Functional Separation of the Na-K Exchange Pump from the Volume Controlling Mechanism in Enlarged Duck Red Cells

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ABSTRACT Previous publications have described a "volume controlling mechanism" in duck erythrocytes that returns both enlarged and shrunken cells to their original isotonic volume. Enlarged cells return to their original size by readjusting their K content. To study the specificity of this aspect of the mechanism for K, we prepared enlarged cells with various Na and K contents. Only cells containing a high K content resume their original size in the standard isotonic medium. The process of regulation resembles that described above. In contrast, cells containing a high Na content fail to reestablish this volume, but shrink instead until they reach a limiting minimal volume (four-fifths of normal). Here, another mechanism, the cation pump rather than the volume controlling mechanism, removes Na and is responsible for the changes in cell size. Enlarged cells with an intermediate Na and K content utilize both mechanisms to reduce their cation content. Only if Na is prevented from leaving the cell and sufficient K is present initially, will these cells reestablish their original size. These studies demonstrate that the cation pump and volume controlling mechanism function independently and, when cells enlarge, only K can effectively traverse the pathway associated with the volume controlling mechanism. This route differs from the one used by the cation pump to eject Na.

INTRODUCTION

Duck erythrocytes can readjust their size by utilizing a ouabain-insensitive "volume controlling mechanism" (1, 2). This mechanism regulates the cation content of cells; the changes in cation content produce, in turn, isosmotic shifts in cell water and changes in cell size (3). Duck red cells are high K cells (K concentration approximately 167 mmol/liter cell H_2O). When they enlarge, either in hypotonic media or after incubation with norepinephrine in isotonic media, they must lose K to shrink. The mechanism releases a sufficient amount of K (as KCl) from the cells to allow them to return to their

original isotonic volume (1, 3). The electrochemical gradient serves as the driving force for this K movement, while a transient increase in a diffusional pathway controls the amount of K lost (1, 3).

This communication examines the response of experimentally enlarged cells incubated in isotonic media. We enlarged cells by increasing their cation content and thus their cell water, utilizing a procedure which allows one to also vary cellular Na and K concentrations. Studies on those modified cells permitted us to test the specificity of the mechanism for K.

MATERIALS AND METHODS

Red cells from Muscovy ducks were obtained by cardiac puncture as described previously (5). The blood was passed through cotton gauze and then centrifuged twice, first at 2,000 *g* for 5 min and then at 17,000 *g* for 15 min. The supernatant fluid and white cell pellet were discarded.

The erythrocytes were then subjected to a modification of the procedure of Garahan and Rega (4). This procedure, or a modified form (6, 7), has been used successfully to vary the Na and K content and concentration of the non-nucleated erythrocytes of sheep and man. Since we were interested in producing enlarged nucleated erythrocytes containing various Na and K contents, the procedure was modified further to meet these specific needs. Packed ice-cold erythrocytes were first suspended and then incubated in an ice-cold solution with the following composition: 2 mM $MgCl_2$, 127.5 mM or 255 mM XCl (X stands for Na or K or a combination of the two), 5 mM X_2HPO_4/XH_2PO_4 (pH 7.45 at 20°C), 30 mM sucrose, and 0.1 mM PCMBS (*p*-chloromercuriphenyl sulfuric acid) (Sigma Chemical Co., St. Louis, Mo.) for 22 h. The suspension had a hematocrit of 1–1.5% and was mixed gently on a Thomas Rotating Apparatus (Arthur H. Thomas Co., Philadelphia, Pa.) during this, the cation-loading stage. At the end of 22 h, the cells were rapidly separated by centrifugation (5 min at 2,200 *g*), care being taken to maintain the cells at 4°C. The next stage, the sealing stage, was accomplished by incubating the cells in a stoppered flask at 41°C for 15 min in a similar medium containing instead of the PCMBS, 5.1 mM DTT (dithiothreitol) (Calbiochem, Los Angeles, Calif.) and 11 mM glucose. The hematocrit of this suspension remained 1–1.5%. Since the cation pump rapidly exchanges intracellular Na for extracellular K during the sealing stage when the medium contains K and the cells a large amount of Na, we shortened this stage to 15 min to produce cells which contained Na as the major monovalent cation (between 50–80%) and K as the minor cation (between 20–50%). These cells have been labeled intermediate cells in the text. Even then, cells which contain Na and K at a ratio of 2:1 at the end of the cation-loading stage have a ratio of 1:1 after separation at the end of the sealing stage. At the end of 15 min the cells were again separated by centrifugation (5 min at 2,200 *g*) and after removing the supernatant fluid were washed once or twice with 50 vol of an isotonic solution, (unless otherwise stated this was the *standard synthetic medium* [see below]). This wash solution was kept at room temperature since an ice-cold solution produced hemolysis; some hemolysis was also present if the cation-loading stage was extended beyond 22 h. When the PCMBS-DTT procedure was performed as described, however, no visible hemolysis occurred. To produce high-K

or high-Na cells (see text) we added all K or Na salts, respectively, to the solutions used in the cation-loading and resealing stages.

When data from untreated cells were required for comparison, lower steady-state (LSS) cells served as these controls. The procedures for obtaining LSS cells ($[K]_c \sim$ 110 mmol/liter, $[Na]_c \sim$ 5 mmol/liter, and $[Cl]_c \sim$ 51 mmol/liter) has been described elsewhere (1, 2).

The standard synthetic medium (5) usually served as the experimental bathing solution. It has the following composition: 2 mM $MgCl_2$, 1 mM $CaCl_2$, 28 mM $NaHCO_3$, 3.8 mM Na_2HPO_4, 3.8 mM NaH_2PO_4, 113 mM NaCl, 2.5 mM KCl, 20 mM dextrose, and 2.5 g/100 ml albumin. Three other isotonic solutions were used; they have been labeled K-free, high-K, and low-Na solutions. In the K-free solution an equivalent amount of NaCl replaced KCl while in the high-K solution K salts of chloride, phosphate, and bicarbonate replaced those of Na. The low-Na (choline) medium has the following composition: 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2.5 mM KH_2PO_4, 28 mM choline bicarbonate (Matheson, Coleman, and Bell, Norwood, Ohio), 121 mM choline Cl, 20 mM dextrose, and 2.5 g/100 ml albumin. All procedures during the experimental period were identical to those used previously (5, 1) including incubating the cells at 41°C in an atmosphere of 95% O_2 and 5% CO_2.

Alterations in cell size were followed by noting the changes with time in the values for (*a*) percent cell H_2O (wt/wt) or (*b*) Hgb absorbancy of a measured volume of cells. Measurements of fractional change in volume, the ordinate in many of the figures, were calculated using values obtained by method *b*. With regard to method *a*, it should be noted that a 2% decrease in the percent cell H_2O (wt/wt) from 72 to 70 corresponds to approximately a 10% decrease in cell size, whereas a 2% decrease from 61 to 59 corresponds to approximately a 5% decrease.

