The Reponse of Duck Erythrocytes to Norepinephrine and an Elevated Extracellular Potassium

Volume regulation in isotonic media

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ABSTRACT This paper presents evidence that duck erythrocytes regulate their size in *isotonic* media by utilizing a previously reported "volume-controlling mechanism." Two different experimental situations are examined. In the first, cells enlarge in a solution containing norepinephrine and an elevated $[K]_o$; and in the second, enlarged cells shrink to their original size if the norepinephrine and excess potassium are removed. As the erythrocytes enlarge, K, Cl, and H_2O accumulate. Shrinkage, in contrast, is accompanied by the controlled loss of K, Cl, and H_2O. These changes and the associated changes in membrane permeability resemble those reported previously when duck erythrocytes incubate in *anisotonic* media. There cells, after first shrinking or swelling, utilize a "volume-controlling mechanism" to reestablish their original size. The mechanism regulates cell size by adjusting the total number of osmotically active intracellular particles. The present studies indicate duck red cells use this mechanism to readjust their total monovalent cation content and thus their solute content in *isotonic* media as well. In addition, evidence is presented which indicates that the "volume-controlling mechanism" and ouabain-inhibitable cation pump differ functionally.

INTRODUCTION

Size is one of the basic characteristics of an animal cell (6); the regulation of cell size is also a fundamental cell function (6, 12). The water content of a cell generally determines size, for water constitutes the major part of most cells—the volume fraction of water in almost all cells varies between 0.7 and 0.9. Since it has been shown that the cytoplasm of vertebrate cells and the suspending medium are isosmotic, a cell's water content is determined, in turn, by its

[1] With the technical assistance of Mary Frances Spears.

solute content (for Reviews, see 11, 12). Thus, in most cells, size is primarily a function of solute content.

The response of duck erythrocytes incubated in *anisotonic* media is therefore of special interest. There, duck red cells, after first swelling or shrinking, return to their original volume (3, 4). An adaptation of this nature requires a "volume-controlling mechanism" which is (*a*) capable of adjusting the total number of osmotically active intracellular particles, and (*b*) sensitive to some parameter associated with cell size.

Duck erythrocytes can also adjust their volume while incubating in *isotonic* media. They swell when incubated with both norepinephrine and an elevated $[K]_o$ (10). This response resembles the pigeon red cell enlargement reported previously by Orskov (8). Conversely, once enlarged, duck red cells shrink if the factors responsible for the enlargement (norepinephrine and an elevated $[K]_o$) are removed from the bathing medium. In this communication we examine both of these responses in detail.

As the cells enlarge and shrink in *isotonic* media, the changes in cation content and membrane permeability resemble those that develop as cells enlarge or shrink, respectively, in *anisotonic* media. These observations indicate that duck erythrocytes, regardless of the tonicity of the medium, utilize the same basic mechanism for readjusting cell size.

This work has been presented before a meeting of the Red Blood Cell Club.[2] Preliminary experiments related to the data in Fig. 1 and Table I have been presented elsewhere (9).

METHODS

The procedures, materials, and methods employed in this paper have been described previously (10, 3, 4). Freshly drawn duck red cells lose approximately 8 mmol of K and 2% of their cell water (wt/wt) during the first 90 min of incubation in a norepinephrine-free synthetic medium before stabilizing at a new *lower steady-state* level (10). Only lower steady-state cells ($[K]_c$ about 110 mmol/liter,[3] $[Na]_c$ about 5 mmol/liter, and $[Cl]_c$ about 51 mmol/liter) were used in this study. The basic isotonic synthetic medium had the following composition (mmol/liter): $MgCl_2$ 2, $CaCl_2$ 1, $NaHCO_3$ 28, Na_2HPO_4 3.8, NaH_2PO_4 3.8, NaCl 115, KCl 2.5, dextrose 20, and albumin[4] (2.5 g/100 ml). The procedure for altering the Na and K concentration of this solution has been described elsewhere (10). A solution with this basic composition will be referred to as a *standard synthetic solution*; unless otherwise designated, the $[K]_o$ is 2.5 mM.

Na and K influx have been calculated by using Eq. 1 (3), while K efflux has been calculated by using Eq. 3 (3). As described previously (3), K influx determinations frequently required multiple brief measurements. During the Na influx determina-

[2] April 1971, Chicago, Ill.

[3] mmol/liter = millimoles per liter of red blood cells.

[4] Bovine albumin powder (fraction V from bovine plasma) from Armour Pharmaceutical Company, Chicago, Ill.

tions, 10^{-4} M ouabain was included in the bathing solution to eliminate any backflux of ^{24}Na through the Na-K exchange pump.

Measurement of ^{24}Na and ^{42}K loss from cells has been presented as the percent tracer released with time since a plot of the logarithm (natural base *e*) of the quantity (1 — percent tracer released with time) was not always linear. Preloaded cells were washed twice with ice-cold tracer-free solutions before the efflux measurements.

As in the experiments with anisotonic media (3, 4), a change in the surface area of the membrane may occur with changes in cell volume. Maximally, a 10% change in volume develops during the experiments in isotonic media. A change in volume of this magnitude would produce a somewhat smaller alteration in surface area, which, in turn, could introduce an error ($<10\%$) in the absolute flux measurement (millimoles per unit area of membrane). No attempt has been made to correct for this possible error.

The osmotic pressures of medium and cells were analyzed by comparing simultaneously the melting point of a microscopic portion of medium and hemolyzed packed cells. Each sample was compared in quadruplicate. The melting points were determined on a Biological Cryostat Nanoliter Osmometer (Clifton Technical Physics, New York) utilizing immersion oil, Type B, (Arthur H. Thomas Co., Philadelphia, Pa.) as the immersing agent. The osmotic pressure of the medium was also determined independently by measuring its freezing point depression on an Aminco Osmometer (American Instrument Co., Inc., Silver Spring, Md.) The freeze-thaw procedure used to hemolyze the cells (13) produced a viscous preparation which contained numerous nuclei when viewed under the light microscope but was devoid of any intact cells.

