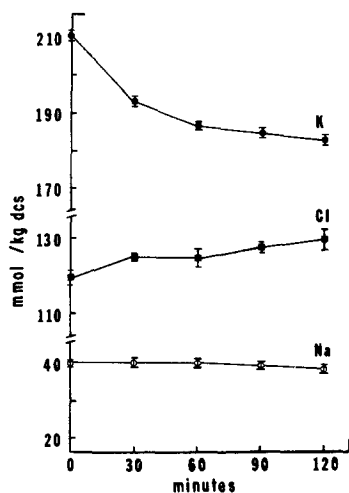
FIGURE 2. Na, K, and Cl content of cells undergoing regulatory volume decrease after transfer from solution A to solution B. Cell ionic content (mmol/kg dcs) is plotted vs. time in minutes. The values are presented as mean ± SEM for 11 experiments. Experimental treatment is described in Materials and Methods and the legend to Fig. 1 (first 120 min). Control values are presented in Table III.

shown in Figs. 2 and 3. Fig. 2 depicts the ion content of cells undergoing RVD. The values presented were obtained by analysis of the same cells whose water content is presented in Fig. 1. These data illustrate that, subsequent to osmotic swelling, the cells lose 20 mmol K/kg dcs during the 2-h sampling period. The

Na content remains virtually unchanged while Cl content increases. The net chloride influx appears to be contrary to requirements for maintenance of electroneutrality, since rather than moving with K, Cl moves in the opposite direction. Thus it appears that some other anion or anions must accompany K as it leaves the cell. A similar lack of correspondence between net cation and anion flux was reported by Roti Roti and Rothstein (10) studying volume regulation by mouse leukemic cells. On the basis of Fugelli's studies of the European flounder erythrocyte (2) and investigations of "isomotic intracellular regulation" by others (13, 14) it seems likely that negatively charged amino acids may serve as counter ions to K^+. The osmolality of the fluid transported during RVD, if one assumes equivalent K and anion loss, is 170 mosmol. The changes in cellular Na, K, and
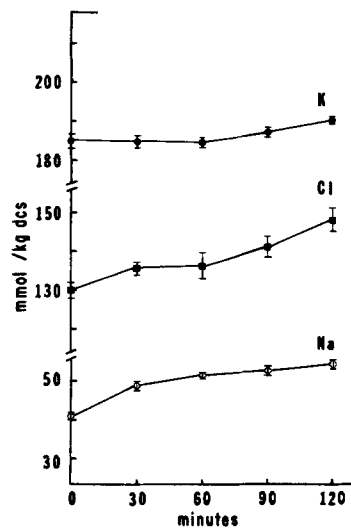

FIGURE 3.   Na, K, and Cl content of cells undergoing regulatory volume increase subsequent to osmotic shrinkage. Cell ion content (mmol/kg dcs, ordinate) is plotted vs. time in minutes (abscissa). The values are mean ± SEM of 11 experiments. Details of treatment are presented in Materials and Methods and the legend to Fig. 1.

Cl content during RVI are presented in Fig. 3. These values were obtained from the same samples whose water content is presented in the second half of Fig. 1. The data show that during RVI cellular Na and K content increases by 19.2 mmol/kg dcs with Na accounting for 75% of the cation uptake. The corresponding increase in cellular chloride content is 16.6 mmol/kg dcs and is thus in good agreement with the increase in intracellular cation content. The slight discrepancy between cation and anion uptake is most likely representative of changes in the charge on hemoglobin as a result of dilution during RVI (15). On the basis of the net cellular uptake of Na, K, Cl, and water the osmolality of the fluid transported during RVI is 275 mosmol. During RVI net Na flux is in the direction of the Na electrochemical gradient (E_{Cl} = −15 mV while E_{Na} = +46 mV), thus Na uptake is a dissipative process. The observed net uptake of K during RVI requires the expenditure of energy since there is a 74-mV gradient

of electrochemical potential favoring K loss (E_{Cl} = −15 mV while E_K = −89 mV). Cellular K content begins to increase 60–90 min after osmotic shrinkage. While water uptake increases at the same time (Fig. 1), the increase in cellular K accounts for only a small portion of the water accumulated during RVI. Further, the net K uptake begins after volume regulation is more than 50% complete and the intracellular ratio of K:Na concentration is less than 69% of control cell values. Thus it seems that the net increase in cellular K content during RVI may be more ion regulatory than volume regulatory.

