The Effect of Norepinephrine and Dibutyryl Cyclic Adenosine Monophosphate on Cation Transport in Duck Erythrocytes

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ABSTRACT Freshly prepared duck erythrocytes, incubated either in plasma or an isotonic synthetic medium containing norepinephrine ([K] of both media $\sim$ 2.5 mM), maintain water and electrolyte composition in the steady state (upper steady state) for at least 90 min. If incubated in the synthetic medium without norepinephrine or in plasma to which a β-adrenergic blocking agent (propranolol) is added, the cells lose both water and electrolyte (predominantly KCl) until a new steady state is reached (lower steady state). Reaccumulation of water and electrolyte from isotonic solutions toward the upper steady-state levels requires the addition of norepinephrine and KCl. Reaccumulation is maximal when the concentration of K and norepinephrine in the medium is 15 mM and 10^{-7} M, respectively. Dibutyryl cyclic-AMP (10^{-2} M) mimics norepinephrine in lower steady-state cells. Although an analogous effect in upper steady-state cells was not established with certainty, it is proposed that the catecholamine-induced net changes in water and electrolyte movement in duck erythrocytes are a consequence of stimulation of the activity of a membrane-bound adenyl cyclase system.

INTRODUCTION

In 1956 Orskov (1) reported that norepinephrine induces an uptake of K from plasma by pigeon erythrocytes in vitro. Subsequently Klainer et al. (2), Davoren and Sutherland (3, 4), and Oye and Sutherland (5) established that the plasma membrane of avian erythrocytes contains a catecholamine-sensitive adenyl cyclase system. Catecholamines are known to affect electrolyte transport in a number of tissues (6, 7) and in the case of liver, Friedman and Park (8) have implicated cyclic-AMP in the genesis of some of these changes. The stimulation of transcellular transport of water and sodium by antidiuretic hormone in toad bladder (9) and of water in the collecting tubule (10), both

epithelial structures, is mediated by cyclic-AMP. In view of the general hypothesis of Sutherland and his coworkers (11) that a variety of hormones including catecholamines exert their characteristic effects in receptor cells via the intermediacy of cyclic-AMP, it was reasonable to assume that electrolyte transport and thus the intracellular electrolyte composition of the avian erythrocyte may also be regulated in part by catecholamine-induced changes in the concentration of cyclic-AMP within the cell. The present studies were designed to investigate this problem in duck erythrocytes.

MATERIALS AND METHODS

Common Muscovy ducks of either sex were used in all studies. Each experiment was performed on blood obtained by cardiac puncture from a single duck.

Media for incubation of erythrocytes were as follows: 1. Normal duck plasma, stored 0–2 hr at 4°C prior to use ([K] ~ 2.5 mM/liter). 2. Standard synthetic medium with the following composition (mM/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[1] (2.5 g%). When required the potassium chloride concentration was altered by substituting an equimolar amount of KCl for NaCl. Sodium-free synthetic media with the following compositions (mM/liter) were also used: (*a*) $MgCl_2$ 2, $CaCl_2$ 1, $KHCO_3$ 28, KH_2PO_4 3.4, choline Cl 121.7, dextrose 20, and albumin (2.5 g%). (*b*) $MgCl_2$ 80, $CaCl_2$ 1, $Mg(HCO_3)_2$ 14, KH_2PO_4 3.4, KCl 16, dextrose 20, and albumin (2.5 g%). $Mg(HCO_3)_2$ was prepared by adding $MgCO_3$[2] to distilled water and gassing with 100% CO_2 until it dissolved. Chloromycetin (0.5 mg%) was added to all experimental solutions incubated at 42°C to prevent bacterial growth. All media were gassed with water-saturated 5% CO_2–95% O_2 for 30–90 min at 42°C prior to their use as either wash or incubation medium. After gassing the pH of all media was 7.40. All synthetic solutions were isotonic to plasma. An isotonic solution is defined as a solution in which added red blood cells retain the same per cent cell H_2O (w/w) as in normal duck plasma. This comparison was made at room temperature on packed cells,[3] analyzed after centrifugation at 4°C (see Analytical methods).

General Experimental Procedures

Blood was drawn into heparinized syringes (approximately 1 cc of panheparin[4] per 100 cc of blood) and routinely poured through a gauze filter to remove any small clots. The blood was then centrifuged at 4°C for 5 or 10 min at 31,000 × *g*. Cells and plasma were separated and the buffy coat removed by suction. Cells were added directly to fresh duck plasma when the latter was to serve as the experimental medium. If a synthetic medium was to be used the cells were first washed one to two times with ice-cold standard synthetic medium to remove any trapped plasma, before add-

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

[2] Magnesium carbonate (Merck & Co., Rahway, N. J.) approximate mol wt 100.

[3] The pH of both plasma and synthetic medium at the time of analysis was 7.70 ± 0.02.