RESULTS

Cell Enlargement

As mentioned previously, duck erythrocytes swell in an isotonic bathing solution which contains norepinephrine and a larger than "normal" $[K]_o$. When the cells enlarge, they accumulate K, H_2O, and a small quantity of Na (10). Fig. 1 illustrates the time-course of these changes; the units used in the ordinate indicate shifts in either the net cation or water content of the cells (see 10). Within 15 min, the major portion of the total gain in cell K and H_2O has already occurred. The cell Na (note the different scale) has also reached a maximum value by this time. During the remainder of the hour, the cells continue to gain K at a slower rate, whereas they lose a small quantity of Na. Thus, although chosen somewhat empirically, the 15 min point divides the overall response into two phases: between 0 and 15 min and 15 and 60 min.

Table I shows the effect of introducing ouabain (10^{-4} M) to the bathing solution used in Fig. 1. Several points are of interest. First we shall consider the changes in cell volume. Cells enlarge identically in the presence or absence of ouabain (as indicated by the change in Δ % cell H_2O [wt/wt]). Nor does ouabain alter the volume of control cells (incubated in the same medium but without norepinephrine) during this interval (4). Therefore the cardiac glyco-

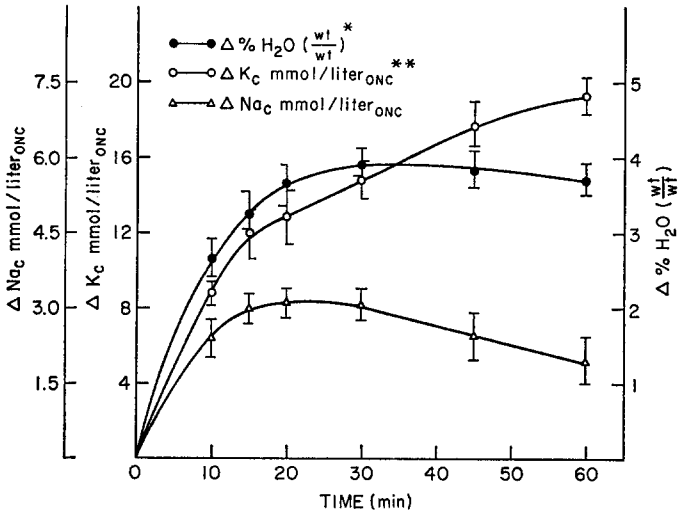

FIGURE 1. The changes in the cell water and cation content of duck erythrocytes during the period of cell enlargment. Duck erythrocytes were incubated in a standard synthetic solution ($[K]_o$ between 16 and 20 mM) which also contained 10^{-6} M norepinephrine. The ensuing changes in cation and water content have been plotted against time. These changes represent the differences between the values of experimental and control cells. Control cells were incubated simultaneously and remained in the lower steady state (see Methods). Vertical lines denote standard deviation.

* % cell H_2O (wt/wt), percent cell water determined gravimetrically.

** $mmol/liter_{onc}$ = millimoles per liter of original number of cells, i.e, millimoles per that number of cells which originally occupied 1 liter.

side does not interfere with the shifts in cell size demonstrated in Fig. 1. If the cells remain at osmotic equilibrium during this enlargement, then one would expect the total number of monovalent cations (Na + K) gained to be the same whether or not ouabain is present. Table I shows that this is true at the end of 60 min. Again, ouabain does not alter this quantity in control cells (4).

In contrast, if one looks at the shifts in Na and K content individually, ouabain has a marked effect. These changes can be examined more easily by noting ouabain's influence during the two phases, 0–15 min and 15–60 min, separately.

In the first phase, the cardiac glycoside produces only a small change. There is a 1.4 mmol reduction in the quantity of intracellular K "normally" gained, while the intracellular Na content increases an additional 2.5 mmol (both designated Δ ouabain). Since control cells, treated with ouabain, lose approximately 1.3 mmol of K and gain 1.7 mmol of Na per 15 min (4), ouabain's effect is only slightly larger in norepinephrine-treated cells. Conse-

TABLE I

OUABAIN'S EFFECT ON CELL ENLARGEMENT IN AN ISOTONIC MEDIUM

	Time period in minutes								
	0–15			15–60			0–60		
	ΔK_c	ΔNa_c	$\Delta\%H_2O$	ΔK_c	ΔNa_c	$\Delta\%H_2O$	ΔK_c	ΔNa_c	$\Delta\%H_2O$
	mmol/liter$_{onc}$		*wt/wt*	*mmol/liter$_{onc}$*		*wt/wt*	*mmol/liter$_{onc}$*		*wt/wt*
Experimental									
10^{-6}M Norepinephrine	+9.0±0.60	+3.6±0.26	+2.4±0.21	+8.7±1.03	−0.1±0.42	+0.9±0.17	+17.7±0.92 (Total +21.2)	+3.5±0.38	+3.3±0.04
10^{-6} M Norepinephrine + 10^{-4} M ouabain	+7.6±0.54	+6.1±0.39	+2.3±0.23	+0.1±0.77	+8.3±0.42	+0.9±0.16	+7.7±0.82 (Total +22.1)	+14.4±0.34	+3.2±0.04
Δ Ouabain	−1.4	+2.5	−0.1	−8.6	+8.4	0	−10.0	+10.9	−0.1
Control									
Δ Ouabain	−1.3	+1.7	−0.0	−4.4	+4.3	−0.1	−5.7	+6.0	−0.1

The standard synthetic solution ($[K]_o$ between 16–20 mM) served as the incubating solution. Experimental flasks also contained 10^{-6} M norepinephrine, while 10^{-4} M ouabain was added to one of two otherwise identical flasks before adding the cells. Results for each time period were obtained by subtracting the cellular value at the beginning of each interval (0 time or 15 min) from the value at the end. Values in the Table represent the mean ± SEM (n = 7). Since control cells, incubated without ouabain, remained in the steady state during the experimental period, only the mean Δ ouabain values have been included in the Table.

quently, most of the K accumulation during the first 15 min is insensitive to ouabain (7.6 mmol).

During the second phase, ouabain affects a greater percentage of the net K uptake. If the cardiac glycoside blocked all of the K uptake during this phase, cells treated with ouabain should contain 13.1 mmol less K (approximately 8.7 mmol normally accumulated +4.4 mmol lost by control cells when they are treated with ouabain during this period) than cells incubated without ouabain. Since experimental cells that have been treated with ouabain contain 8.6 mmol rather than 13.1 mmol less K, the difference or 4.5 mmol accumulates by a process which is not affected by the cardiac glycoside.