Since during RVD the cells lose K and water at constant Na, the intracellular concentration ratio of K:Na decreases progressively. Thus it was hypothesized that cells which have lost K during RVD subsequently adjust intracellular
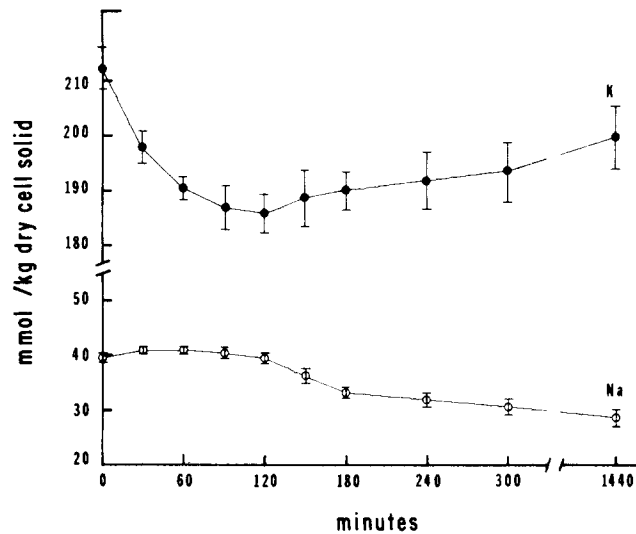

FIGURE 4. Na and K content of cells subsequent to osmotic swelling. Cell ion content (mmol/kg dcs, ordinate) is plotted against time in minutes (abscissa). The values presented are mean ± SEM for five experiments. The experiment is an extension of that represented in Fig. 2 (regulatory volume decrease) in that sampling is carried out for 1,440 min as opposed to 120 min.

[K]:[Na] as volume begins to stabilize. In order to test the hypothesis cells were osmotically swollen and their Na and K content was monitored for a 24-h period. The results of the experiments are shown in Fig. 4. The Na and K values represented in the first 120 min are very similar to those shown in Fig. 2. These data clearly show that after an initial K loss from the cells, K content begins to rise, while Na content decreases. Although results are not presented here, the cell water content demonstrated no significant change after 120 min.

Effect of Ouabain on Cell Water Na, K, and Cl Content during RVD and RVI

Ouabain (10^{-4} M) was added to suspensions of flounder red blood cells to evaluate the alterations in ion fluxes via the various conductance pathways during the volume regulatory response. In addition, ouabain was expected to

help in identifying net K uptake during RVI as a volume- or ion-regulating function of the Na-K pump. The effect of ouabain upon the water content of osmotically perturbed (closed circles) and volume static (open circles) cells is presented in Fig. 5. The data clearly show that volume regulation is unaffected by the presence of ouabain (see Fig. 1). Transfer of cells from solution A (325 mosM) to solution B (200 mosM) at T = 0 resulted in osmotic swelling to 142.2% ± 2% of control cell volume. The data show that cell water content began to decrease immediately and after 60 min of RVD it was 121% ± 2% of control values. After RVD in solution B the experimental cells were resuspended in
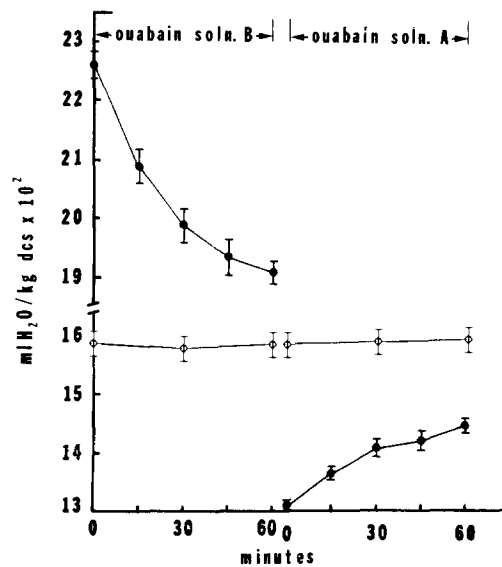

FIGURE 5. Effect of ouabain (10^{-4} M) upon osmotically perturbed (closed circles) and volume static (open circles) cell water content. The intracellular water content (ml/kg dcs × 10^{-2}) is plotted against time in minutes. Values are expressed as mean values ± SEM for seven experiments. The experimental treatment of cells represented in this figure differs from treatment of cells represented in Fig. 1 in that 10^{-4} M ouabain was added to cell suspensions and samples were taken for only 60 as opposed to 120 min.

solution A and underwent rapid osmotic water loss to 82% ± 1% of control values. Osmotic shrinkage was immediately followed by RVI and cell water content increased to 91% ± 1% of control in 1 h. Since the net water movements characteristic of the volume regulatory response are not impaired by ouabain, they cannot be directly dependent upon ion movements through the Na-K pump.