The concentration of Na, K, and Cl in most of these studies is expressed as millimoles per liter of that number of cells which initially occupied 1 liter ($mmol/liter_{ONC}$ = millimoles per liter of original number of cells). The reference point for this calculation is the number of lower steady-state cells occupying 1 liter. Measurements of Hgb absorbancy, a quantity directly related to cell number, were used to refer all measurements back to the same number of cells. This calculation corrects for changes in cell size and permits expression of the results as the electrolyte content per unit number of cells. Thus, a change in the cation or anion concentration/$liter_{ONC}$ represents a net gain or loss of electrolyte by the cell.

The procedures for measuring and calculating K influx have been described previously (Eq. 1 in reference [1]). This same reference (1) also contains a description of the procedures for measuring the rate of ^{24}Na and ^{42}K loss from cells. Efflux measurements have usually been presented as the percent tracer released from the cells with time. Before beginning the efflux measurements, preloaded cells were always washed twice with 40 vol of tracer-free media (standard synthetic medium).

The procedures for measuring Na, K, and Cl, percent cell H_2O (wt/wt), Hgb, pH, and the radioactivity associated with ^{24}Na and ^{42}K have also been described previously (5, 1).

Special care was required when mixing the packed cell pellet before removing an aliquot of cells for analysis since some heterogeneity was present in those cell populations containing a high Na content. A similar small amount of heterogeneity has been

reported in human red cells produced by the original method (4). When it was not possible to mix adequately, the entire packed cell pellet was analyzed at each sampling. Cell heterogeneity was assayed by centrifuging the cells and allowing them to separate according to density, dividing the packed cell column into three equal portions and then analyzing each portion separately. The top and bottom portion of a population of high-Na cells differed in Na content by as much as 10 mmol/liter$_{ONC}$; this degree of heterogeneity could be reduced but not eliminated by mixing the cell suspension frequently during the cation-loading stage. The heterogeneity present in high-K cells was not appreciably different from that in freshly isolated duck erythrocytes (<2.5 mmol/K/liter$_{ONC}$ difference between the top and bottom portion of cells.) The experiment illustrated in each figure is representative of at least five others.

RESULTS

High-K Cells

Using the procedure described in the Methods, one can obtain cells with a K content as high as 250 mmol/liter$_{ONC}$ and a Na content as low as 2 mmol/liter$_{ONC}$. Such cells have a volume approximately 63% larger than control (LSS) cells. Enlarged high-K cells with a cation content and volume of about this magnitude serve as one of the two groups of cells used in the studies demonstrated in Fig. 1 and Table I; the other group contains a K content approximately two-thirds this value (moderately enlarged cells). Both groups shrink when initially incubated in an isotonic medium ($[K]_o$, K concentration of medium = 2.5 mM). The cells shrink rapidly at first, but as they approach their original volume, the rate decreases and finally ceases. The reduction in size is osmotic in nature, resulting from a loss of KCl and H_2O (Table I). K moves toward its electrochemical equilibrium.[1]

Thus, the mechanism responsible for the K loss and volume regulation seen here resembles that reported previously (1, 3). This is illustrated further by the observation that introducing ouabain (10^{-4} M) to the bathing medium has little effect on the changes in cell size (Fig. 1) or the primary event, the net K loss (Table I), while eliminating the gradient for K by incubating the cells in a high-K medium, prevents shrinkage (Fig. 1) and K loss (Table I). And, as before the K loss develops because of a transient increase in K efflux. Fig. 2 shows the initial elevation in the flux and rate constant for K efflux and the subsequent reduction in both with time as the cells approach their

[1] The largest high-K cells have a chloride ratio $[Cl]_o/[Cl]_i$ as small as 1.15. As they lose KCl and shrink, the chloride ratio increases until it approximates the value in control cells (~1.7, see references 1 and 2) when the cells reacquire their original volume. Thus, these cells have a small but variable electrical gradient (calculated membrane potential varies between 3–14 mV, inside negative) that is vectorially opposite to the net K loss. However, a steep outwardly directed concentration gradient exists (intracellular concentration exceeds the extracellular concentration by a factor of 60 or more) which results in an electrochemical gradient that is always in the same direction as the net K loss.

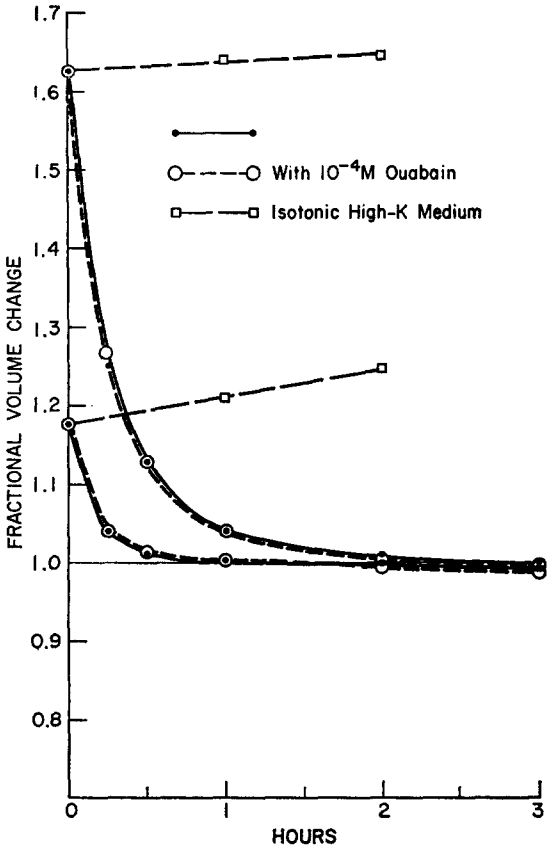

FIGURE 1. The fractional volume changes in swollen high-K cells as they incubate in the standard synthetic medium ●——●; in the same solution with 10^{-4} M ouabain, O- - - -O, and in an isotonic high-K medium (153 mM KCl), □— —□ (hematocrit 7–8%). All changes in cell volume were referred to the volume of (LSS) control cells which have a fractional volume of 1.0. In experiments using the standard synthetic medium, the rise in medium K as the cells lose K necessitated separating the cells at 80 min and resuspending them in fresh media. Cells were separated by centrifugation in a procedure which lasted 3–4 min. For cells incubated in the high-K medium, we have not recorded values beyond 2 h, since by this time these cells showed evidence of dysfunction. The suspension takes on a darker red appearance at 1 h and in the case of the larger cells, a small percentage gradually assume a round appearance rather than the usual oval shape. The cells with the largest original volume had the following composition at 0 time: percent cell H_2O (wt/wt) 73.2; Na = 3.2, and K = 253 mmol/liter$_{ONC}$; while the smaller cells had a percent cell H_2O (wt/wt) of 65.3% and a Na and K of 1.9 and 159 mmol/liter$_{ONC}$, respectively.

original volume. The ouabain-insensitive component of K influx is elevated by a small amount initially, but, at each interval the total K influx measurement (ouabain-sensitive + ouabain-insensitive) is equivalent to or less than the 2-h K efflux measurement. Thus, these tracer measurements remain con-