The remaining portion of the net K uptake during the second phase (4.2 mmol) is blocked by ouabain. It is apparent from Table I that during this period the loss of K, produced by ouabain, is accompanied by an equivalent gain in Na (both designated Δ ouabain). These changes are approximately twice those seen when control cells are treated with the cardiac glycoside for this period (loss of 4.4 mmol of K and gain of 4.3 mmol of Na). One generally attributes cation changes of this nature to ouabain's inhibition of the cation pump. Thus, during this phase, the Na-K exchange pump operates more rapidly than normal, but at an exchange rate that approximates 1:1. Since it is known that increasing the Na content of cytoplasm affects the pump in this manner, it is probable that the net uptake of K (that blocked by ouabain) originates from a preceding increase in Na content. The newly acquired Na, in the absence of ouabain, is then exchanged for K by a more rapidly operating pump.

Raising the concentration of ouabain to 10^{-3} M or preincubating cells in 10^{-4} M ouabain for ½ h before the addition of norepinephrine does not alter the above results. As expected, however, cells preincubated with ouabain contain approximately 2–3 mmol more Na and less K at the start of the experimental period (0 time).

One can now summarize ouabain's effect. As expected, the cardiac glycoside modifies appreciably the Na and K concentration of cells, but it does not interfere to any great extent with either the total quantity of electrolyte transferred across the membrane or with the shifts in cell size that are associated with norepinephrine treatment.

It is now also possible to compare the process of cell enlargement in *isotonic* media with that in *hypertonic* media (4). As shown previously (10, 4), in both responses the changes in electrolyte content are nearly identical, the requirement for extracellular Na and an increase in "normal" $[K]_o$ are similar, and the concentration curves relating $[K]_o$ to an increase in cell size are the same. The data presented in Table I and elsewhere (4) also demonstrate that ouabain does not alter the rate at which cells enlarge in either response. But experiments using ouabain do divide the uptake of K into two components: one

is ouabain-sensitive, the other ouabain-insensitive. In both responses the ouabain-insensitive component is the larger of the two.

Radioactive tracers were used to measure the changes in the unidirectional movements of Na and K associated with the net changes in Na and K demonstrated in Fig. 1. To distinguish the changes in fluxes produced by norepinephrine alone from those associated with the norepinephrine-dependent cellular enlargement, measurements were performed in two media: *low K* [*standard synthetic solution* ($[K]_o$ = 2.5 mM) with 10^{-6} M norepinephrine] and *high K* [*standard synthetic solution* ($[K]_o$ = 17.0 mM) with 10^{-6} M norepinephrine]. Only cells incubated in the high K medium swell in the presence of norepinephrine.

Initially, norepinephrine produces a 3- to 5-fold increase in Na influx (Fig. 2) and a 6- to 10-fold increase in K influx (most of which is insensitive to ouabain) (Fig. 3). Fig. 2 shows that the norepinephrine-dependent portion of the 0–5 min Na influx value in the high K medium exceeds that in the low K medium. A similar comparison of the initial K influx values in Fig. 3 is not possible—in part because of potential errors in these measurements (see Methods). Figs. 2 and 3 also show how these fluxes vary with time. As the cells enlarge in the high K medium, both Na and K influx decrease until at 1 h they are ap-

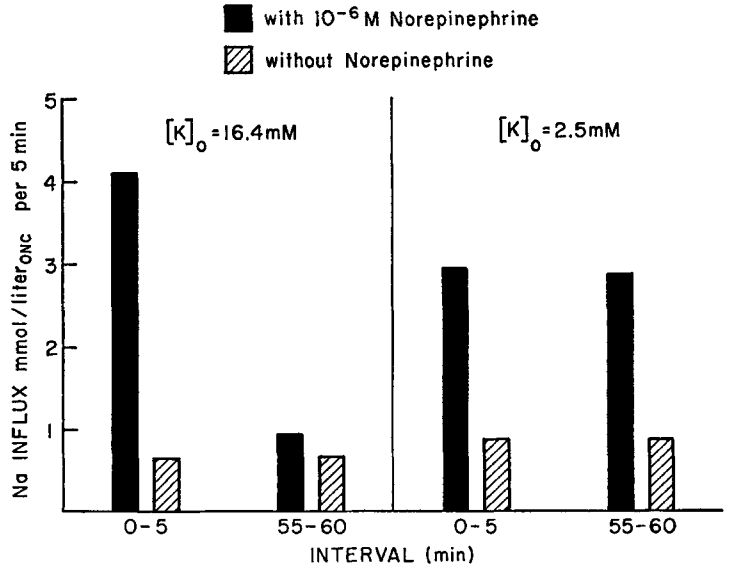

FIGURE 2. The changes with time in the Na influx of norepinephrine-treated duck erythrocytes. Duck erythrocytes began the incubation at 0 time in one of four different experimental solutions (standard synthetic solution: $[K]_o$ = 2.5 or 16.4 mM; with or without 10^{-6} M norepinephrine). At the beginning of each interval (0 time or 55 min) 10^{-4} M ouabain and a tracer quantity of ^{24}Na were introduced into a tracer-free portion of each cell suspension.