The ion content of ouabain-treated volume-static cells is shown as a function of time in Fig. 6, while H_2O, Na, K, and Cl content of paired ouabain-free controls are presented in Table IV. The data in Fig. 6 illustrate that incubation in solution A containing 10^{-4} M ouabain caused slight but significant alterations

in cell Na and K content: K decreased by 5 mmol/kg dcs in 1 h, while Na increased by the same amount.

The ion conent of ouabain-poisoned cells undergoing RVD is presented as a function of time in Fig. 7. The ouabain-treated osmotically swollen cells demon-
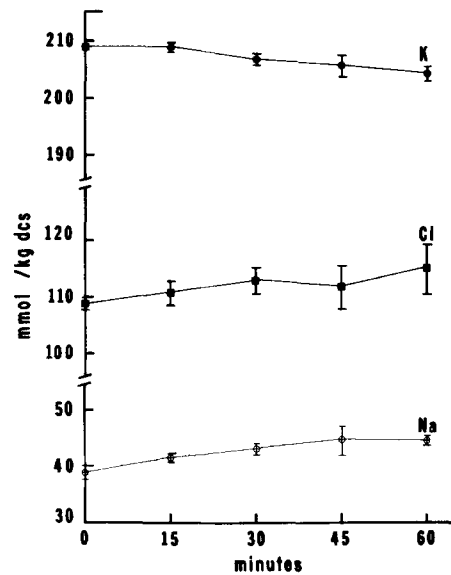
FIGURE 6.   Na, K, and Cl content of cells exposed to 10^{-4} M ouabain. The ion content, expressed (mmol/kg dcs) is plotted against time in minutes. All values are expressed as mean value of seven experiments ± SEM. The cells were preincubated in solution A (refer to Materials and Methods), and finally suspended in solution A containing 10^{-4} M ouabain. The ion content data correspond to the data on water content of volume static ouabain-treated cells in Fig. 5. Ion and water content of ouabain-free controls are presented in Table IV.

TABLE IV

WATER AND ION CONTENT/kg dcs OF CONTROLS
ASSOCIATED WITH OUABAIN (10^{-4} M) POISONED VOLUME
STATIC CELLS

H$_2$O	Na	K	Cl
ml	mmol	mmol	mmol
1,590.0*	38.0	210.0	109.0
±13.0	±1.5	±4.6	±4.75
(34)	(34)	(34)	(34)

* Mean ± SEM (n).

strated a large net K loss (cf. Fig. 2). In contrast to the ouabain-free case, net K loss was greater in the presence of ouabain (RVD + ouabain, −19%; RVD, −10%). In addition, during RVD plus ouabain cellular Na content increased by 37%, while in ouabain's absence cell Na content was invariant. Thus in the presence of ouabain RVD is characterized by large net movements of both Na

and K (cf. Fig. 2). However, if Na gain (19 mmol/kg dcs) is subtracted from K loss (42 mmol/kg dcs), it is obvious that the net decrease in intracellular cation content during RVD is unaffected by ouabain's presence.

The alterations in intracellular Na, K, and Cl content during RVI in the presence of ouabain are illustrated in Fig. 8. These data illustrate a net Na gain of 32 mmol/kg dcs in 1 h while K decreases by 19 mmol/kg dcs in 1 h. In contrast, cells undergoing RVI in the absence of ouabain gained 13 mmol Na/kg dcs in the 1st h after shrinkage while cell K content remained constant. Thus, while the magnitude of the net Na and K fluxes associated with RVI in the presence of
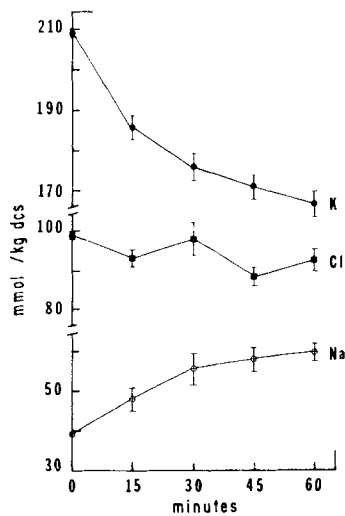

FIGURE 7. Na, K, and Cl content of cells undergoing regulatory volume decrease in the presence of 10^{-4} M ouabain. The data are shown as mmol/kg dcs (ordinate) vs. time in minutes (abscissa). All values are expressed as mean ± SEM ($n = 7$). With the exceptions of 10^{-4} M ouabain addition and a shorter sampling period, these cells were treated the same as those represented in Fig. 2. The values presented correspond to water content data illustrated for experimental cells in the first 60 min of Fig. 5. For further experimental details, see Materials and Methods and Fig. 1.

ouabain are elevated relative to identically treated ouabain-free cells, the net increase in intracellular cation content is 13 mmol/kg dcs during the 1st h in both cases. Clearly then, the net cation fluxes and therefore net water flow during RVI are not directly dependent upon a functional Na-K pump.