[4] 1000 units/ml sodium heparin (Abbott Laboratories, North Chicago, Ill.).

ing the cells to the appropriate bathing solution. In those studies in which a preincubation period was necessary, the cells after washing were placed in the standard synthetic medium (K concentration 2.5 up to 40 millimolar) for 90 min. At the end of this period they were either reisolated rapidly and placed in another medium or permitted to remain in the original solution to which appropriate additions were made. Details of the specific experimental protocols are presented in the Results section. Continuous water-saturated 95% O_2–5% CO_2 was passed via a manifold over each experimental flask during the incubation period. The flasks were incubated in a shaker bath[5] at a temperature of 42°C. Both a constant flow of the O_2–CO_2 mixture as well as thorough mixing was necessary for reproducible results. A hematocrit of 10% was used in all incubation procedures. There was no visible hemolysis (<0.5%) in any of the experiments.

Aliquots for analysis were removed at appropriate times as indicated below by pouring a sample from the mouth of the experimental flask after disconnecting the flow of O_2 and CO_2. The sampling procedure required less than 1 min. Samples were centrifuged in special Lucite tubes at 31,000 × *g* for 10 min at 4°C in a Sorvall RC-2 centrifuge. An aliquot of the supernatant was pipetted volumetrically for analysis. The remainder of the supernatant was then removed by careful suction and discarded. After thorough mixing of the red cell pellet, a sample of cells was transferred volumetrically for analysis utilizing a 250 λ Misco[6] blood pipet. Another aliquot of cells was removed for the determination of cell water content. All manipulations were performed at room temperature. A similar procedure was used in the preparation of freshly drawn cells for Na, K, and cell H_2O analysis.

Analytical Methods

NA$^+$ AND K$^+$ ANALYSIS Cell samples were hemolyzed by diluting them 1:200 in demineralized water. The hemolysate was centrifuged at 31,000 × *g* for 5 min either directly or after adding 2 drops of concentrated NH_4OH,[7] in order to remove residual cellular elements or the gel which formed after adding NH_4OH. This procedure did not affect the analytical results. Na^+ and K^+ were determined with an internal Li standard flame photometer (IL Model 143). Cell sodium concentration was corrected for a trapped medium content of 1.3% (see below).

CL$^-$ ANALYSIS Volumetric samples of packed cells and plasma were diluted appropriately with demineralized water. After deproteinization in a $ZnSO_4$–NaOH solution, the samples were read in duplicate on a Cotlove chloridometer.[8] Cell chloride concentration was also corrected for a trapped medium content of 1.3%.

CELL WATER DETERMINATION Cell water was determined gravimetrically by the difference between the wet and dry weight of an aliquot of cells. The sample of centrifuged cells was weighed initially and again after drying to constant weight for 24 hr at 56°C. The vial containing the dried cells was allowed to reach room

[5] Eberbach Corp., Ann Arbor, Mich.
[6] Microchemical Specialties Co., Berkeley, Calif.
[7] Fisher Scientific Company, Silver Spring, Md.
[8] Laboratory Glass & Instrument Corp., New York.

temperature in a desiccator prior to reweighing. No correction was made for trapped plasma.

SPECIFIC GRAVITY OF CELLS The specific gravity of cells was determined by weighing a known volume of packed cells and distilled H_2O in a special Misco micropipet at room temperature.

TRAPPED MEDIUM DETERMINATION Trapped medium was estimated by adding 6 μl of carboxyl ^{14}C-inulin[9] to each centrifuge tube prior to pouring the sample from the experimental flask. The solutions were thoroughly mixed and centrifuged as before. The samples of medium and cells were deproteinated with 6% PCA, added to a mixture of toluene and Triton χ-100 (Packard Instrument Co., Downers Grove, Ill.) (3.5:1) containing 5.5 g PPO (Packard) and 0.15 g dimethyl POPOP (Packard) per liter, and then counted in a liquid scintillation spectrometer (Packard Tricarb model 3003). Quenching corrections, when necessary, were made by using the internal standard technique. The per cent trapped medium in all the experimental designs was 1.3% ± 0.1%. This value is in close agreement with the value determined previously for duck erythrocytes using a comparable technique (12).

Materials

(-)Arterenol bitartrate hydrate (B grade) was obtained from Calbiochem, Los Angeles, Calif. N^6,2'0 dibutyryl 3',5' AMP was obtained from Boehringer Mannheim Corp., New York. Propranolol was a gift of Ayerst Laboratories, Inc., New York, while chloromycetin was donated by the Parke-Davis Company, Towson, Md.