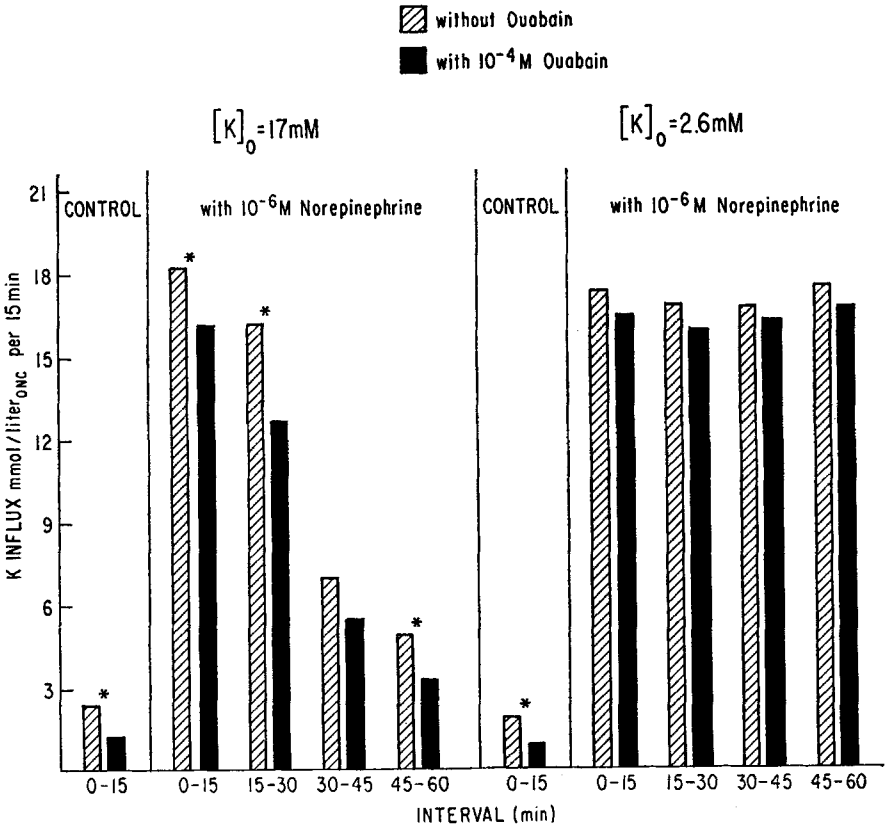

FIGURE 3. The changes with time in the K influx of norepinephrine-treated duck erythrocytes. Duck erythrocytes began the incubation at 0 time in one of four different experimental solutions (standard synthetic solution: $[K]_o$ = 2.6 or 17 mM; with or without 10^{-6} M norepinephrine). At the beginning of each 15 min interval (0, 15, 30, and 45 min), portions from each of the four different suspensions were removed and reintroduced into separate flasks for the flux assay. Each 15 min value, recorded in the figure, required two flasks, since the 15 min value is the sum of two measurements, one during the first 7.5 min, and the other during the last 7.5 min. At the beginning of each 7.5 min interval, then, a tracer quantity of ^{42}K was introduced into one of the two flasks. When ouabain (10^{-4} M) was used, it was introduced into both flasks at the beginning of the 15 min interval. The K influx of control cells, incubated without norepinephrine, did not change with time; therefore only the measurements for the first 15 min period are shown.

* The K influx in the presence of ouabain is significantly different from the influx in the absence of ouabain. $P < 0.01$ (n = 5).

proximately twice that of control cells. In contrast, the initial elevated Na and K influx values of cells incubated in the low K medium do not decrease with time, but remain elevated.

Norepinephrine also stimulates Na and K efflux several-fold. Let us con-

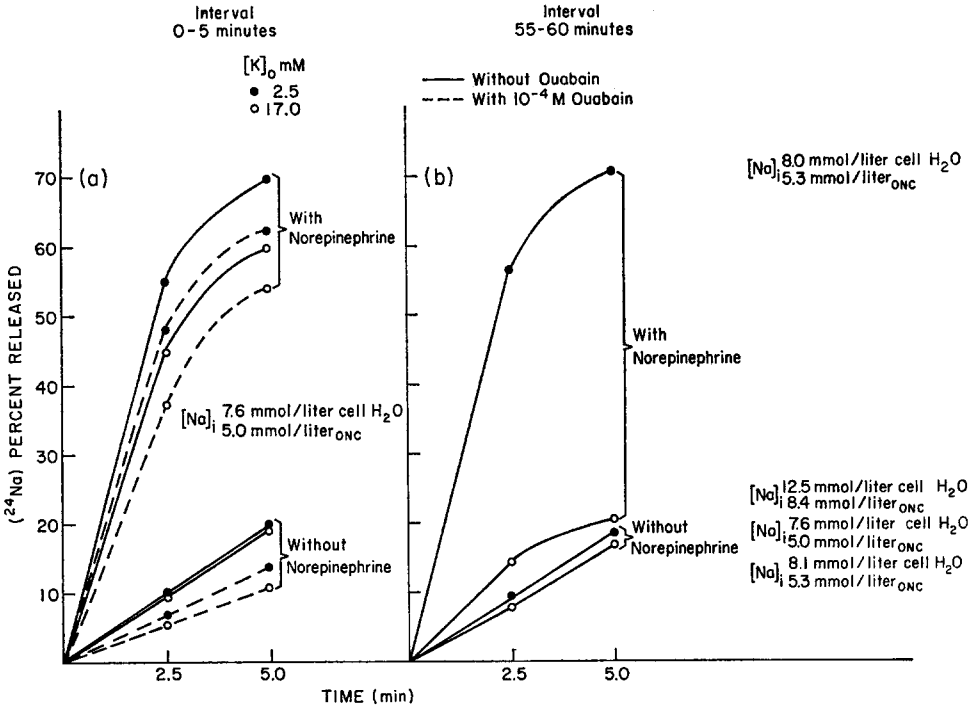

FIGURE 4. The changes with time in the rate of ^{24}Na loss from norepinephrine-treated duck erythrocytes in the presence and absence of ouabain. Duck erythrocytes were preloaded with ^{24}Na and washed with a nonradioactive solution before being introduced into one of four different nonradioactive experimental solutions (standard synthetic solution: $[K]_o$ = 2.5 or 17.0 mM; with or without 10^{-6} M norepinephrine) for the flux assay. In (*a*), cells were preloaded during the 90 min period routinely used to obtain lower steady-state cells (see Methods). The standard synthetic solution ($[K]_o$ = 2.5 mM) served as the medium for this procedure and the subsequent washing. In (*b*), cells which had already reached the lower steady state were loaded during a subsequent 55 min incubation (0–55 min). Here, the solutions used during the efflux measurements also served as the media for loading and washing cells. In (*a*), ouabain (10^{-4} M) was added to one of two otherwise identical flasks before adding the washed radioactive cells. The Na concentrations included in the figure correspond to the cellular values at the beginning of the tracer measurement.

sider the changes in the high K medium first and the changes with Na before those with K.

