The Response of Duck Erythrocytes to Hypertonic Media

Further evidence for a volume-controlling mechanism

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ABSTRACT The addition of a hypertonic bathing medium to duck erythrocytes results in an initial instantaneous phase of osmotic shrinkage and, when the $[K]_o$ of the hypertonic solution is larger than "normal," in a second, more prolonged phase, the *volume regulatory phase*. During the latter, which also requires extracellular Na, the cells swell until they approach their initial isotonic volume. The increase in cell volume during the volume regulatory phase is accomplished by a gain in the cell content of K, Cl, and H_2O. There is also a smaller increase in the Na content of the cell. Potassium is accumulated against an electrochemical gradient and is therefore actively transported into the cell. This accumulation is associated with an increase, although dissimilar, in both K influx and efflux. Changes in cell size during the volume regulatory phase are not altered by 10^{-4} M ouabain, although this concentration of ouabain does change the cellular cation content. The response is independent of any effect of norepinephrine. The changes in cell size during the volume regulatory phase are discussed as the product of a volume controlling mechanism identical in principle to the one reported in the previous paper which controls cell volume in hypotonic media. Similarly, this mechanism can regulate cell size, when the Na-K exchange, ouabain-inhibitable pump mechanism is blocked.

INTRODUCTION

The previous paper demonstrated that duck erythrocytes are capable of reestablishing their approximate isotonic volume in nonhemolytic hypotonic media after an initial phase of swelling (Kregenow, 1971). In this communication the response of duck erythrocytes to hypertonic media was examined and compared to that in hypotonic media. Duck red cells can also readjust their size in hypertonic media, returning toward their initial volume after first shrinking. This, however, occurs only if the $[K]_o$ of the hypertonic

bathing solution is elevated above "normal levels." The cell's capacity to regulate its volume is, therefore, latent, dependent also upon a change in the bathing solution. There are a number of other important differences between the two responses, besides the fact that the changes in cell volume are in opposite directions, and the hypertonic response requires an increase in the $[K]_o$. These differences will be documented and discussed.

As with the response in hypotonic media, volume regulation in hypertonic media can be divided empirically into two phases: an initial rapid osmotic phase and a more prolonged volume regulatory phase. The over-all response of duck erythrocytes in hypertonic media is considered as another example of "isosmotic intracellular regulation" and the volume regulatory phase as the product of a volume controlling mechanism with receptor, transmitter, and effector properties. The nonidentical nature and character of the K changes in hypertonic media when compared to those in hypotonic media indicates that the effector portion of the proposed mechanism is dissimilar in the two media. The cell, therefore, utilizes different membrane pathways to affect the final cation changes.

MATERIALS AND METHODS

The essential features of the materials and methods employed in this paper have been described previously (Riddick et al., 1971; Kregenow, 1971). The NaCl content of the standard synthetic medium (π = 323 mosmols) was increased to produce two hypertonic solutions (π = 373 mosmols and 435 mosmols). These will be referred to as the mildly and the moderately hypertonic medium, respectively. Low sodium media were prepared by replacing the NaCl and Na bicarbonate with an isosmotic quantity of $MgCl_2$ and $Mg(HCO_3)_2$ or choline chloride and choline bicarbonate.[1] Medium potassium concentration was increased, when necessary, by replacing an isosmotic quantity of NaCl, $MgCl_2$, or choline Cl with KCl. A value of 1.3 ± 0.1% using inulin-^{14}C was found to represent the percentage of medium trapped between the cells during centrifugation in all media. The standard error of the mean for the trapped plasma measurement in the moderately hypertonic medium, although still within the standard error of the mean given above, was greater than in either of the other two media. The methods for calculating the theoretical and experimental changes in cell volume (Fig. 1) were described in the previous paper (Kregenow, 1971). Placing cells in a hypertonic solution, however, causes the cells to shrink, a volume change in the opposite direction from the change in hypotonic media.

At the time of the chloride analysis, the possible variations in the pH of the supernatant fraction were as described previously (Kregenow, 1971). The errors in the chloride ratio $[Cl]_o/[Cl]_i$ introduced by these variations are less in the hypertonic media than they were in the hypotonic media since both the numerator and denominator are now larger. The errors are maximally 0.02 units in both the mildly and moderately hypertonic solutions at zero time and 0.05 units for any of the values analyzed serially (Table I).

[1] Choline bicarbonate, 44% aqueous solution; Matheson, Coleman, and Bell, Norwood, Ohio.

Freshly drawn duck erythrocytes lose approximately 8 mM K and 2% of their cell water (w/w) during the first 90 min of incubation in the standard synthetic medium before stabilizing at a new lower steady-state level (Riddick et al., 1971). Upon continued incubation, the cells remain in this lower steady state for at least an additional 90 min. Only lower steady-state cells ($[K]_c \sim 110$ mM/L, $[NA]_c \sim 5$ mM/L, and $[Cl]_c \sim 51$ mM/L)[2] were employed in this study. They were obtained by preincubating freshly drawn cells for 90 min as described previously. The centrifugation procedure required to remove the preincubation medium from the cells before adding the cells to the experimental media was limited to 1 min.