Presentation of Data

Cell water is expressed as a percentage of the original wet weight of an aliquot of cells (% cells H_2O w/w). This value was multiplied by the specific gravity of the cells to determine the per cent cell water on a volume basis (% cell H_2O V/V). Chloride concentration per liter of cell water (mM/liter cell H_2O) was obtained by dividing the chloride concentration per liter of cells (mM/liter cells) by the % cell H_2O (V/V). This value was used in the calculation of the chloride concentration ratio Cl_o:Cl_i where o refers to the outside bathing solution and i, the intracellular phase. The concentration of sodium, potassium, and chloride in most of the studies is expressed as millimoles per liter of that number of cells which initially occupied 1 liter (mM/liter onc = millimoles per liter of original number of cells), where

$$\text{mM/liter onc} = (\text{mM/liter cells}) \times \frac{\frac{W_{D\,to}}{W_{W\,to}}}{\frac{W_{D\,t}}{W_{W_t}}} \times \frac{P_t}{P_{to}}$$

$W_{D_{to}}$ and $W_{w_{to}}$ = dry and wet weight of an aliquot of packed cells at t_o.
W_{D_t} and W_{w_t} = dry and wet weight of an aliquot of packed cells at any time t.
P_t and P_{to} = specific gravity of packed cells obtained at time t and t_o.

[9] New England Nuclear Corp. (Boston, Mass.). 320 mg inulin = 1 mCi.

This calculation corrects for changes in cell size and permits expression of results as the electrolyte content per unit weight of cell solids. Since a direct relationship exists between the dry weight of cells and the number of cells present, this is analogous to expressing the results as millimoles per that number of cells originally present in 1 liter of cells. A change in the cation concentration/liter onc thus represents a net loss or gain of electrolyte by the cell.

RESULTS

The concentration of potassium[10] in freshly drawn red cells (isolated 10–20 min after cardiac puncture) is 110 ± 1 mM per liter; that of sodium[11] is 6.09 ± 0.11 mM per liter and the per cent cell water (w/w)[12] is 61.8 ± 0.1. The cells remain in the steady state for at least 1½ hr when incubated in their own plasma (Table I) (K concentration ~2.5 mM per liter). This steady state will henceforth be referred to for identification purposes as the upper steady state (USS). Cells added to the standard synthetic medium lose potassium and water (see below) but this change in composition is prevented by the addition of 10^{-6} M norepinephrine (Table I). Under these circumstances the cells remain in the USS virtually as when incubated in their own plasma. No effect of epinephrine is evident if it is added to cells incubated in fresh plasma with this potassium concentration.

Table II illustrates the net losses of potassium, sodium, and water from erythrocytes incubated for 90 min in standard synthetic medium without norepinephrine. Shrinkage and loss of cation were not prevented by increasing the K concentration of the medium to 15 millimolar. It was assumed that the absence of electrolyte and water loss from red cells incubated either in synthetic medium containing norepinephrine or in normal plasma (noted in Table I) indicated that endogenous catecholamine was present in fresh plasma. This conclusion was supported by the observation that the addition of a β- adrenergic blocking agent (10^{-4} M propranolol) to plasma resulted in electrolyte and water losses from red cells similar to those in synthetic medium without norepinephrine. These results are summarized in Table II. At this point it cannot be determined whether endogenous catecholamine is normally present in high concentration in duck plasma and serves to maintain intracellular composition at the upper steady-state level or whether it was released in consequence of cardiac puncture or manipulations in the intact duck prior to removal of blood from the body.

The results of studies designed to determine whether cyclic-AMP plays an integral role in the maintenance of the upper steady state were inconclusive. In three of six studies 10^{-2} M dibutyryl cyclic-AMP (13, 14), when added to

[10] SEM $N = 25$.
[11] SEM $N = 25$.
[12] SEM $N = 30$.

TABLE I

UPPER STEADY-STATE Na, K, Cl, AND WATER CONTENT OF DUCK ERYTHROCYTES INCUBATED IN PLASMA AND IN STANDARD SYNTHETIC MEDIUM PLUS 10^{-6} M NOREPINEPHRINE

Medium	K_c Time, min			Na_c Time, min			% cell H_2O (w/w) Time, min			Cl_c Time, min		
	0	60	90	0	60	90	0	60	90	0	60	90
	mM/liter RBC			*mM/liter RBC*						*mM/liter RBC*		
Fresh duck plasma $[K]_o \sim 2.5$ mM/liter	106	106	—	6.5	6.3	—	61.0	61.1	—	60	58	—
	109	110	—	5.9	5.7	—	61.4	61.5	—	55	55	—
	115	—	115	6.5	—	6.4	62.5	—	62.6	50	53	—
	113	—	114	6.3	—	6.1	61.5	—	61.8			