Na efflux was approximated by following the rate of ^{24}Na loss from prelabeled cells. Fig. 4 *a* shows that most of the initial increase in the rate of ^{24}Na loss in the high K medium is insensitive to ouabain. Figure 4 *b* shows, in addition, that the earlier rapid rate of ^{24}Na loss has decreased with time; at 55 min it approaches that from control cells. Values for K efflux were calculated

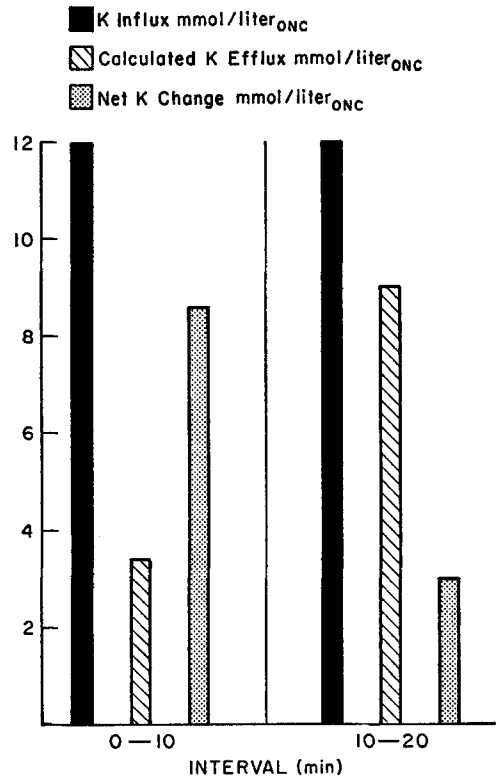

FIGURE 5. The relationship between K influx and efflux of duck erythrocytes during the first 20 min of the response to norepinephrine and an elevated $[K]_o$. Duck erythrocytes were incubated in the high K medium (standard synthetic solution: $[K]_o$ = 17 mM which also contained 10^{-6} M norepinephrine). At the beginning of each interval (0, 10 min), a portion from this suspension was removed and reintroduced into separate flasks for the influx assay. Each 10 min influx, recorded in the figure, required two flasks, since the 10 min value is the sum of two measurements, one during the first 5 min, and the other during the last 5 min. At the beginning of each 5 min interval, then, a tracer quantity of ^{42}K was introduced into one of the two flasks. The net K changes represent the differences in cellular K content at the beginning and end of each 10 min interval.

from simultaneous measurements of K influx and net K change (see Methods). Fig. 1 showed previously that the rate at which K accumulates in this medium decreases after 10 min. Since the rapid K influx, shown in Fig. 3 remains constant for the first 20 min,[5] K efflux must gradually increase to explain this net change. This is shown in Fig. 5, where the K influx, net K change, and calculated K efflux are analyzed at 10-min intervals for the first 20 min. Later K efflux decreases from this earlier elevated value as the influx (see Fig. 3) and net change (see Fig. 1) gradually decrease.

[5] Measurements of K influx remain elevated and constant during the first 20 min when the influx is calculated by using either of the two methods (Eqs. 1 and 2 in ref. 3).

In contrast, in low K medium the initial rapid rate of ^{24}Na loss (Fig. 4 *a*) remains elevated even after 55 min (Fig. 4 *b*). Similarly, the initial elevated rate of K efflux persists at the end of 55 min. Measurements of K efflux have not been illustrated, for they are identical to K influx measurements in Fig. 3 since there is no net K change under these circumstances (10).

Propranolol (10^{-4} M), a beta adrenergic blocking agent, prevents duck red cells from gaining K or enlarging in the presence of norepinephrine (10). If this agent is added to the bathing medium with norepinephrine, K and Na influx no longer increase. This is true in both the low and high K media. In addition, propranolol does not alter the corresponding influxes of control cells. Thus, this beta adrenergic blocking agent also prevents the catecholamine-induced changes in unidirectional movement.

In summary, then, the data presented in Figs. 2–5 indicate a generalized increase in permeability develops whenever norepinephrine is introduced to an isotonic bathing medium; both Na and K enter and leave the cell more rapidly. As the cells in the high K medium enlarge, this generalized increase in permeability subsides. A previous publication (4) reports a similar phenomenon when duck erythrocytes incubate in hypertonic media. This parallelism reinforces the comparative nature of the cell enlargement in hypertonic and isotonic media.

Cell Shrinkage

As mentioned in the Introduction, enlarged norepinephrine-treated cells shrink when they reincubate in an isotonic medium which is free of norepinephrine and an elevated extracellular K. Before investigating cell shrinkage, however, it was necessary to standardize the procedure for preparing enlarged erythrocytes. This was routinely accomplished by preincubating cells for 80 min in modified standard synthetic solution which contained 10^{-6} M norepinephrine and had a $[K]_o$ of approximately 15 mM. By 80 min, maximal cell enlargement has occurred and the elevated flux values have returned toward control levels. At the end of the preincubation period, the cells were washed twice with 8 vol of an ice-cold standard synthetic solution ($[K]_o$ = 2.5 mM, free of norepinephrine). The experimental period started (time 0) when the washed cells began reincubation (42°C) in a medium identical to the wash solution. To eliminate any residual beta adrenergic norepinephrine activity, propranolol (10^{-4} M) was included in this solution; this precautionary measure, however, was subsequently found to be unnecessary. The procedure for preparing control cells was essentially identical—only the medium used in the preincubation period differed. It was free of norepinephrine and, when necessary, had a $[K]_o$ of approximately 2.5 or 15 mM.