Propranolol (10^{-4} M) was introduced to all experimental bathing solutions just before adding the cells. The addition of propranolol was necessary to insure that the system was not under the influence of norepinephrine. Duck erythrocytes, when incubated with the catecholamine, norepinephrine, gain K^+, Na^+, Cl^-, and H_2O from an isotonic medium in a somewhat identical fashion to the uptake demonstrated in this paper. This concentration of propranolol, a beta adrenergic blocking agent, is sufficient to inhibit the norepinephrine effect (Riddick et al., 1971).

Potassium and sodium influxes were calculated by use of equation 1 and potassium efflux by use of equation 3 (see Kregenow, 1971). As in the experiments in hypotonic bathing solutions, a membrane surface area change may occur as the cells undergo volume changes in the anisotonic media. The possible decrease in surface area associated with the initial shrinkage in the most hypertonic solution would introduce only a 5% error in the absolute value of the K influx measurement (millimoles per unit area of membrane). An error of this magnitude would not alter any of the conclusions drawn from the influx and efflux measurements.

RESULTS

The initial portion of the response of duck erythrocytes to hypertonic media has been labeled the osmotic phase. When duck erythrocytes are placed in solutions of increasing osmolality, they behave as osmometers. Their reduction in cell volume is proportional to the increase in the tonicity of the bathing medium. The decrease in cell volume however is less than one would expect if the cells behaved as "ideal osmometers" (Fig. 1). The decrease in cell volume in both experimental hypertonic solutions is only slightly more than half (55%) of the expected theoretical decrease. This is in contrast to the 66% discrepancy found when duck red cells were introduced to hypotonic media (Kregenow, 1971). When analyzed in this fashion, the changes in cell size during the osmotic phase are relatively smaller in hypertonic media than in hypotonic media. A similar relationship exists in human erythrocytes when the magnitude of "bound water" is compared in hypotonic and hypertonic media (for a review, see Dick, 1969).

Fig. 2 demonstrates that duck erythrocytes, after first shrinking in hypertonic media, are able to gain water and approach the volume existent in

[2] mM/L = mM/liter of red blood cells.

isotonic media. This portion of the response has been labeled the volume regulatory phase. The readjustment is not perfect, but approaches the original (lower steady-state) cell volume by 90 min if the increase in tonicity does not exceed 110 mosmols. Cells incubated in solutions more hypertonic than this fail to reach this volume even when incubated for 150 min. In contrast, no such limit, short of hypotonic hemolysis, appears to exist for the hypotonic volume regulatory phase. Fig. 2 also demonstrates the dependence of the

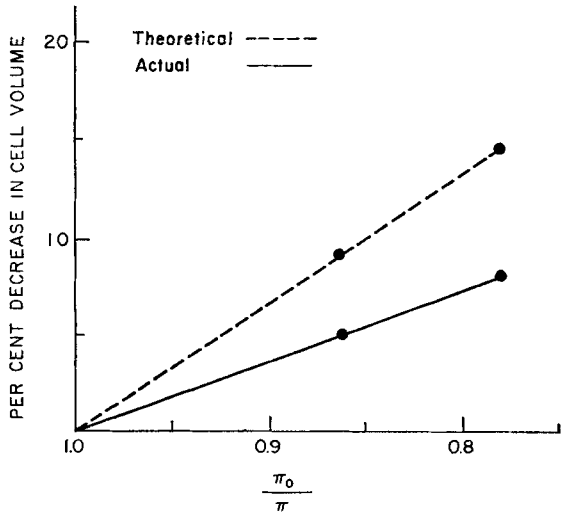

FIGURE 1. The initial decrease in the theoretical and experimental cell volume of duck erythrocytes incubated in hypertonic media. Duck erythrocytes were introduced into isotonic or hypertonic media. Determinations of cell water, cell specific gravity, and osmolality of the supernatant were performed on an aliquot of the cells and bathing medium obtained at zero time (approximately 2 min after introducing the cells to the experimental solutions).

hypertonic volume regulatory phase upon an elevation of the potassium concentration in the bathing medium. Simply increasing the tonicity without raising $[K]_o$ (two lower dashed lines), or raising $[K]_o$ without increasing the tonicity (upper solid line), fails to produce any cell enlargement.

Table I demonstrates the net electrolyte changes associated with the movement of cell water demonstrated in Fig. 1 and 2. The electrolyte alterations at zero time are associated with the loss of cell water illustrated in Fig. 1, the osmotic phase. The net electrolyte changes associated with the volume regulatory phase, Fig. 2, can be obtained by subtracting the zero time sample from the 30, 60, and 90 min values.

During the osmotic phase, there is a significant gain in the total (Na + K) monovalent cation content of the initial cell sample only in the moderately

hypertonic bathing solution (3.3 ± 0.9[3] mM/L_{onc}). However, there is a net gain in chloride, the major monovalent anion in both hypertonic media. Cook (1967) and Gary-Bobo and Solomon (1968) have demonstrated that the human erythrocyte gains chloride under similar circumstances. The gain in cell chloride demonstrated here, if accompanied by a gain in cell H_2O sufficient to maintain osmotic equilibrium, would explain only part (approximately ¼) of the discrepancy between the initial experimental decrease in cell volume and that of an ideal osmometer. This initial gain in chloride content tends to reduce the expected increase in the chloride ratio ($[Cl]_o/[Cl]_i$) as the cells shrink.