TABLE I

CHANGES IN ELECTROLYTE CONTENT ASSOCIATED WITH VARIATIONS IN SIZE OF ENLARGED HIGH-K CELLS

Medium	$\Delta[K]_e$		$\Delta[Na]_e$		$\Delta Na + K$		$\Delta[Cl]_e$		Cell H_2O (wt/wt)		
	¼ h	2 h	¼ h	2 h	¼ h	2 h	¼ h	2 h	0	¼ h	2 h
	mmol/liter$_{ONC}$		*mmol/liter$_{ONC}$*		*mmol/liter$_{ONC}$*		*mmol/liter$_{ONC}$*		%	%	%
Enlarged cells											
Exp. 1 Standard synthetic	−65	−126	+4	+7	−61	−119	−57	−105	73.0	67.5	58.4
Standard synthetic + 10^{-4} M ouabain	−67	−141	+9	+19	−58	−122			73.0	67.3	58.8
High-K		+4		+1		+5			73.1		73.5
Exp. 2 Standard synthetic	−57	−114	+4	+7	−53	−107	−51	−89	71.6	66.6	58.8
Standard synthetic + 10^{-4} M ouabain	−55	−126	+6	+15	−49	−111			71.6	66.2	58.2
High-K		+3		+1		+4			71.8		72.0
Moderately enlarged cells											
Exp. A Standard synthetic	−23	−41	+2	+7	−21	−34	−21	−32	64.6	60.7	58.8
Standard synthetic + 10^{-4} M ouabain	−22	−55	+2	+21	−20	−34			64.6	60.5	57.7
High-K		+1		+12		+13			64.8		66.3
Exp. B Standard synthetic	−20	−36	+3	+7	−17	−29	−16	−28	64.7	60.4	58.4
Standard synthetic + 10^{-4} M ouabain	−24	−48	+6	+16	−16	−32			64.8	60.6	58.0
High-K		+1		+15		+16			65.0		66.8

The changes in electrolyte content associated with the variations in cell size illustrated in Fig. 1. The experimental protocol is identical to that described in the legend of Fig. 1. Δ values were obtained by subtracting the values at 15 min and 2 h from the values at 0 time. The Na, K, and Cl content of enlarged and moderately enlarged cells at 0 time were, respectively: exp. 1, 3.2, 244, and 154 mmol/liter$_{ONC}$; exp. 2, 3.0, 224, and 139 mmol/liter$_{ONC}$; exp. A, 3.9, 162, and 83 mmol/liter$_{ONC}$, and exp. B, 2.8, 151, and 77 mmol/liter$_{ONC}$.

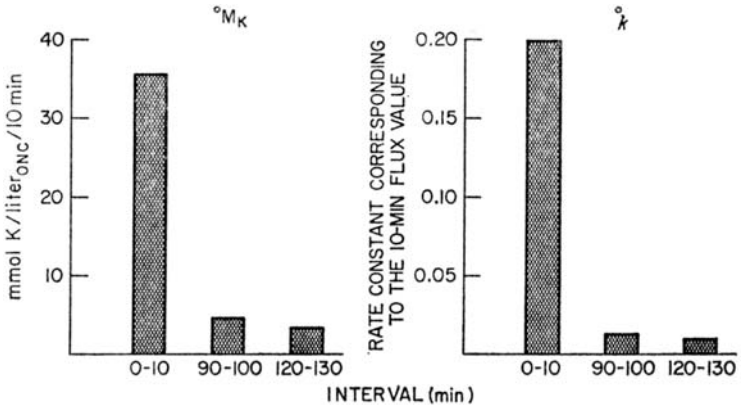

FIGURE 2. The changes in K efflux and the corresponding rate constant as enlarged high-K cells regulate their volume in the standard synthetic medium. The experimental protocol resembles that described in the legend of Fig. 1, with the following exceptions. Cells were preloaded with ^{42}K by incubating them with tracer during the entire cation-loading and resealing stages, and, when necessary, during part of the experimental period. At the times indicated, 0, 90, 120 min, two portions of the radioactive cell suspension were removed and the cells separated by centrifugation. After washing the cells with tracer-free medium, one group was analyzed for Na, K, percent cell H_2O (wt/wt), and Hgb; the K concentrations obtained from these values were taken as that present at the beginning of each efflux measurement. The other group was resuspended in tracer-free medium for the efflux measurements. Samples were removed at 0, 3, 7, and 10 min; a plot of the quantity ln (1 minus percent tracer released) against time produced a straight line in each case. K efflux was calculated by multiplying the 10-min ln value by the concentration (mmol/liter$_{ONC}$) found at the beginning of the measurement. The corresponding rate constant was obtained by dividing this flux value by the appropriate K concentration (mmol/liter cell H_2O). At 0 time the cellular values were: percent cell H_2O (wt/wt) = 73.2, $[Na]_c$ = 4.0 mmol/liter$_{ONC}$, $[K]_c$ = 232 mmol/liter$_{ONC}$, $[K]_c$ = 179 mmol/liter cell H_2O. At 90 min the cellular values were: percent cell H_2O (wt/wt) = 60.1, $[Na]_c$ = = 8.0 mmol/liter$_{ONC}$, $[K]_c$ = 118 mmol/liter$_{ONC}$, $[K]_c$ = 173 mmol/liter cell H_2O. At 120 min the cellular values were: percent cell H_2O (wt/wt) = 59.3, $[Na]_c$ = 9.1 mmol/liter$_{ONC}$, $[K]_c$ = 115 mmol/liter$_{ONC}$, $[K]_c$ = 172 mmol/liter cell H_2O.

sistent with the hypothesis that K leaves the cell because of a transient increase in an apparent diffusional pathway.

Table II shows that high-K cells, after incubating for 2 h, have reached a steady state. For changes in Na and K content and concentration fail to occur when incubation is continued in the standard synthetic medium despite the presence of steep concentration gradients across the membrane for Na entry and K loss. Table II also shows that at 2 h high-K cells resemble control (LSS) cells. (See Methods for the composition of LSS cells.) Their volume and K content are similar; only the Na content is different, being several millimoles higher in treated cells. It is not surprising then, to find that tracer measurements on these cells demonstrate a ouabain-sensitive component for K

TABLE II

THE ELECTROLYTE AND H_2O CONTENT OF HIGH-K CELLS WHICH HAVE RETURNED TO THEIR ORIGINAL SIZE FROM A PREVIOUSLY ENLARGED STATE

	$[K]_c$		$[Na]_c$		Cell H_2O (wt/wt)	
	2 h	3 h	2 h	3 h	2 h	3 h
	mmol/liter$_{ONC}$		*mmol/liter$_{ONC}$*		%	
Enlarged cells						
Exp. 1	120	119	11.3	12.2	58.4	58.1
Exp. 2	119	113	8.9	11.0	58.7	57.5
Exp. 3	110	107	10.6	10.1	58.8	58.0
Exp. 4	120	118	10.3	11.7	59.1	58.3
Exp. 5	115	112	9.9	11.1	59.3	58.7
Average	116.8	113.8	10.2	11.1	58.9	58.2
Moderately enlarged cells						
Exp. *a*	120	118	14.1	13.3	58.0	57.9
Exp. *b*	107	105	10.1	9.9	58.4	58.4
Exp. *c*	118	118	14.7	13.9	58.9	58.3
Exp. *d*	115	114	13.0	12.3	58.4	58.2
Exp. *e*	116	115	8.0	8.4	58.1	58.4
Average	115.2	114.0	12.0	11.6	58.4	58.3