Previously enlarged duck erythrocytes shrink rapidly when reincubated in the standard synthetic solution (Fig. 6). Shrinkage continues until the cells

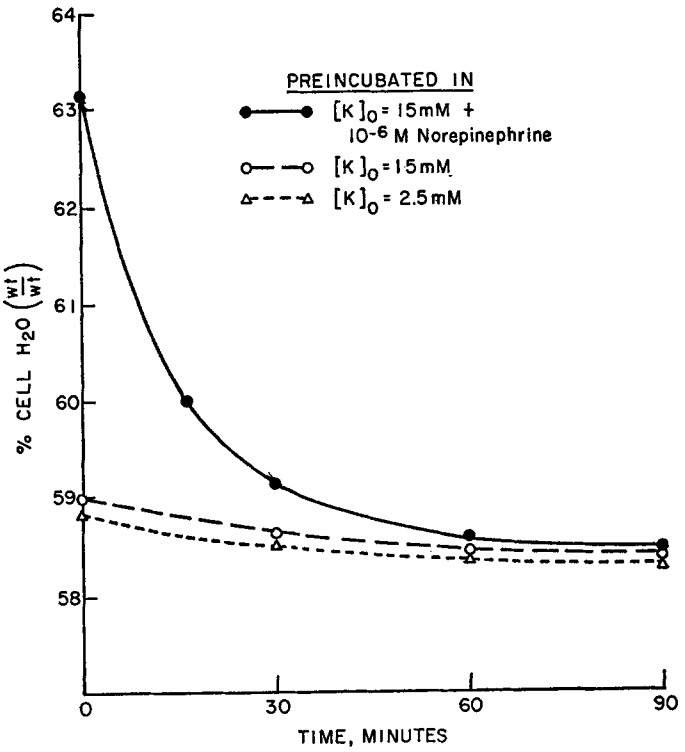

FIGURE 6. Cell shrinkage in an isotonic medium. The experimental protocol has been described in the text.

approach their original lower steady-state volume, when cell size stabilizes again. In Fig. 6, the approximate volume of lower steady-state cells corresponds to the volume of control cells[6] which have been preincubated in a standard synthetic solutions with a $[K]_o$ of 2.5 or 15 mM.

Table II demonstrates the electrolyte loss associated with cell shrinkage. Cells lose potassium and chloride. There were no significant changes in Na content. The loss of K takes place in the same direction as its electrochemical gradient and the total loss of electrolytes (K + Cl) is sufficient to account for the loss of cell water if the cells remain in osmotic equilibrium with their environment as they shrink.

Introducing ouabain (10^{-4} M) to the bathing solution does not alter the rate at which cells shrink. In either the presence or absence of ouabain, the shape of the curve relating percent cell H_2O (wt/wt) to time is the same and is

[6] The control cells in this study did not always remain in the lower steady state during the experimental period, at least as defined previously (10). This may be the result of the more extensive wash procedure or the longer total incubation period. In the present experiments, however, any changes in cell size or electrolyte content (see Legend of Table II) in control cells were minor when compared to the extensive alterations in experimental cells.

TABLE II
NET K^+ AND Cl^- CHANGES ASSOCIATED WITH CELL SHRINKAGE IN AN ISOTONIC MEDIUM

Exp	ΔK^+ at time (min)			ΔCl^- at time (min)		
	30	60	90	30	60	90
	mmol/liter$_{onc}$			*mmol/liter$_{onc}$*		
1	−17	−18.8	−18	−14	−16	−16
2	−13.6	−15	−16	−10	−11	−12.5
3	−16	−17.6	−17.6	−16	−18	−18

The experimental protocol was identical to the one used in Fig. 6. Δ values were obtained by subtracting the value of experimental cells at 0 time from the value at each subsequent time. At 0 time, experimental cells contained in millimoles/liter of red blood cells: $[K]_c$ = 118.8 ± 1.0, $[Na]_c$ = 6.8 ± 0.7 and $[Cl]_c$ = 56.9 ± 2.7; and a % H_2O (wt/wt) of 62.6 ± 0.4 (mean ±SD, n = 6). Control cells never lost more than 3.2 mmol of K or 3.0 mmol of Cl nor gained more than 2.5 mmol of Na during the 90 min experimental period.

identical to that in Fig. 6. However, ouabain does influence the cells, and has its expected effect on their Na and K content. The changes in the electrolyte content of treated cells resembles those reported previously for treated control cells (3). There is a 6–9 mmol loss of K and a 6–9 mmol gain in Na in 90 min. The gain in Na compensates for the loss of K so that the total number of monovalent cations lost during the response remains the same. It should be pointed out, though, that during the first 15 min, when most of the K loss occurs, ouabain has an almost negligible effect on K content.

Presented in the first portion of Table III (part A) are two experiments which demonstrate that the loss of cell K (described in Table II) results from a large transient increase in K efflux (see also Fig. 7). In contrast, the Na influx and rate of ^{24}Na loss do not differ from the values in control cells. (A difference of 15% or more must be present to be statistically significant.)

Thus, the shrinkage of enlarged cells in an isotonic medium, reported here, resembles in almost all respects the reduction in cell size reported in hypotonic media (3). Because the response in isotonic medium is somewhat variable, it corresponds to a response in a hypotonic medium with an osmolality between 247 and 260 mosmol. Of the parameters mentioned so far, only the changes in Na may not be identical. This questionable difference remains in part because of the inherent difficulties that exist when one tries to compare measurements of Na content and permeability in the two situations.

Continuing this comparison, raising the $[K]_o$ blocks cell shrinkage in both conditions. If the $[K]_o$ is increased to 75 mM in a hypotonic medium (234 mosmol), K influx becomes equivalent to K efflux and the cells fail to shrink (3). A larger $[K]_o$ (108 mM) is required, however, to block the isotonic response, reported here. The requirement for more potassium is to be expected if the physical-chemical force responsible for the net K loss is the same in both

TABLE III

CHANGES IN K INFLUX AND EFFLUX ASSOCIATED WITH CELL SHRINKAGE IN ISOTONIC MEDIA

Part	Exp	Preincubated with norepinephrine (enlarged cells)	$[K]_o$ concn of experimental medium	K Influx at time (min)				Net K Change at time (min)				Calculated K Efflux at time (min)			
				15	30	60	90	15	30	60	90	15	30	60	90
			mM	*mmol/liter$_{onc}$*				*mmol/liter$_{onc}$*				*mmol/liter$_{onc}$*			
A	1	−	2.8	2.9	5.6	12.2	18.0	0	0	−1.1	−0.8	2.9	5.6	13.3	18.8
		+	2.9	2.5	7.1	14.6	21.8	−15.9	−17.1	−19.0	−18.7	19.4	24.2	33.6	40.5
	2	−	2.7	3.0	6.8	14.6	22.3	−1.0	−1.5	−2.1	−3.0	4.0	8.3	16.7	25.3
		+	2.9	4.8	8.5	17.2	26.0	−12.7	−16.6	−17.6	−17.8	17.5	25.1	34.1	43.8
B	1	−	2.9	2.4	5.7			+0.5	+0.5			2.1	5.2		
		−	108.0	3.6	8.1			0	+0.6			3.6	7.5		
		+	2.7	4.3	7.0			−14.0	−17.7			18.3	24.7		
		+	108.0	17.2	35.4			−1.0	+1.0			18.2	34.4		
	2	−	2.8	2.3	4.4			−1.0	−1.0			3.3	5.4		
		−	107.0	3.5	7.8			+0.5	+0.5			3.0	7.3		
		+	3.0	3.9	7.1			−13.2	−16.0			17.1	23.1		
		+	107.0	19.5	41.4			0	−1.1			19.5	42.5		