DISCUSSION

The data presented describe a volume regulatory response by osmotically perturbed flounder red blood cells. After osmotic swelling to 145% of control volume, the cells lose water and osmotically active particles until volume stabilizes at 120% of control volume. When osmotically shrunken, the flounder red blood cell will gain osmotically active particles and water. As a result, volume increases from 88% to 98% of control values in 2 h. The net water movements

during volume regulation are primarily the result of net inorganic ion fluxes. During RVD the cells lose K^+ and presumably anion, while cellular NaCl gain is responsible for water uptake during RVI. Since all the water transported cannot be accounted for on the basis of net inorganic ion (Na, K, Cl) flux, it is suspected that organics play a role. Studies of volume regulation by red cells of the European flounder *P. flesus* (2) showed that one-eighth of the particles lost during RVD are ninhydrin positive. If one assumes that the same relationship applies in the present study (cells lose both inorganic and organic molecules during RVD), the calculated osmolarity of the fluid transported during RVD is 194 mosM, a value in good agreement with the predicted value of 200 mosM. During RVI the calculated osmolarity of the fluid gained, solely upon the basis
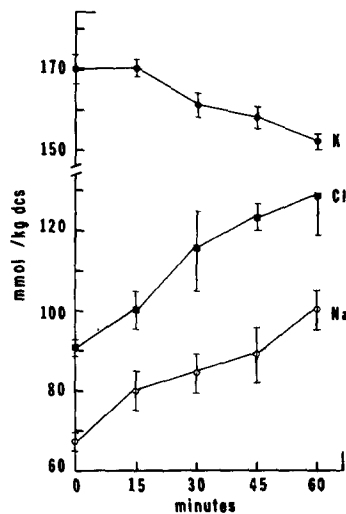

FIGURE 8. Na, K, and Cl content of cells undergoing regulatory volume increase in the presence of 10^{-4} M ouabain. The data are presented as mmol/kg dcs (ordinate) plotted against time in minutes (abscissa). All values are means ± SEM for seven experiments. The points indicated correspond to experimental points in the second 60 min of Fig. 5. Data concerning identically treated ouabain-free cells are presented in Fig. 3.

of inorganic ions, is 275 mosM while the external media is 325 mosM. If one assumes that organics account for one-eighth of the increase in intracellular osmotically active particles,[1] the osmolarity of the transported fluid is 312 mosM, a value in good agreement with an external osmolarity of 325 mosM.

During volume regulation the net cation fluxes responsible for net water flow occur through dissipative pathways. Cells which are osmotically swollen lose net

[1] Although there are no amino acids in solution A, studies of " isosmotic intracellular regulation" by crustacea (13, 14) demonstrate that adaptation to hypertonic media is accompanied by increases in the cellular content of newly synthesized amino acids. The authors also state that upon adaptation to hypotonic medium intracellular particle number decreases due to changes in both membrane permeability and biosynthetic-degradation pathways. Thus it is possible that organics participate in both RVD and RVI by flounder erythrocytes.

K at constant Na while osmotically shrunken cells gain Na with only a slight increase in intracellular K content. Since during RVD $E_{Cl} \approx E_m = -20$ mV, while $E_K = -84$ mV, there is a 64 mV driving force favoring K loss. Volume regulation by osmotically shrunken cells is the result of increases in cellular Na, Cl, and osmotically obliged water. Since during RVI $E_{Cl} = -15$ mV while $E_{Na} = +45$ mV, there is a 60-mV force driving Na into the cell. Therefore the net Na and K movements responsible for water flow during volume regulation are driven by their respective electrochemical gradients. The net conservative K influx during RVI and Na efflux and K influx subsequent to RVD are consistent with known ion regulatory functions of the Na-K pump (16–21). The above findings, when viewed together with the ouabain insensitivity of the volume regulatory response, lead to the conclusion that the Na-K pump does not, in a direct sense, contribute to the volume regulatory response.