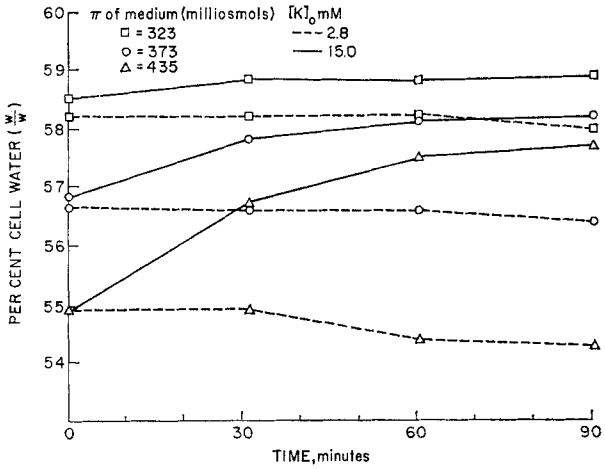

FIGURE 2. The volume regulatory phase of duck erythrocytes in hypertonic media. Duck erythrocytes were incubated in an isotonic or hypertonic synthetic media with a $[K]_o$ of either 2.8 or 15.0 mM. The experiment illustrated here is representative of five others.

During the volume regulatory phase, potassium is the major cation and chloride the major anion accumulated. The accumulation of potassium is against an electrochemical gradient (membrane potential is less than 15 mv—inside negative)[4] and as such is an example of active transport. This is in contrast to the loss of potassium in the hypotonic volume regulatory phase which was with the electrochemical gradient. There is also a small gain in the cell sodium content. The uptake of chloride together with the additional expected uptake of a small quantity of bicarbonate is sufficient or more than sufficient to maintain electroneutrality across the membrane as cation movement occurs. If all of the accumulated potassium and sodium is osmotically

[3] SE of the mean, where number of experiments (N) = 9, $P < 0.05$.

[4] This potential has been calculated from the chloride ratio and is probably in excess of the actual in vivo value since the loss of CO_2 from the cell suspension before the analysis increases this ratio.

TABLE I

NET Na^+, K^+, AND Cl^- CHANGES ASSOCIATED WITH THE VOLUME RESPONSE OF DUCK ERYTHROCYTES IN HYPERTONIC MEDIA WITH A $[K]_o$ OF APPROXIMATELY 18 mM

Exp.	Medium osmolality	$[K]_o$	ΔK in mM/L_{cells} Time (min)				ΔNa in mM/L_{cells} Time (min)				ΔCl in mM/L_{cells} Time (min)				$[Cl]_o/[Cl]_i$ Time (min)			
			0	30	60	90	0	30	60	90	0	30	60	90	0	30	60	90
No.	*mosmols*	*mM*																
1	373	17.2	+1.1	+4.7	+8.4	+8.7	+0.1	+2.1	+2.6	+1.9	+4.8	+7.4	+9.2	+11.3	1.78	1.71	1.71	1.68
	435	17.4	+3.2	+10.3	+14.8	+18.6	+0.3	+4.4	+4.0	+3.4	+5.9	+13.7	+19.1	+20.5	1.84	1.73	1.62	1.63
2	373	18.1	+1.1	+ 6.0	+9.8	+11.7	+0.6	+3.0	+2.9	+2.8	+5.1	+10.0	+13.8	+15.2	1.74	1.66	1.61	1.51
	435	18.8	+2.2	+11.8	+17.5	+17.4	+0.9	+5.2	+4.7	+4.3	+8.2	+16.8	+20.7	+24.2	1.73	1.64	1.59	1.54

Duck erythrocytes were incubated in isotonic (π = 323 mosmols) or hypertonic media (π = 373 or 435 mosmols). The $[K]_o$ was approximately 18 mM in the hypertonic media and 2.5 mM in the isotonic medium. Cells incubated in the isotonic medium remained in the lower steady state during the experimental period and served as controls. Δ values were obtained by calculating the difference between the value for cells in the hypertonic medium and the control value at each time. The control steady-state chloride ratio $[Cl]_o/[Cl]_i$ for Exp. 1 was 1.71 and was 1.69 for Exp. 2.

active and there is an additional uptake of an osmotically equivalent number of monovalent anions, the net increase in osmotic particles is larger than what would be expected if the water uptake were an isosmotic adjustment. The calculated osmolality of the additional water accumulated in the mildly hypertonic medium (π = 373 mosmols) is 600 ± 90[5] mosmols and 540 ± 40[5] mosmols in the moderately hypertonic medium (π = 435 mosmols). This differs from a similar measurement in the hypotonic volume regulatory phase which showed that the changes in cell volume were essentially isosmotic adjustments at least in two of the three hypotonic solutions. There are a number of explanations for this apparent osmotic disequilibrium; the present evidence does not help to eliminate any of the possibilities. The chloride ratio $[Cl]_o/[Cl]_i$ increases initially in hypertonic media and then decreases as the cells readjust their volume, indicating a change in the membrane potential that is the inverse of the changes that develop during the hypotonic volume regulatory phase.