The experimental protocol is identical to that described in the legend of Fig. 1; the cellular values are those found at the second and third hour of incubation. These values are expressed in mmol/liter$_{ONC}$ but can also be expressed in mmol/liter cells since they now have a volume similar to the original (LSS) cells.

influx and Na outflux not unlike those seen in (LSS) cells. Fig. 3 shows the rate of ^{24}Na loss from 2-h-treated cells in the presence and absence of ouabain (10^{-4} M). These measurements should be compared to similar measurements on LSS cells reported previously (1, 2, 3) (See footnote also.)[2]

High Na Cells

In contrast, Fig. 4 and Table III show what happens when we incubated swollen high-Na cells in isotonic media. Two groups of cells were also used in these studies. In one group, labeled enlarged cells, the cells have a Na

[2] In a series of five paired experiments in which the rate of ^{24}Na loss from LSS and 2-h-treated cells were compared, the $[Na]_c$ of LSS cells was 6.1 mmol/liter$_{ONC}$ while that of 2-h-treated cells was 8.9. Accordingly, the mean ouabain-sensitive flux from LSS cells was 9.4 ± 2.6 mmol/liter$_{ONC}$/h. (Rate constant calculated from the first two points, 0 and 3 min) compared to 11.5 ± 2.1 for 2-h-treated cells. This difference, however was not statistically significant ($P > 0.05$.) In both groups of cells, the ouabain-insensitive flux showed considerable variability 7.2 ± 4.1 mmol/liter$_{ONC}$/h in LSS cells compared to 11.6 ± 6.4 in 2-h-treated cells. All flux values have been expressed as the mean ± standard deviation.

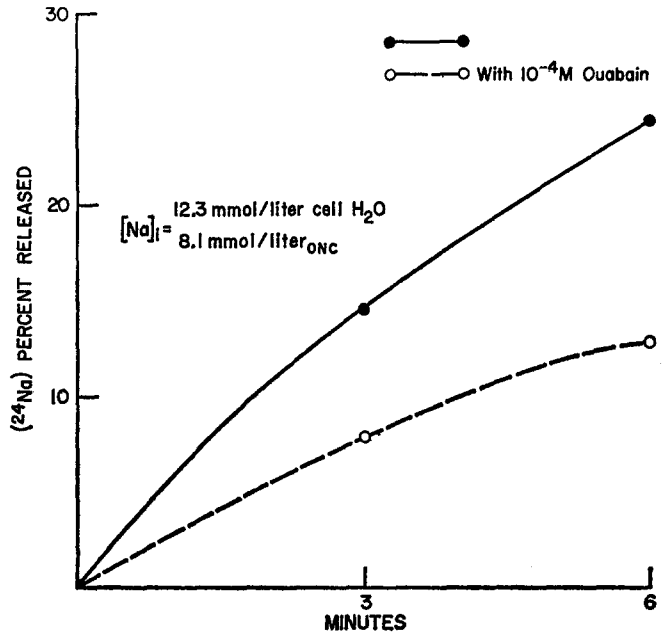

FIGURE 3. Ouabain's effect on the rate of ^{24}Na loss from enlarged high-K cells after they have reached their original volume. Cells attain their original volume by incubating in the standard synthetic medium for 2 h as described in the legend of Fig. 1; during this period cells were preloaded with ^{24}Na. The 2-h cells were then washed and incubated in tracer-free standard synthetic medium. The cellular values at the beginning of the efflux measurements have been included in the figure.

content as high as 240 mmol/liter$_{ONC}$ and a K content as low as 2 mmol/liter$_{ONC}$; such cells have a volume approximately 60% larger than control (LSS) cells. The cation content of the other group is approximately three-fifths of this value (moderately enlarged cells). Fig. 4 demonstrates that the two groups shrink as did the high-K cells; the shrinkage is also osmotic in nature resulting from the loss of NaCl and H_2O (Table III). But both the shrinkage process and the mechanism for removing cations (Na) differ. Now both result from the action of the cation pump.

The shrinkage process differs in two ways. First, cells shrink at a slower rate (compare the initial slope of the curves in Fig. 4 with those in Fig. 1). Second, cells shrink below their original volume only to stabilize at a size that is approximately 80% of control values. The latter volume has been labeled the *minimal volume*. Thus, although the cells shrink, the process is no longer regulatory; that is, the cells no longer reestablish their original isotonic volume as they did with the volume controlling mechanism.

The cation pump, responding to an increase in the internal Na concentration removes the Na. For either the addition of ouabain (10^{-4} M) to the

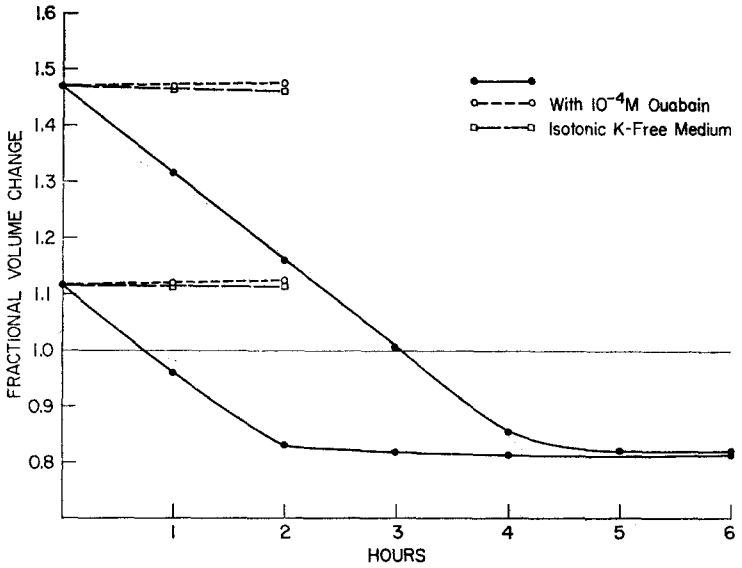

FIGURE 4. The fractional volume change in swollen high-Na cells as they incubate in the standard synthetic medium. ●——●, in the same solution with 10^{-4}M ouabain O- - - - -O, and in isotonic K-free medium □— — □ (hematocrit 7–8%). All changes in cell volume were referred to the volume of (LSS) control cells which have a fractional volume of 1.0. In those experiments where cells shrink, K uptake by the cells reduced the medium concentration. To maintain the medium K concentration above 2.5 mM, it was necessary to separate and resuspend the cells in fresh medium periodically (approximately every 40 min). Cells incubated with ouabain show evidence of metabolic dysfunction; the suspension takes on a darker red color by 1½ h. The cells with the larger original volume had the following composition at 0 time: percent cell H_2O (wt/wt) = 71.0, $[Na]_c$ = 198 and $[K]_c$ = 2.9 mmol/liter$_{ONC}$; while the smaller cells had a percent cell H_2O (wt/wt) of 63.2 and a $[Na]_c$ and $[K]_c$ of 144 and 2.4 mmol/liter$_{ONC}$, respectively.

bathing medium or removing the extracellular K, procedures known to inhibit the cation pump, block cell shrinkage (Fig. 4) and also prevent Na loss (Table III). In addition, most of the Na leaves the cell against an electrochemical gradient rather than with the gradient. For by 1 h and often earlier, both a concentration and electrical gradient exist which are vectorially opposite to the net Na movement.[3]

In human erythrocytes, with an elevated internal Na concentration, the cation pump does not function as a perfect exchanger, but rather more Na is removed than K taken up (8, 9). Presumably, a similar uncoupling occurs

[3] As with high-K cells (see footnote 1) high-Na cells initially have a small chloride ratio $[Cl]_o/[Cl]_i$ which increases as the cells shrink. Thus, there is a small but gradually increasing electrical gradient across the membrane that is in the opposite direction to the net Na loss. However, initially, there is also a small outwardly directed concentration gradient so that the initial electrochemical gradient approximates zero. Nevertheless, as the cells lose Na and shrink, the intracellular Na concentration gradually declines; by ½ h, intra- and extracellular concentrations are nearly equivalent resulting in an electrochemical gradient that is vectorially opposite to the net Na loss from this point onward.