Two groups of duck erythrocytes were incubated in one of two experimental solutions (standard synthetic solution: $[K]_o$ approximately 2.5 or 108 mM) for the influx assay. Experimental solutions also contained propranolol (10^{-4} M). The two groups of cells correspond to enlarged (preincubated with norepinephrine) and control (preincubated without norepinephrine) cells of Fig. 6. Since the influx determinations required separate measurements every 15 min (0–15, 15–30, 30–45, 45–60, 60–75, 75–90), a trace quantity of ^{42}K was introduced into a tracer-free portion from each cell suspension every 15 min. The values from the measurements at the earlier time periods were added to the values from the later time periods to arrive at the cumulative influx value for 30, 60, and 90 min recorded in the Table. Net K changes represent differences in the K content of cells between 0 time and the time indicated. K efflux has been calculated from the measurements of influx and net change (see Methods).

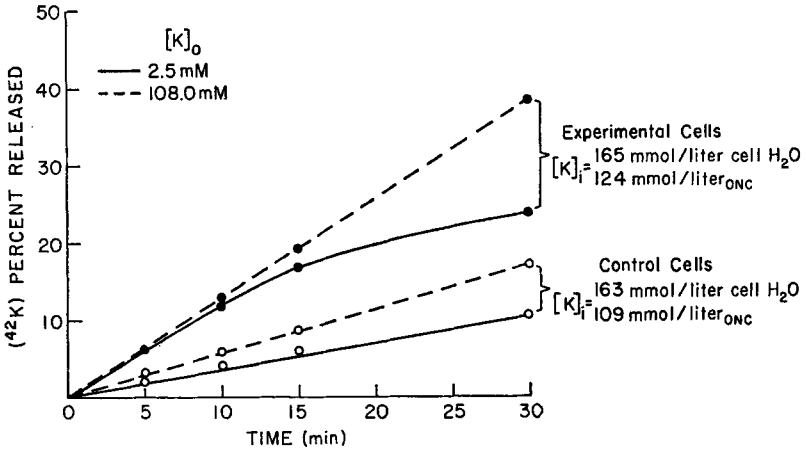

FIGURE 7. The effect of raising the $[K]_o$ on the rate of ^{42}K loss from duck erythrocytes during cell shrinkage in isotonic media. Duck erythrocytes were divided into two groups, labeled with ^{42}K, and then washed with a nonradioactive solution before being introduced into one of two nonradioactive experimental solutions (standard synthetic solution: $[K]_o$ of either 2.5 or 108.0 mM) for the flux assay. The two experimental solutions also contained propranolol (10^{-4} M). The two groups of cells correspond to the enlarged and control cells of Fig. 6. The procedures used to produce these two groups of cells were also utilized to label and wash the cells. Cells were labeled by including a tracer quantity of ^{42}K in the preincubation media; the routine washes with the standard synthetic solution removed medium containing ^{42}K from around the radioactive cells. The K concentrations included in the figures correspond to the cellular values at the beginning of the tracer measurement.

situations, i.e., the electrochemical gradient (see 3). Initially cells in the isotonic solution have a higher $[K]_c$, 167 mmol/liter cell H_2O, than cells in the hypotonic medium, 144 mmol/liter cell H_2O. Thus, the concentration gradient for the outward movement of K is greater; and, therefore, a larger concentration gradient for the inward movement of K is necessary if K influx and efflux are to be equivalent. Table III (part B) shows that, as expected, the initial 15 min K influx is increased and equivalent to K efflux when enlarged cells incubate in medium with a $[K]_o$ of approximately 108 mM.

Table III (part B) and Fig. 7 also show that enlarged cells, incubated in medium with a $[K]_o$ of approximately 108 mM, have a persistent increase in K efflux during the second 15 min compared to the reduced K efflux in a standard synthetic solution ($[K]_o$ = 2.5 mM). This persistent increase in K efflux could, along with the continued increase in K influx, represent an evolving K exchange diffusion process since now both the inward and outward movement of K have apparently become $[K]_o$ dependent. A similar phenomenon occurs during the same period in the hypotonic response when the $[K]_o$ is 75 mM (3).

DISCUSSION

The similarities between the processes by which cells readjust their volume in isotonic and anisotonic media have been discussed previously and will not be mentioned again. They are, however, too numerous to be coincidental, thus, suggesting that a similar mechanism operates to control cell size in both kinds of media.

Nevertheless, when cells enlarge in isotonic and hypertonic media, two major differences exist. The first difference is that norepinephrine is required to initiate enlargement in an isotonic medium (10), whereas simply introducing cells to a hypertonic medium produces the response (4). This suggests that introducing cells to a hypertonic solution produces a change in the cell which is similar to the one brought about by norepinephrine via the adenyl cyclase system (10). This change, whatever its nature, would then trigger a response which continues until the cell acquires its new volume (if sufficient extracellular K is present). An alternate explanation is that hypertonicity, like norepinephrine, causes cyclic AMP to accumulate. The second difference deals with the speed of the response. Norepinephrine initiates a more rapid response; it is nearly complete by 15 min (see Fig. 1). In a hypertonic medium, a comparable change in cell size requires nearly 60 min (4).

We shall now analyze all the responses in an attempt to gain additional insight into the mechanism. To simplify the analysis, we assume that in each response the shifts in cell size are *osmotic* in nature. There is, in fact, good agreement between the quantity of electrolyte lost or gained during each response and the quantity expected to be gained or lost if the response *were* osmotic in nature (see also 3 and 4).