Fig. 3 shows the calculated total monovalent cation concentration (millimoles Na + K per liter of cell H_2O) for cells incubated in the moderately hypertonic media. After the initial stage of osmotic shrinkage and the concomitant rise in this value, there is a further small increase with time in those cells which swell and regulate their volume (hypertonic medium, $[K]_o$ = 19.0 mM), but not in those that remain shrunken (hypertonic medium, $[K]_o$ = 2.6 mM). An analysis of the Na and K concentration values separately enlarges upon this observation. In both groups of cells the Na concentration value is elevated. The Na concentration of the shrunken cells increases 4.4 ± 0.5[6] mM/liter cell H_2O by 60 min while the comparable value for cells which have reverted towards their original volume is 3.6 ± 0.5[6] mM/liter cell H_2O. The potassium concentration, on the other hand, decreases in the shrunken cells while it either remains the same or increases slightly in the enlarging cells. It is the latter response combined with the increase in the sodium concentration which causes the concentration of the total monovalent cations to increase as the cells readjust their volume. Such a response is to be expected if there is an apparent hypertonic uptake of monovalent cations.

Since an increase in the extracellular potassium concentration is a requirement for the volume regulatory phase, this dependence was examined further in the moderately hypertonic medium (Fig. 4). As the $[K]_o$ is increased from 2.5 to 15 mM, the cells swell. The volume of cells at 30 min is greater the higher the potassium concentration. The response reaches a plateau at a $[K]_o$ of 15 mM.

Table II shows the changes in the cellular monovalent cation content associated with the response of cells illustrated in Fig. 4. As the $[K]_o$ increases,

[5] SE of the mean, N = 6.

[6] SE of the mean, N = 6, $P < 0.01$.

the potassium and water movements accompany one another. The time when the rate at which the increase in Na content begins to decline is also $[K]_o$ dependent. A $[K]_o$ of 10 mM is required before a decrease becomes apparent during the 30–60 min interval. Only at a $[K]_o$ approaching 15 mM does the Na content remain unchanged or fall slightly during this period.

The results of the experiments summarized in Table III demonstrate that the volume regulatory phase is $[Na]_o$ dependent as well. Cells were incubated

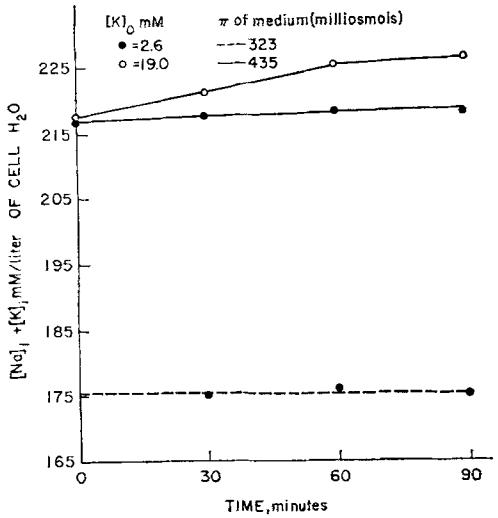

FIGURE 3. The total monovalent cation ($Na^+ + K^+$) concentration (millimoles per liter cell H_2O) of duck erythrocytes during the volume regulatory phase in the moderately hypertonic medium. Duck erythrocytes were incubated in the isotonic or moderately hypertonic medium (π = 323 and 435 mosmols, respectively). The $[K]_o$ of the latter solution was either 2.5 or 19 mM. The experiment illustrated here is representative of six others.

in hypertonic solutions (π = 435 mosmols) with two different extracellular potassium concentrations. The experimental hypertonic solutions have a $[K]_o$ of 19 mM, a maximally effective concentration, while the control hypertonic solutions have a $[K]_o$ of approximately 2.5 mM. As seen previously, when sodium is present in the bathing solution, cells incubated in the former approach their original isotonic volume while cells incubated in the latter do not. Substitution of either choline or Mg for sodium in the experimental hypertonic media eliminates the otherwise expected accumulation of electrolytes and water.

Since the important cellular change during the volume regulatory phase appears to be a $[K]_o$-dependent active accumulation of potassium, cells were incubated with the cardiac glycoside, ouabain, an agent known to alter the Na and K content of duck erythrocytes (Allen, 1967) and to inhibit the Na-K

exchange pump mechanism in erythrocytes generally. The results in Fig. 5 show that ouabain (10^{-4} M) does not interfere with the changes in cell volume that develop during the volume regulatory phase nor does it appreciably alter the volume of control cells either in hypertonic medium ($[K]_o$ = 2.5 mM) or in standard isotonic medium ($[K]_o$ = 2.5 or 19.0 mM). (For the effect of 10^{-4} M ouabain on cells incubated in an isotonic solution, $[K]_o \sim 2.5$ mM, see Table V and Fig. 6 of the previous paper [Kregenow, 1971].) However, ouabain does change the Na and K content of these cells (Table IV). Control

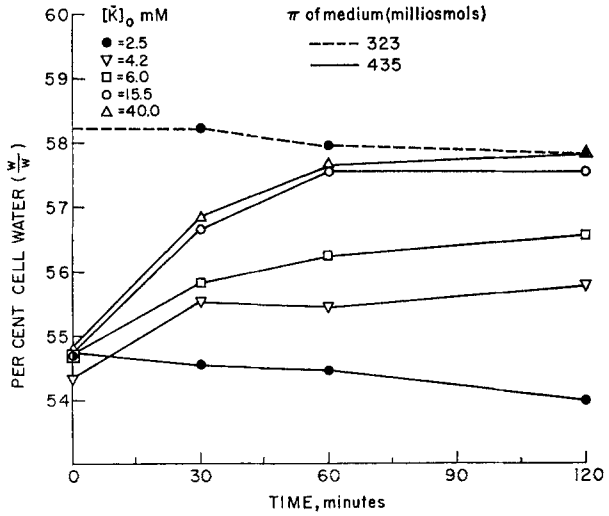

FIGURE 4. The increase in the cell H_2O of duck erythrocytes incubated in hypertonic media (π = 435 mosmols) in response to raising the $[K]_o$ from 2.5 to 40.0 mM. Duck erythrocytes were incubated in the isotonic or moderately hypertonic media. The $[K]_o$ of the latter medium was varied between 2.5 and 40.0 mM. The experiment illustrated here is representative of four others.