TABLE III

CHANGES IN ELECTROLYTE CONTENT ASSOCIATED WITH VARIATIONS IN SIZE OF ENLARGED HIGH-Na CELLS

Medium	$\Delta[K]_c$		$\Delta[Na]_c$		$\Delta[Cl]_c$		Cell H_2O (wt/wt)		
	2 h	6 h	2 h	6 h	2 h	6 h	0	2 h	6 h
	mmol/liter$_{ONC}$		*mmol/liter*$_{ONC}$		*mmol/liter*$_{ONC}$		%	%	%
Enlarged cells									
Exp. 1									
Standard synthetic	+14	+30	−90	−186	−74	−122	73.5	64.6	50.4
Standard synthetic + 10^{-4} M ouabain	+2		+2				73.4	73.5	
K-free	−1		+1				73.5	73.4	
Exp. 2									
Standard synthetic	+26	+42	−81	−161	−52	−109	70.0	63.2	50.2
Standard synthetic + 10^{-4} M ouabain	−1		+4				70.0	70.2	
K-free	−2		−2				70.1	69.8	
	1 h	4 h	1 h	4 h	1 h	4 h	0	1 h	4 h
	mmol/liter$_{ONC}$		*mmol/liter*$_{ONC}$		*mmol/liter*$_{ONC}$		%	%	%
Moderately enlarged cells									
Exp. A									
Standard synthetic	+20	+36	−41	−89	−21	−43	62.1	56.9	49.3
Standard synthetic + 10^{-4} M ouabain	−1		0				62.2	62.2	
K-free	−2		−1				62.3	62.1	
Exp. B									
Standard synthetic	+12	+33	−38	−90	−24	−43	62.4	56.7	49.8
Standard synthetic + 10^{-4} M ouabain	0		0				62.6	62.6	
K-free	−1		0				62.9	62.7	

The changes in electrolyte content associated with the variations in cell size illustrated in Fig. 4. The experimental protocol is identical to that described in the legend of Fig. 4. Δ values were obtained by subtracting the values at 1, 2, 4, or 6 h from the values at 0 time. The Na, K, and Cl content of enlarged and moderately enlarged cells at 0 time were, respectively: exp. 1, 230, 3.1, and 149 mmol/liter$_{ONC}$; exp. 2, 192, 3.4, and 133 mmol/liter$_{ONC}$; exp. A, 133, 3.6, and 67 mmol/liter$_{ONC}$; exp. B, 129, 3.0, and 65 mmol/liter$_{ONC}$.

in high-Na duck cells, and serves to explain the net Na loss. It is also possible that the net cation loss is aided by the fact that K taken up by the pump is subsequently lost through the volume controlling mechanism. However, to explain the large K concentrations found in some minimal volume cells, (as high as 70 mmol/liter cell H_2O; see Table III), it is necessary to postulate that the transfer of K out of the cells through the volume controlling mechanism ceases once the cells reach their original volume.

Why cells cannot shrink beyond the minimal volume remains unclear. Tracer measurements still indicate that the cation pump remains operative (Fig. 5) and is stimulated by an elevation in $[Na]_o$. These cells also have an elevated membrane potential (as indicated by the chloride ratio). Their chloride ratio $(Cl]_o/[Cl]_i$ varies between 2.0–3.0 corresponding to a mem-

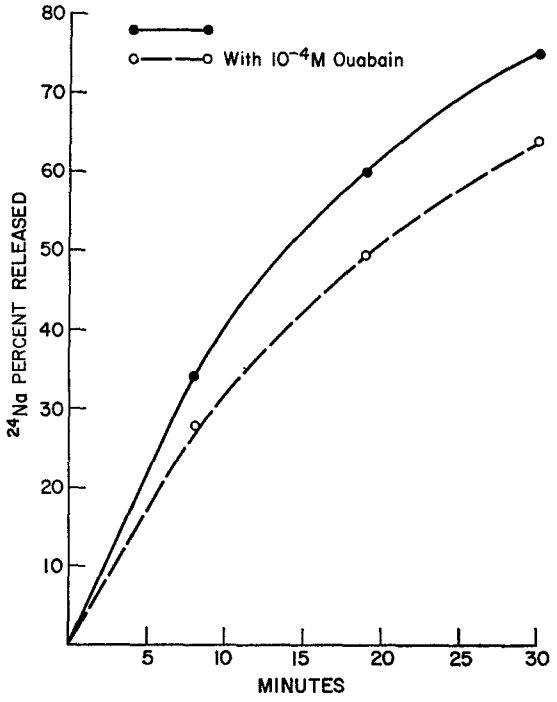

FIGURE 5. Ouabain's effect on the rate of ^{24}Na loss from minimal volume cells. We incubated moderately enlarged high-Na cells for 4 h until they had attained the minimal volume. The procedure has been described in the legend of Fig. 4; cells were preloaded with ^{24}Na during this period. The standard synthetic medium served as both the wash and experimental bathing solution. If the first two points in each curve (0 and 8 min) are used to calculate a rate constant, the flux (mmol/liter$_{ONC}$/h) corresponding to the upper curve is 122 and the lower curve 95. The difference, 27, represents the ouabain-sensitive flux. At 0 time, the cells had the following composition: percent cell H_2O (wt/wt) = 49.8, $[Na]_c$ and $[K]_c$ = 39.7 and 36.3 mmol/liter$_{ONC}$, respectively.

brane potential between 17 and 28 mV. This phenomenon (i.e., an apparent minimal volume for intact cells) will be discussed later.

Intermediate (Na and K) Cells

From Table I one can calculate the amount of cation and specifically the amount of K enlarged high-K cells must lose to return to their original volume. Using exp. 1 as an example, the cells lose 119 mmol of monovalent cat-

ion by 2 h; and gain 7 mmol of Na, therefore they lose 126 mmol of K during the regulatory process. Of course, the magnitude of this quantity will depend, in each instance, on the magnitude of the initial cell volume and the corresponding change in Na content. It corresponds to the minimal amount of K cells must contain to return to their "normal" isotonic volume via the volume controlling mechanism.