The following experiment was performed to provide additional evidence that the shifts in size are osmotic in nature. In such an osmotic shift, an alteration in membrane permeability first affects the total solute content of cells, which in turn reduces or increases the activity of water in the cytoplasm, whereupon inward or outward movement of water leads to an increase or decrease in cell volume. Since the changes in solute content are gradual, and the movement of water rapid, measurements of the activity of water in the intra- and extracellular phase should be essentially the same at any given moment. To test whether the osmolality of the intra- and extracellular phase are always the same, we compared the osmotic pressure of cells and medium by analyzing the melting point of a microscopic portion of each (see Methods). This comparison was performed in hypotonic medium (234 mosmol), in hypertonic medium (435 mosmol), and as cells enlarged or shrunk, in isotonic media. Measurements were obtained at the onset of the experiment when the disparity between the initial and final cell size was largest; after the cells had readjusted their size halfway between the initial and final values, and after the cells had returned to their control size, or had, when incubated with

norepinephrine enlarged maximally. Changes in cell size were estimated from measurements of cell water content. The values for the mean of the differences in osmolality between cells and medium ranged from $+2.4 \pm 3.9$ mosmol[7] to -2.2 ± 3.8 mosmol. The values for control cells (lower steady-state cells incubated in a standard synthetic solution, $[K]_o = 2.5$ or 18.0 mM) ranged between -1.9 ± 3.5 mosmol and $+1.2 \pm 2.9$ mosmol. In no case were the values for experimental cells significantly different from the values for the corresponding control cells. Thus, as cells regulate their size in media with markedly different tonicity, they remain essentially isosmotic with the medium in which they are suspended. The error inherent in our measurements does not rule out the possibility, however, that small differences in osmolality or pressure are present during the responses.

As shown previously (3, 4, 10), the solutes, transferred in and out of the cell, are primarily Na^+, K^+, and Cl. Since Cl permeability (P_{Cl}) in red cells is seldom rate limiting when compared to P_{Na} and P_K, we must examine the control of cation movement to understand how these cells regulate their volume.

Insight into the way in which the monovalent cations move across the membrane can be obtained by analyzing the studies of cell shrinkage presented in this paper and elsewhere (3). In these studies, changes in K account for almost all of the cation loss. The driving force for this loss of K^+ can be defined by the electrical and chemical gradients. For, the movement of K^+ is toward its electrochemical equilibrium, results almost solely from an increase in K efflux, and is blocked by raising the $[K]_o$ to a level which would be expected to eliminate the electrochemical gradient. On the other hand, the decrease in Na permeability in hypotonic media (3) and the response to raising $[K]_o$ seen here and in hypotonic media (3) are incompatible with an alternative hypothesis—that the membrane squeezes out the necessary solute. Although the driving force for K movement is almost certainly the electrochemical gradient, the process by which K traverses the membrane is not clear. K movement of the kind in question is usually explained by simple diffusion. Nevertheless, K might interact with a carrier or some other membrane component and still display these characteristics. An example of the evidence that suggests that such an interaction may exist is found in the bidirectional $[K]_o$-dependent potassium fluxes that develop during the second 15 min both in a hypotonic medium with a $[K]_o$ of 75 mM (3) and an isotonic medium with a $[K]_o$ of 108 mM (see Table III). However, in the absence of more convincing evidence, it seems reasonable at this point to assume that the net K loss responsible for the apparent osmotic shrinkage develops because of a controlled increase in the K leak pathway.

It is tempting to suggest that when cells enlarge, a similar driving force is

[7] Mean ± standard deviation (n = 5).

responsible for the accumulation of cations. Under these circumstances, passive forces—this time the electrochemical gradient for sodium—can indeed account for some of the cation accumulation, for there is a net gain of Na during this process (see Fig. 1 and Reference 4). The net Na flux is downhill toward electrochemical equilibrium. And, at least in the isotonic response, the Na accumulated by 15 min (see Fig. 1) can be accounted for by the increase in that portion of Na influx which is dependent upon both $[K]_o$ and norepinephrine (see Fig. 2). (This is true if the Na influx, measured in Fig. 2 over a 5 min period, persists at the same rate for the next 10 min.) It should be mentioned here that McManus (7) and Allen (1) have presented indirect evidence that under somewhat different conditions (95% N_2 + 5% CO_2) the Na influx of duck red cells can become sensitive to $[K]_o$. However, the driving force for much of the cation accumulation during cell enlargement does not appear to be passive since most of the gain in K is accompanied by a rapid bidirectional K movement, which is not consistent with a process of simple diffusion .Nevertheless, it is possible that the driving force in this instance is also the electrochemical gradient if the membrane potential has been increased by a change in the relationship of P_{Cl} to P_K. For, as first theoretically shown by Glynn and Warner (2) and discussed elsewhere by Kregenow and Hoffman (5), if P_K approaches or exceeds P_{Cl}, then the resultant increase in membrane potential can produce an acceleration in K influx. This and the other possibility suggested previously (4)—that the rapid bidirectional movement of K represents a process of facilitated diffusion—are currently under investigation.

The cellular change which initiates the response remains unknown, although a discussion of some of its characteristics is possible. One of its most unusual features is the manner in which relatively small changes in cell size bring about sufficient modifications to stimulate very different cell responses. The response to a 6% increase in cell size (3) differs dramatically from the response to a 6% decrease in size (4). It seems unlikely that a small change in the concentration of some substrate created by this slight amount of swelling or shrinking could serve as the sensitive variable. Excluded also are the Na or K concentration or content, total (Na + K) concentration or content, and the intracellular osmolality, for they fail to vary as the cells regulate their volume, or vary so much as to lack the necessary consistency required of the initiating event (see also 3 and 4). As suggested previously (3), a membrane component which responds with a conformational change to variations in cell size, could be the underlying event.

One final comment seems appropriate. There are apparently different roles played by the cation pump and "volume-controlling mechanism." In this study and others (3, 4), the cation pump (defined as the cause of that portion of Na and K movement blocked by ouabain) appears to regulate the relative

concentrations of Na and K; while the volume-controlling mechanism controls the total (Na + K) content.

The author wishes to express his gratitude to Dr. Jack Orloff for his encouragement during the study.

Received for publication 25 October 1971.

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