cells gain between 3 and 7 mmole Na and lose between 4 and 7 mmole K by 90 min. For cells undergoing the volume regulatory phase, sodium instead of potassium is now the major cation accumulated. These cells contain between 12 and 15 mmole more Na and 12–15 mmole less K after incubating 90 min with ouabain. Identical results are obtained when the concentration of ouabain is increased to 10^{-3} M. A gain in the Na content and a fall in the K content of a cell incubated in the presence of ouabain is usually considered to be the consequence of inhibition of the Na-K exchange pump mechanism. In the duck erythrocyte, then, the pump mechanism operates at an exchange ratio of approximately 1:1. The greater accumulation of Na in the ouabain-treated cells during the volume regulatory phase suggests the following: (*a*) approximately 6 mmole Na (difference between the experimental [hypertonic,

$[K]_o$ = 19 mM] and isotonic and hypertonic control values) accumulates during the response under normal circumstances. (*b*) In the absence of ouabain this accumulation of Na is converted to a gain in potassium by the pump mechanism through an Na-K exchange. (*c*) The pump mechanism operates more rapidly than normal during the volume regulatory phase but at an identical exchange ratio.

A large proportion of the K uptake however is still ouabain insensitive. For

TABLE II

THE CHANGE IN Na^+, K^+, AND H_2O CONTENT OF DUCK ERYTHROCYTES IN RESPONSE TO VARYING THE $[K]_o$ OF A HYPERTONIC MEDIUM

Exp.	$[K]_o$	ΔK_c, mM/L_{one} At 90 min	ΔNa_c, mM/L_{one} Time (min) 30	60	90	Δ% H_2O (w/w) At 90 min
No.	mM					
1	2.8	−4.7	+1.2	+2.0	+4.5	−0.6
	6.1	+10.6	+2.4	+3.0	+3.1	+2.0
	16.3	+20.2	+3.4	+2.9	+2.8	+2.9
	38.9	+21.3	+5.5	+3.9	+2.3	+3.1
2	2.9	+1.1	+1.7	+1.9	+2.9	0
	6.3	+10.9	+2.1	+2.7	+2.1	+2.4
	16.5	+16.8	+3.9	+3.4	+1.7	+3.2
	39.9	+16.5	+3.8	+2.7	+2.3	+3.0
3	3.0	−2.7	+1.1	+2.4	+2.7	0
	6.3	+12.6	+3.3	+3.2	+3.3	+2.5
	20.5	+19.7	+3.7	+3.6	+3.5	+3.2
	39.5	+18.7	+3.8	+3.5	+3.2	+3.3

Duck erythrocytes were incubated in the moderately hypertonic medium (π = 435 mosmols) whose $[K]_o$ varied between 2.5 and 40 mM. Control cells incubated in the isotonic medium ($[K]_o$ = 2.5 mM) remained in the lower steady state during the experimental period. Δ values were obtained by using the zero time sample as a base line.

instance in the first experiment in Table IV, ouabain might be expected to inhibit all of the normal K uptake (18 mM/L_{one}) plus an additional 5 mM/L_{one}, the quantity inhibited in either isotonic or hypertonic control cells. This amounts to approximately 23 mM/L_{one} instead of the 12 mM/L_{one} found experimentally. Therefore, 10–12 mmole of the normal 18 mmole of potassium normally accumulated during the response is ouabain insensitive. This is an uptake of K that still satisfies the criteria for active transport (accumulation against an electrochemical gradient). It is also dependent upon the presence of Na in the bathing medium (see Table III). It is by definition, then, an $[Na]_o$-sensitive, ouabain-insensitive, active accumulation of potassium.

TABLE III

THE EFFECT OF REPLACING $[Na]_o$ ON THE VOLUME REGULATORY PHASE OF DUCK ERYTHROCYTES IN THE MODERATELY HYPERTONIC MEDIUM

Exp.	Hypertonic medium, 435 mosmols	$[K]_o$	ΔK_c, mM/L one At 90 min	ΔNa_c, mM/L one At 90 min	Δ% cell H_2O (w/w) At 90 min
		mM			
A	Na^+	2.5	−0.3	+2.0	+0.3
		19.0	+15.8	+4.0	+3.0
	Mg^{++}	2.5	−2.1	−2.1	−0.2
		19.0	−0.9	−1.0	−0.2
	$Choline^+$	2.5	−2.2	−3.6	−0.9
		19.0	−3.0	−1.7	−0.7
B	Na^+	2.5	−0.6	+3.0	+0.3
		19.0	+13.8	+3.1	+3.1
	Mg^{++}	2.5	−3.0	−3.0	−0.9
		19.0	−1.7	−1.7	−0.5
	$Choline^+$	2.5	−4.4	−3.4	−1.1
		19.0	−1.7	−2.5	−0.9

Duck erythrocytes were incubated either in the standard or low sodium (Mg^{++} or $choline^+$) moderately hypertonic medium. The potassium concentration was either 2.5 or 19.0 mM. The Na concentrations of the Mg and choline media were always less than 4 mM. Δ cellular values were obtained by calculating the difference in the values at 90 min from the values at zero time.