Fig. 6 and Table IV show the response of swollen cells which contain ap-

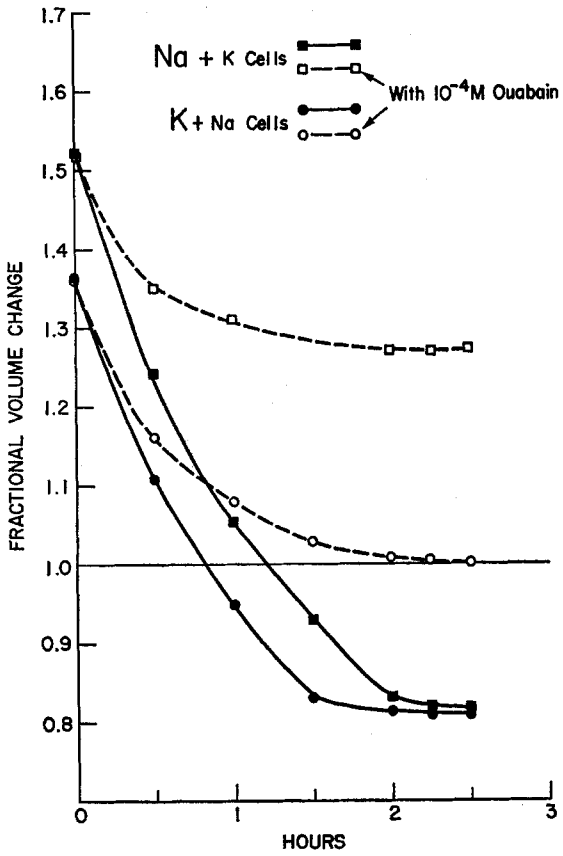

FIGURE 6. The fractional volume change in enlarged intermediate cells as they incubate in the standard synthetic medium with or without 10^{-4} M ouabain (hematocrit 7–8%). Intermediate cells were produced which contain either Na (**Na** + K cells) or K (**K** + Na cells) as the major monovalent cation. All changes in cell volume were referred to the volume of (LSS) control cells which have a fractional volume of 1.0. The rise in $[K]_e$ as the cells lost K necessitated separating and resuspending the cells in fresh media at 80 min. Only those ouabain-treated (**Na** + K) cells which remain enlarged showed evidence of metabolic dysfunction by 1½ h, the color of the cell suspension turns dark red. (**Na** + K) cells had the following composition at 0 time: percent cell H_2O (wt/wt) 72.2, $[Na]_c$ = 154, and $[K]_c$ = 58 mmol/liter$_{ONC}$, while the (**K** + Na) cells had a percent H_2O (wt/wt) of 69.9 and a $[Na]_c$ and $[K]_c$ of 80 and 113 mmol/liter$_{ONC}$, respectively.

TABLE IV

DECREASE IN MONOVALENT CATION CONTENT ASSOCIATED WITH CHANGES IN SIZE OF ENLARGED INTERMEDIATE CELLS

Additions to the standard synthetic medium	$\Delta[K]_c$		$\Delta[Na]_c$		Cell H_2O (wt/wt)		
	½ h	2¼ h	½ h	2¼ h	0	½ h	2¼ h
	mmol/liter$_{ONC}$		*mmol/liter*$_{ONC}$		%	%	%
Na + K cells							
Exp. 1 None	−18	−19	−22	−99	68.6	64.2	50.1
+ 10^{-4} M Ouabain	−25	−42	0	+6	68.6	66.3	65.0
Exp. 2 None	−13	−17	−22	−101	71.8	68.2	51.2
+ 10^{-4} M Ouabain	−18	−40	+1	−2	71.8	70.0	67.6
K + Na cells							
Exp. A None	−34	−78	−15	−60	69.9	63.5	51.1
+ 10^{-4} M Ouabain	−42	−88	+5	+21	69.9	64.2	60.3
Exp. B None	−21	−55	−10	−51	67.8	64.3	50.9
+ 10^{-4} M Ouabain	−30	−63	+10	+15	67.8	64.9	60.1

The decrease in monovalent cation content associated with the changes in cell size illustrated in Fig. 6. The experimental protocol is identical to that described in the legend of Fig. 6. Δ values were obtained by subtracting the values at ½ and 2¼ h from the value at 0 time. At 0 time the Na and K concentration of enlarged (**Na + K**) cells were, respectively: exp. 1, 133 and 53 mmol/liter$_{ONC}$; exp. 2, 151 and 49 mmol/liter$_{ONC}$; while those of enlarged (**K + Na**) cells were: exp. A, 80 and 113 mmol/liter$_{ONC}$; exp. B, 67 and 115 mmol/liter$_{ONC}$.

preciable quantities of both Na and K. Alterations in cell volume are presented in Fig. 6, while Table IV shows the changes in monovalent cation content. Fig. 6 shows that cells containing more K than Na (**K + Na cells**), regulate their volume[4] if the mechanism for removing Na (i.e., the cation pump) has been blocked with ouabain provided the quantity of K is sufficient to allow the volume controlling mechanism to operate (see preceding paragraph). In contrast, cells containing a K content less than this value (**Na + K** cells) fail to regulate their volume and remain swollen under the same experimental conditions. Thus, enlarged cells containing intermediate amounts of Na and K reestablish their original volume only if they contain sufficient K and the mechanism (cation pump) for removing Na has been blocked.

On the other hand, if ouabain is omitted from the bathing medium, so that the cation pump remains operative, both groups of intermediate cells (**K + Na** and **Na + K** cells) act as high-Na cells continuing to shrink below

[4] Although these cells reduce the rate of K loss and stabilize their size by 2 h as they approach the LSS volume, it should be pointed out that the 2-h "stabilized condition" is not entirely similar to that of the 2-h high-K cells illustrated in Fig. 1 and Table II. The two cell populations differ in their cation content, transport characteristics, and metabolism. For instance the net Na and K movements associated with the cation pump are absent in the ouabain-treated cells illustrated here.

their original volume until either they attain the minimal volume or $[Na]_e$ approaches its normal value.[5] In Fig. 6 and Table IV both groups of cells shown reach the minimal volume. Notice that intermediate cells shrink at a rate between that seen in high-K cells and high-Na cells (See Figs. 1 and 4.)

Thus, in the absence of ouabain, cells containing both Na and K lose electrolyte through both mechanisms, but the cation pump, responding to an elevation in the internal Na concentration, predominates. These studies also establish that the two mechanisms (cation pump and volume controlling mechanism) are functionally separate entities. Earlier publications suggested this previously (1, 2, 3).

Studies on the Membrane Pathway

Although the preceding studies demonstrate that the two mechanisms differ, the possibility remains that they share a portion of the same membrane pathway yet are otherwise spatially separate. Accordingly, experiments were designed to answer whether the volume controlling mechanism and cation pump used the same or different pathways. If the same pathway were used by both, one would expect some competition between Na and K for this route. The presence or absence of K exiting through the volume controlling mechanism should alter the rate at which Na leaves through the cation pump and vice versa.