The net Na and K fluxes presented in Figs. 2, 3, 6, 7, and 8 provide information concerning the Na and K pump and leak pathways during volume regulation. These data are summarized in Table V in an attempt better to

TABLE V
NET FLUXES OF Na AND K (mmol/kg dcs IN 1 h)

	K			Na		
	Control	Ouabain	Ouabain difference	Control	Ouabain	Ouabain difference
Volume static	0	−5	−5	0	+5	+5
RVD	−23	−42	−19	0	+19	+19
RVI	0	−19	−19	+12	+32	+20

The values were taken from Figs. 2, 3, 6, 7, and 8 and represent net changes which occurred during the first 60 min of treatment. The plus and minus signs preceding the numbers denote cellular uptake and loss, respectively.

evaluate the changes which occur in the flux pathways during volume regulation. The data in the columns labeled "ouabain" represent net Na and K movements in the direction of their respective electrochemical gradients. These data illustrate that during volume regulation net Na gains and K losses are at least four times and as many as eight times greater than values obtained by studying volume-static cells. Yet while passive Na and K fluxes increase dramatically in response to osmotic perturbation, the driving forces ($\Delta \bar{\mu}_{Na}$ and $\Delta \bar{\mu}_K$) favoring Na gain and K loss change by only a few percent. Since the net flux of an ion is equal to the product of the driving force acting upon the ion and the membrane's conductance to the ion, the differences in net Na and K fluxes are reflections of changes in the membranes Na and K conductances and therefore permeabilities. As such, comparisons of the net Na and K fluxes associated with osmotically perturbed and volume static cells illustrate that Na permeability (P_{Na}) and K permeability (P_K) increase in response to osmotic swelling and shrinkage. It appears that the increases in P_{cation} are weighted in such a manner that during RVD P_K increases to a greater extent than P_{Na}, while during RVI the converse is true.

The net flux values presented in the columns labeled ouabain difference are

an indication of the Na and K fluxes which occur via the Na-K pump. The data illustrate that the ouabain difference for osmotically perturbed cells is about four times values obtained from the volume-static cells. Therefore we must conclude that pump rate increases in response to osmotic perturbation.

While not reflected in Table V it is clear from Figs. 2, 3, 7, and 8 that the net Na and K fluxes associated with volume regulation are not linear with time. In fact, the fluxes are highest immediately after osmotic perturbation and decay as some function of cell volume. In this regard, the electrochemical gradients for K during RVD and Na during RVI decrease by only a few percent while net K loss during RVD and Na gain during RVI decrease dramatically. Again, since the net flux of an ion is equal to its conductance times the electrochemical potential difference for the ion, decreases in the rate of K loss during RVD and Na gain during RVI reflect decreased P_K and P_{Na}, respectively. In addition, the rate of Na uptake by ouabain-treated cells during RVD (Fig. 6) decreases from an initial extrapolated hourly rate of 40 mmol/kg dcs × h to a final rate of 5 mmol/kg dcs × h. Accompanying this 88% decrease in net flux rate is a 27% decrease in $\Delta \bar{\mu}_{Na}$. Therefore the decreased rate of Na uptake is the result of decreased P_{Na}. Further, the constancy of Na_C during RVD in ouabain's absence (Fig. 2) suggests that the rate of Na pumping decreases in parallel with P_{Na}. As discussed in relation to Table V, P_K, P_{Na}, and Na-K pump rate increase in response to osmotic perturbation, while the above discussion illustrates that these parameters decrease as cell volume is regulated. Taken as a whole, the behavior of the Na and K pump and permeability pathways suggests a functional dependence during cell volume regulation.

The present study has demonstrated that the flounder erythrocyte exhibits a volume regulatory response subsequent to osmotic perturbation. Volume regulation occurs as a result of net inorganic cation fluxes and osmotically obliged water flow. The net Na and K fluxes characteristic of the volume regulatory response are in the direction of their respective electrochemical gradients and occur in response to increased P_K and P_{Na}. Associated with increased permeability, the rates of Na and K pumping are also increased. As volume is regulated, both Na and K permeabilities and pump rates decrease as some function of cell volume. Thus it appears that ion fluxes via active and passive pathways, if not linked due to interactions with a similar membrane component, are functionally linked through the volume regulatory mechanism. The passive nature of the ion movements associated with the volume regulatory response has been stressed, since ion fluxes via dissipative pathways are ostensibly responsible for volume regulation. Yet it should be borne in mind that the driving force for the net ion fluxes responsible for volume regulation is the energy stored in the Na-K electrochemical gradients as a result of the pump's activity.

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