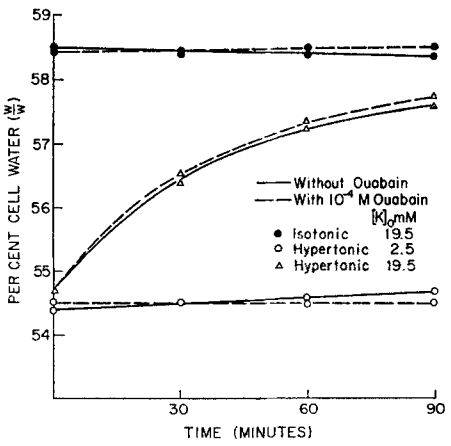

FIGURE 5. The effect of ouabain on the changes in cell volume during the volume regulatory phase in the moderately hypertonic medium. Duck erythrocytes were incubated in isotonic or moderately hypertonic media with a $[K]_o$ of either 2.5 or 19.5 mM. Ouabain (10^{-4} M) was added to the appropriate flask just before adding the cells. The experiment was performed in duplicate and is representative of a total of four experiments. The points represent the average value from the duplicate experiments; the range at each point is ±0.15 or less.

The ouabain experiment demonstrates in addition that the changes in cell volume, the primary effect of the volume controlling mechanism, are not altered by inhibiting the Na-K exchange pump mechanism. As in the hypotonic volume regulatory phase (Kregenow, 1971), Na appears to replace an equivalent amount of K as the osmotically active intracellular cation when

TABLE IV

OUABAIN'S EFFECT ON THE Na AND K CONTENT OF DUCK ERYTHROCYTES DURING THE VOLUME REGULATORY PHASE IN THE MODERATELY HYPERTONIC MEDIUM

Exp.	Medium osmolality	$[K]_o$	Ouabain, 10^{-4} M	ΔK_c mM/L_{one} 90 min	ΔNa_c mM/L_{one} 90 min	Δ Ouabain mM/L_{one} 90 min K	Na
	mosmols	mM					
A	323	2.5	−	0	+0.3	(−6.7	+6.3)
	323	2.5	+	−6.7	+6.6		
	323	19.0	−	−1.0	+0.3	(−6.5	+6.9)
	323	19.0	+	−7.5	+7.2		
	435	2.5	−	−1.0	+4.7	(−4.9	+3.1)
	435	2.5	+	−5.9	+7.8		
	435	19.0	−	+18.2	+2.5	(−14.1	+12.0)
	435	19.0	+	+4.1	+14.5		
B	323	2.5	−	+1.0	+0.2	(−5.8	+5.8)
	323	2.5	+	−4.8	+6.0		
	323	19.0	−	0	0	(−6.7	+6.9)
	323	19.0	+	−6.7	+6.9		
	435	2.5	−	−1.0	+3.0	(−7.0	+4.8)
	435	2.5	+	−8.0	+7.8		
	435	19.0	−	+16.7	+3.1	(−13.3	+12.3)
	435	19.0	+	+3.4	+15.4		

The experimental procedure was identical to the one described in Fig. 5. The values for ΔK_c and ΔNa_c were obtained by subtracting the value at zero time from those at 90 min. The values for Δouabain are the differences in the Na and K content of cells in each medium after a 90 min incubation with ouabain and are obtained from the differences in the columns labeled ΔK_c and ΔNa_c.

the cells regulate their volume in the presence of ouabain. The cellular parameter associated with cell size which serves to modulate changes in cell volume, cannot be either the individual Na or K content or concentration, since these values differ widely when ouabain is either absent or present, yet both groups of cells regulate their volume identically.

^{24}Na and ^{42}K were used to examine the alterations in membrane permeability during the volume regulatory phase. Experiments were performed in isotonic and hypertonic media at two different external potassium concentrations, 2.5 and 19 mM. As previously shown, cells incubated in a hypertonic

solution (π = 375 or 435 mosmols) with a $[K]_o$ of 19 mM revert to their original volume, while cells incubated in a hypertonic solution with a $[K]_o$ of 2.5 mM remain shrunken and serve as controls.

The alterations in K influx and efflux under these circumstances are presented in Table V. Increasing the osmolality of the bathing solution produces an increase in K influx regardless of the $[K]_o$. The magnitude of the initial increase in K influx is larger the more hypertonic the bathing solution.