Experimentally the problem can be approached in two ways. In the first, we utilized enlarged cells with nearly equivalent quantities of Na and K. These cells lose both Na (~20 mmol by ½ h) and K (~30 mmol by ½ h) at rapid rates; Na leaves via the cation pump and K through the volume controlling mechanism. Fig. 7 shows that eliminating Na loss by inhibiting the cation pump with ouabain does not affect the initial rate at which K ions leave the cells (measured here by using ^{42}K). The rate of K loss is identical in the presence or absence of ouabain. The dotted line indicates the expected increase in rate of ^{42}K loss from the ouabain-treated cells, if eliminating Na movement by using ouabain had allowed an equivalent amount of K to enter the pathway. For this calculation, Na loss through the cation pump was determined by noting the difference in the Na content of cells treated with and without ouabain during the 15-min interval.

In the second approach, we compared the initial rate of Na loss through the cation pump in enlarged high-Na cells and enlarged cells containing an equivalent amount of Na and K (Na + K cells). High-Na cells do not lose K through the volume controlling mechanism, but actually gain several millimoles during the first ½ h; in contrast, (Na + K) cells lose appreciable quantities of K (>30 mmol by ½ h). Na loss through the cation pump was de-

[5] <16 mmol/liter cell H_2O. This value is approximate and has been determined from observations on both high-Na and intermediate cells.

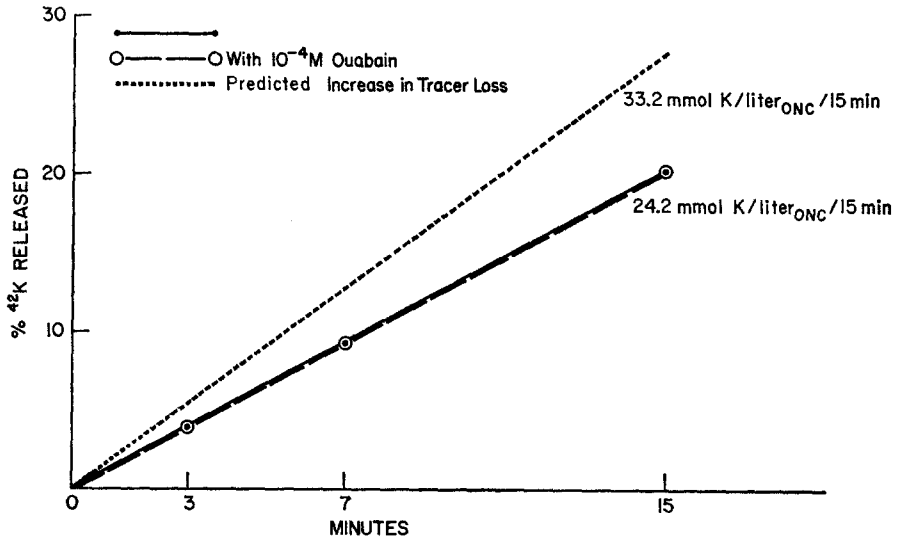

FIGURE 7. Ouabain's effect on the rate of ^{42}K loss from enlarged cells containing nearly equivalent amounts of Na and K. Cells were preloaded with tracer by adding a small quantity of isotope to the media used during the cation-loading and resealing stages. The standard synthetic medium served as both the wash and experimental bathing solution. The 15-min values at the end of each line represent calculated flux values. At 0 time the cells had a percent cell H_2O (wt/wt) of 68.9 and a Na and K content, respectively, of 93 and 104 mmol/liter$_{ONC}$.

termined in both groups by noting the difference in Na content of cells treated with and without ouabain after 30-min incubation. The $[Na]_c$ in both groups of cells is in the range where the effect of $[Na]_c$ on cation pumping rate is maximal.[6] Table V demonstrates that Na loss is the same in both groups of cells despite the marked difference in net K loss.

Thus, both experimental approaches suggest that the pathway used by the cation pump to eject Na is separate from the route used by the volume controlling mechanism to remove K.

To decide whether K but not Na has a privileged relationship with the volume controlling mechanism and its associated pathway, we performed the following experiment on enlarged cells containing equivalent quantities of Na and K. Tracer measurements showed that the rate constant for the initial 10-min ouabain-insensitive component of Na efflux was 0.018 ± 0.002 compared to a value of 0.191[7] ± 0.006 for the corresponding K efflux rate constant. It was assumed that Na would exit as part of the ouabain-insensi-

[6] In duck erythrocytes as in human red cells (9, 10) ouabain-inhibitable Na loss is a function of $[Na]_c$. In both, this effect plateaus at high $[Na]_c$. Table V shows that in duck red cells ouabain-inhibitable Na loss rises three- to fourfold with increasing $[Na]_c$, plateauing at a $[Na]_c$ below 81 mmol/liter cell H_2O (the smallest concentration present in any of these cells).

[7] Mean ± SD n = 3.

TABLE V

A COMPARISON OF THE RATE OF Na LOSS THROUGH THE Na-K EXCHANGE PUMP IN ENLARGED HIGH-Na AND INTERMEDIATE (Na + K) CELLS

	Enlarged intermediate (Na + K) cells net Na loss	Enlarged high-Na cells net Na loss
	mmol	*mmol*
Exp. 1	20.4	21.4
Exp. 2	15.7	14.2
Exp. 3	18.6	17.5
Exp. 4	19.6	19.2
Average	18.5	18.1
Corresponding net K loss through the volume controlling mechanism (>30 mmol by ½ h).		Corresponding net K loss through the volume controlling mechanism (essentially zero).

Enlarged cells, containing mostly Na (<3 mmol K) or intermediate cells containing nearly equivalent amounts of Na + K were incubated in the standard synthetic medium ($[K]_o$ between 2.5–3.5 mM). This concentration of K is in the maximum stimulatory range for ouabain-sensitive Na outflux under these conditions. Na loss through the cation pump was determined by noting the difference in the Na content of cells treated with and without ouabain, during the first 30-min interval. K loss through the volume controlling mechanism was assayed by noting the loss in K content during the same interval. Each experiment was paired, the cells originating from a single duck; each measurement was the average of duplicate determinations. The Na concentration (mmol/liter cell H_2O) at 0 time for intermediate cells in exp. 1 through 4 were, respectively: 81, 84, 88, and 94; the corresponding Na concentration of all high-Na cells varied between 168–172.

tive component of Na efflux if it were to use the volume controlling mechanism. This component of Na efflux has a rate constant an order of magnitude smaller than K efflux which as shown in Fig. 2 largely represents K loss through the volume controlling mechanism.

Thus, this experiment suggests that K but not Na can effectively use this aspect of the volume controlling mechanism and its associated pathway in the duck red cell. It should be pointed out, however, that this does not mean Na is excluded from this pathway. For such a statement would require experiments, not yet feasible, involving precise measurements and separation of those components of ouabain-insensitive Na efflux.

DISCUSSION

One can now conceptualize some of the membrane events that occur when enlarged duck red cells regulate their volume. Fig. 8 depicts how this aspect of the volume controlling mechanism might operate.