TABLE V

CHANGES IN K INFLUX AND EFFLUX ASSOCIATED WITH THE INCUBATION OF DUCK ERYTHROCYTES IN HYPERTONIC MEDIA WITH A NORMAL OR ELEVATED $[K]_o$

Exp.	Medium osmolality	$[K]_o$	Influx, mM/L$_{cells}$ For interval (min)			Net change in mM/L$_{cells}$ For interval (min)			Cal. efflux in mM/L$_{cells}$ For interval (min)		
			0–30	30–60	60–90	0–30	30–60	60–90	0–30	30–60	60–90
No.	*mosmols*	mM									
1	323	2.8	3.4	3.7	3.8	+0.6	0	−0.2	2.8	3.7	4.0
		20.2	4.3	4.5	4.7	+0.1	0	+0.2	4.2	4.5	4.5
	375	2.9	12.8	14.0	17.2	−0.2	+1.5	−1.0	13.0	12.5	18.2
		19.6	12.6	9.1	7.2	+4.1	+3.4	+1.2	8.5	5.7	6.0
	435	2.8	30.8	40.0	43.0	+0.5	−0.9	+1.6	30.3	40.9	41.4
		19.4	28.2	16.5	9.5	+7.8	+4.2	0	20.4	12.3	9.5
2	323	2.9	4.0	3.5	3.6	0	−0.2	0	4.0	3.7	3.6
		19.0	4.9	4.4	4.6	−0.4	0	−0.2	5.3	4.4	4.8
	375	3.0	9.6	13.3	13.5	0	+0.3	−0.2	9.6	13.0	13.7
		18.8	9.5	8.0	5.7	+3.7	+2.0	+0.2	5.8	6.0	5.5
	435	2.8	33.3	36.8	35.0	−0.7	+0.6	0	34.0	36.2	35.0
		19.2	28.8	16.2	9.5	+6.8	+4.4	0	22.0	11.8	9.5

Duck erythrocytes were incubated in isotonic or hypertonic media with a $[K]_o$ of approximately 2.5 or 19 mM. Each 30 min influx value is the sum of two measurements, one during the first 15 min interval, and a second during the last 15 min. A tracer quantity of ^{42}K was introduced to a tracer-free sample of cell suspension at the beginning of each measurement.

In the enlarging cells ($[K]_o$ = 19 mM), K efflux, although elevated, initially is less than K influx since there is an appreciable net gain of potassium during the first 30 min. The increase in influx and efflux is transient; both decrease by 2 hr to a point where they are identical and approximately twice the control isotonic values. On the other hand, in the shrunken cells ($[K]_o$ = 2.5 mM), the increase in K influx is associated with an equivalent increase in K efflux since there is no change in the potassium content of these cells. The increase in K influx and efflux persists during the 2 hr incubation period.

Changes in the Na influx and rate of ^{24}Na loss were also examined under these circumstances and these results are presented in Table VI and Fig. 6. Table VI demonstrates that an increase occurs in the initial Na influx value in the moderately hypertonic medium when the $[K]_o$ is either 2.5 or 19 mM.

The initial rate of ^{24}Na loss, plotted as the per cent ^{24}Na released *versus* time, is also increased in both media (Fig. 6). This increase develops primarily in an ouabain (10^{-4} M)–insensitive pathway.

The modifications in Na and K fluxes must remain in large part descriptive. There is a generalized increase in both Na and K permeability which develops initially in both groups of cells. In the case of potassium, the generalized increase in permeability decreases and returns to normal as the cells swell to their original volume but persists in those cells that remain shrunken. This

TABLE VI

THE Na INFLUX OF DUCK ERYTHROCYTES DURING THE INITIAL 5 MIN OF INCUBATION IN ISOTONIC OR MODERATELY HYPERTONIC MEDIUM WITH A NORMAL OF ELEVATED $[K]_o$

Exp.	Medium osmolality	$[K]_o$	Na/influx in mM/L_{cell} for 5 min
No.	*mosmols*	*mM*	
1	323	2.8	0.7
		18.4	0.6
	435	2.7	2.1
		18.6	2.4
2	323	2.7	0.7
		19.5	0.4
	435	2.7	2.5
		20.4	2.7
3	323	2.6	0.6
		18.0	0.5
	435	2.5	2.3
		17.8	2.8

Duck erythrocytes were incubated in isotonic or moderately hypertonic medium (π = 323 and 435 mosmols, respectively) with a $[K]_o$ of approximately 2.5 or 19 mM.

characteristic serves as one of the factors that identifies a norepinephrine-dependent volume adjustment in isotonic media as a process mediated by the same volume controlling mechanism (Riddick et al., 1971; Kregenow and Orloff, manuscript in preparation).[7]

DISCUSSION

The major finding in this paper is the demonstration that duck erythrocytes are capable of readjusting their volume in hypertonic media after first shrinking. This cellular adaptation is similar to the response in hypotonic media in

[7] Kregenow, F. M., and J. Orloff. 1971. The response of duck erythrocytes to norepinephrine and an elevated $[K]_0$. Volume regulation in an isotonic medium. Manuscript in preparation.

that it can be divided empirically into two stages, an initial instantaneous osmotic phase and a more prolonged volume regulatory phase. However, the characteristics of the two phases differ from those in hypotonic media. These differences have been discussed previously and will not be mentioned again other than to emphasize that the effector portion of the volume controlling