Tosteson and Hoffman (11, 12) consider the steady-state volume and cation composition of cells to result from the action of a Na-K exchange pump working in parallel with leaks for both of these ions. This generally accepted view is presented schematically here. The Na + K exchange pump, desig-

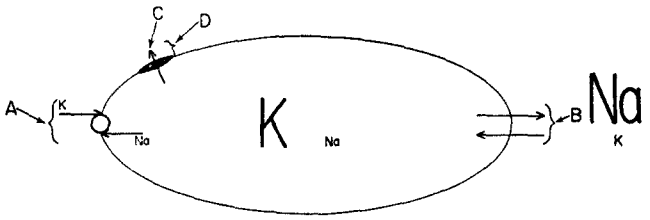

FIGURE 8. Depiction of some of the membrane events that might occur when enlarged duck red cells use the volume controlling mechanism to regulate their volume.

nated A in the drawing, is shown as a coupled mechanism moving Na out of and K into the cell, both net movements occurring against their respective electrochemical gradients. The diffusional (leak) pathway or pathways for Na and K are designated B in the drawing.

The findings presented in this paper and elsewhere (1, 3) show that under unsteady-state conditions a membrane pathway, C, becomes apparent in duck erythrocytes. It is through this pathway that the volume controlling mechanism, designated D, controls K loss from enlarged cells. C has been drawn as part of the volume controlling mechanism, in this case as part of the effector portion. Note that the pathway C is different from the route taken by Na as it is ejected by the Na-K exchange pump. It should be pointed out that we can't at the present time exclude the possibility that pathway C is identical to the diffusional pathway for K, labeled B in the drawing.

Recent studies indicate that other vertebrate cells, besides avian erythrocytes, contain the volume controlling mechanism. The studies of Roti Roti and Rothstein (13) show that the mechanism is present in mammalian cells. These authors have reached a similar mechanistic explanation of how swollen mouse lymphoblasts, incubated in hypotonic media, reacquire their original volume. Recent studies by Parker (14, 15) indicate that the volume-dependent permeability changes of dog and cat red cells, known from earlier studies (16–19) may, in fact, represent a similar mechanism. However, there are marked differences between the responses of enlarged duck red cells, normally high-K cells, and enlarged dog red cells, normally high-Na cells, as they reacquire their original volume.

Our finding that Na is not the preferred cation in enlarged duck erythrocytes coupled with new findings on volume regulation in dog red cells (20), raises the interesting possibility that dog red cells have the effector portions of the mechanism reversed. If this hypothesis were correct, the transport process responsible for reducing the size of enlarged duck erythrocytes would cause shrunken dog red cells to swell. (In both cells, these transport processes appear to be diffusional in nature.) Similarly, the process responsible for enlarging shrunken duck erythrocytes would function instead to shrink swollen dog red cells. (In both cells, these transport processes are similar in that they

now produce net cation movement against an electrochemical gradient. Both processes also depend, in some way, upon the presence of an extracellular cation.)

The observation that cells reach a minimal volume and shrink no further is in itself interesting, for it indicates that duck red cells, incubating in isotonic media, contain a mechanism for limiting their reduction in size. It is therefore worthwile to examine some of the characteristics of these shrunken cells especially those related to the possible control of electrolyte content.

Minimal volume cells contain 73[8] ± 7.2 mmol/liter$_{ONC}$ of total (Na + K) monovalent cations. Cells have been obtained in which either Na (150 mmol/liter cell H_2O) or K (121 mmol/liter cell H_2O) was the predominant cation. Data, presented in Results, show that these cells have an elevated chloride ratio and therefore an elevated membrane potential and that they also display the Na and K fluxes associated with an active cation pump. Perhaps most interesting, however, is the presence of an enormous bidirectional increase in ouabain-insensitive Na and K movements (often >100 mmol/liter$_{ONC}$/h). These movements resemble in many respects those seen when duck red cells incubate in hypertonic media (2). The control of cation content and movement in minimal volume cells is the subject of a future publication.

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REFERENCES

1. KREGENOW, F. M. 1971. The response of duck erythrocytes to non-hemolytic hypotonic media. Evidence for a volume-controlling mechanism. *J. Gen. Physiol.* **58**:372.
2. KREGENOW, F. M. 1971. The response of duck erythrocytes to hypertonic media. Further evidence for a volume-controlling mechanism. *J. Gen. Physiol.* **58**:396.
3. KREGENOW, F. M. 1973. The response of duck erythrocytes to norepinephrine and an elevated extracellular potassium. Volume regulation in isotonic media. *J. Gen. Physiol.* **61**:509.
4. GARRAHAN, P. J., and A. F. REGA. 1967. Cation loading of red blood cells. *J. Physiol. (Lond.)*. **193**:459.
5. RIDDICK, D. H., F. M. KREGENOW, and J. ORLOFF. 1971. The effect of noradrenaline and dibutyryl cyclic-AMP on cation transport in duck erythrocytes. *J. Gen. Physiol.* **57**:752.
6. SACHS, J. R. 1970. Sodium movements in the human red blood cell. *J. Gen. Physiol.* **56**:322.
7. HOFFMAN, P. G., and D. C. TOSTESON. 1971. Active sodium and potassium transport in high potassium and low potassium sheep red cells. *J. Gen. Physiol.* **58**:438.
8. MAIZELS, M. 1949. Cation control in human erythrocytes. *J. Physiol. (Lond.)*. **108**:247.
9. POST, R. L., and P. C. JOLLY. 1957. The linkage of sodium, potassium and ammonium active transport across the human erythrocyte membrane. *Biochim. Biophys. Acta.* **25**:118.
10. POST, R. L., C. R. MERRITT, C. R. KINSOLVING, and C. D. ALBRIGHT. 1960. Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte membrane. *J. Biol. Chem.* **235**:1796.
11. TOSTESON, D. C., and J. F. HOFFMAN. 1960. Regulation of cell volume by active transport in high and low potassium sheep red cells. *J. Gen. Physiol.* **44**:169.
12. TOSTESON, D. C. 1964. Regulation of cell volume by sodium and potassium transport. *In*

[8] Mean ± SD (n = 12).

The Cellular Function of Membrane Transport. J. F. Hoffman, editor. Prentice-Hall, Inc., Englewood Cliffs, N. J.

13. ROTI ROTI, L. W., and A. ROTHSTEIN. 1973. Adaptation of mouse leukemic cells (L5178Y) to anisotonic media. I. Cell volume regulation. *Exp. Cell Res.* **79**:295.
14. PARKER, J. C. 1973. Dog red blood cells. Adjustment of density in vivo. *J. Gen. Physiol.* **61**:146.
15. PARKER, J. C. 1973. Dog red blood cells. Adjustment of salt and water content in vitro. *J. Gen. Physiol.* **62**:147.
16. DAVSON, H. 1940. Ionic permeability. The comparative effects of environmental changes on the permeability of the cat erythrocyte membrane to sodium and potassium. *J. Cell Comp. Physiol.* **15**:317.
17. DAVSON, H. 1942. The haemolytic action of potassium salts. *J. Physiol.* (*Lond.*). **101**:265.
18. HOFFMAN, J. F. 1966. The red cell membrane and the transport of sodium and potassium. *Am. J. Med.* **41**:666.
19. SHA'AFI, R. I., and J. J. HAJJAR. 1971. Sodium movement in high sodium feline red cells. *J. Gen. Physiol.* **57**:684.
20. PARKER, J. C. 1974. Calcium-dependent volume regulation by dog red cells. *Fed. Proc.* **33**:360.