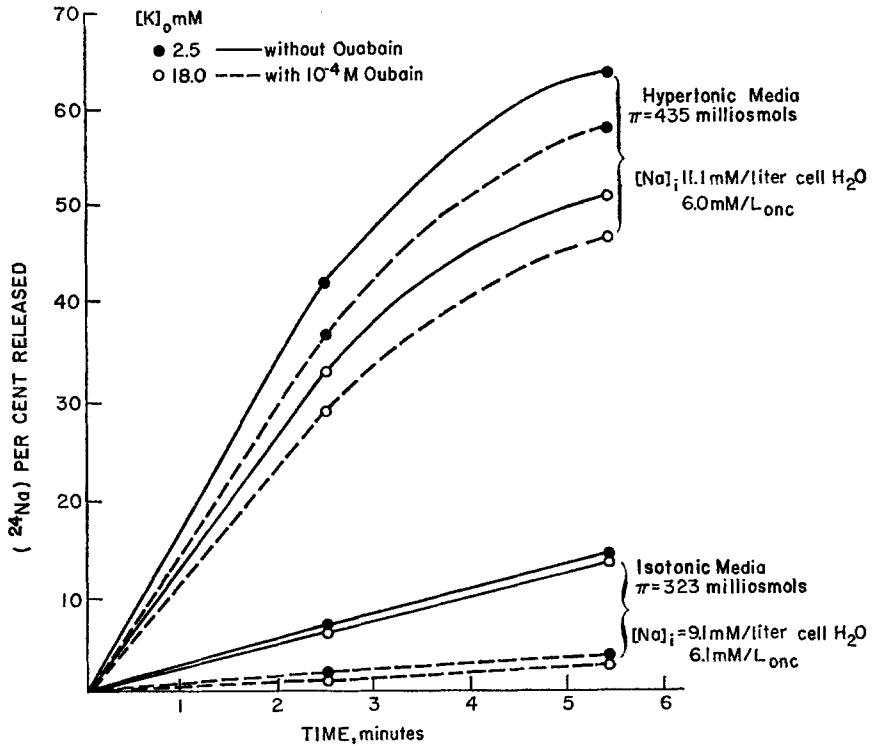

FIGURE 6. A comparison of the rate of ^{24}Na loss from duck erythrocytes incubated in isotonic or moderately hypertonic medium with a normal or elevated $[K]_o$. Duck erythrocytes, prelabeled with ^{24}Na, were incubated in isotonic or moderately hypertonic medium (π = 323 and 435 mosmols, respectively), whose $[K]_o$ was either 2.5 or 18 mM. 10^{-4} M ouabain was introduced to the appropriate experimental flask just before adding the cells.

mechanism, responsible for the volume regulatory phase, must differ in the two responses and that the response in hypertonic media is delitescent at "normal" extracellular potassium concentrations.

No single membrane event can explain the response during the hypertonic volume regulatory phase. This is in contrast to the finding during the hypotonic volume regulatory phase (Kregenow, 1971), where it was possible to consider a single hypothesis (transient increase in K leak) as an explanation for the major permeability and electrolyte changes. However, the following

analogy may be useful. The changes in K content and permeability during the hypertonic volume regulatory phase parallel in some respects the active accumulation of amino acids by vertebrate cells (Schultz and Curran, 1970). In both, active transport requires the presence of extracellular sodium. In each, the consequence of a strict application of the pump-leak hypothesis (leak implying simple diffusion) to the flux measurements leads to difficulties (Wilbrandt and Rosenberg, 1961). This comparison is strengthened when one considers that isosmotic intracellular regulation, which is exemplified by the hypertonic response in duck erythrocytes (see next paragraph), is largely accomplished in invertebrates by changes in amino acid content rather than potassium content of cells.

The volume regulation reported in this paper appears to represent another example of isosmotic intracellular regulation (Jeuniaux et al., 1961). Numerous invertebrates are capable of this cellular adaption when similarly exposed to a hypertonic bathing solution (for a review, see Potts and Parry, 1964). The volume regulating phase of invertebrates differs from that of duck erythrocytes in several respects. In invertebrates, the major intracellular osmotic adjustment is an increase in the quantity of intracellular organic acids, mainly amino acids. The amino acids appear to originate from preexisting intracellular protein (Florkin and Schoffeniels, 1969). On the other hand, in the duck erythrocyte response, the osmotic adjustment is associated with an increase in the intracellular electrolyte content. The source of the newly acquired electrolyte is the extracellular medium.

The literature contains several examples of an erythrocyte response to hypertonic media which may represent isosmotic intracellular regulation. Orskov (1954 and 1956) has shown that pigeon, hen, and frog red cells remove potassium from plasma and swell when the latter is made hypertonic by the addition of a concentrated solution of sodium chloride. There are many similarities between Orskov's observations and the volume regulatory phase of duck erythrocytes reported in this paper. Davson (1940) has shown that a hypertonic solution of KCl inhibits the gain of potassium while accelerating the loss of Na in cat erythrocytes, a nonnucleated high Na red blood cell. He was aware of the fact that these permeability changes, if present when the cells were incubated in hypertonic plasma, would reverse the initial decrease in cell volume.

Tosteson and Hoffman (1960) have presented the concept of a model cell which controls its volume in the steady state by the action of Na and K leaks working in parallel with an Na-K exchange ouabain-inhibitable pump. At first glance, the experiments with ouabain reported here and in the previous paper appear to contradict this concept. However, these volume adjustments and membrane permeability changes in duck erythrocytes are not germane to their model since the measurements were obtained for the most part in the

unsteady state. Furthermore, although these studies indicate that the volume controlling mechanism can operate when the ouabain-inhibitable pump mechanism is blocked, this does not imply that the former is completely independent of the latter, for the high potassium content of duck erythrocytes is maintained in part by the Na-K exchange mechanism. The cell's high K content and the resultant concentration gradient between the cells and medium serves as the driving force for the net loss of potassium when the proposed increase in the K leak pathway develops during the hypotonic volume regulatory phase.

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