	K_c Time, min				Na_c Time, min				% cell H_2O (w/w) Time, min			
	0	60	120	180	0	60	120	180	0	60	120	180
	mM/liter RBC				*mM/liter RBC*							
Standard synthetic medium $[K]_o$= 2.5 mM/liter + 10^{-6} M norepinephrine	116	118	117	—	5.7	5.6	5.6	—	61.5	61.5	61.3	—
	105	104	104	—	6.9	7.2	6.9	—	60.6	60.5	60.3	—
	105	106	107	107	6.3	6.3	6.0	6.2	61.5	61.5	61.5	61.2
	113	113	112	113	6.5	6.3	6.1	6.3	61.3	61.3	61.4	61.4

Chloride values were determined in separate experiments.

the synthetic medium, mimicked norepinephrine in that maintenance of the water and electrolyte composition of upper steady-state cells was achieved for 3 hr. In three other experiments, dibutyryl cyclic-AMP did not prevent the losses of electrolyte and water. We have no explanation for the differences between the two sets of studies.

The loss of cell water and electrolyte in the absence of added hormone virtually ceases within approximately 90 min. Following this time period, cells remain in a new steady state which henceforth will be referred to as the lower steady state (LSS) for at least another 90 min. This is illustrated in Table III. It should be noted that the development of the lower steady state is not prevented by increasing the K concentration of preincubation and ex-

TABLE II

CHANGES IN ELECTROLYTE AND WATER CONTENT OF DUCK ERYTHROCYTES AFTER 90 MIN INCUBATION IN SYNTHETIC MEDIUM WITHOUT NOREPINEPHRINE OR IN PLASMA CONTAINING 10^{-4} M PROPRANOLOL

Medium	N	ΔK_c	ΔNa_c	Δ% cell H_2O (w/w)*
		mM/liter$_{onc}$	mM/liter$_{onc}$	
Standard synthetic medium $[K]_o$=2.5 mM/liter	5	−8.5 (±0.5)	−0.83 (±0.08)	−1.6 (±0.3)
Standard synthetic medium $[K]_o$=15 mM/liter	6	−7.5 (±0.4)	−0.6 (±0.1)	−2.0 (±0.3)
Plasma ($[K]_o$~2.5 mM/liter)		−10.0	−0.7	−2.6
+10^{-4} M propranolol		−8.0	−0.8	−1.9

Control cells were maintained in the upper steady state during the 90 min experimental period by incubating them in plasma or synthetic medium I ($[K]_o$=2.5 mM/liter) with 10^{-6} M norepinephrine. Δ cellular changes were obtained by calculating the difference in the values at 90 min from the steady-state values of control cells at this time. Numbers in parentheses are SEM.
* A 2% decrease in % cell H_2O (w/w) corresponds approximately to a 5% decrease in cell volume.

perimental bathing media to 15 millimolar. In other studies 40 millimolar K was also ineffective.

The characteristics of the lower steady state were investigated in the following series of studies. In these experiments, the cells were first permitted to reach the lower steady state by preincubation for 90 min in synthetic medium containing no norepinephrine. At the end of this time period and immediately prior to obtaining a zero time sample, the experimental manipulation was instituted. In general, it was observed that the cation-"depleted," shrunken cell in the lower steady state reverted toward "normal" composition if bathed in standard synthetic medium containing 15 mM K and norepinephrine. No reconstitution occurred in the standard synthetic medium (K = 2.5 mM) in

TABLE III

LOWER STEADY-STATE ELECTROLYTE AND WATER CONTENT OF DUCK ERYTHROCYTES IN STANDARD SYNTHETIC MEDIUM WITHOUT NOREPINEPHRINE

	K_c		Na_c		% cell H_2O (w/w)			
	Time, min		Time, min		Time, min			
	0	90	0	90	0	90	Cl_c	
	mM/liter RBC		*mM/liter RBC*		*mM/liter RBC*		*mM/liter RBC*	
Standard synthetic medium	110	111	6.7	6.8	59.3	59.6		
$[K]_o$=2.5 mM/liter	110	110	5.3	5.3	59.7	59.6		
	108	109	4.7	5.0	59.5	59.4		
	Time, min		Time, min		Time, min		Time, min	
	0	60	0	60	0	60	0	60
Standard synthetic medium	109	110	5.8	5.9	60.4	60.3	57	58
$[K]_o$=15 mM/liter	106	106	5.7	5.8	60.0	59.8	55	54
	104	103	3.7	3.4	60.5	60.3	61	60

Cells were preincubated in the standard synthetic medium ($[K]_o$=either 2.5 mM/liter or 15 mM/liter) without norepinephrine for 90 min. At this time, the initial sample was obtained (time 0). The 60 and 90 min values were obtained from analyses of cells that had been incubated in their respective bathing solutions for 150 and 180 min. Cells were considered to be at a steady state if their concentration (mM/liter$_{onc}$) of Na, K, and Cl did not vary by more than ±0.3, ±0.2, ±2, respectively and the % cell water (w/w) did not vary by more than ±0.3 during the experimental interval.

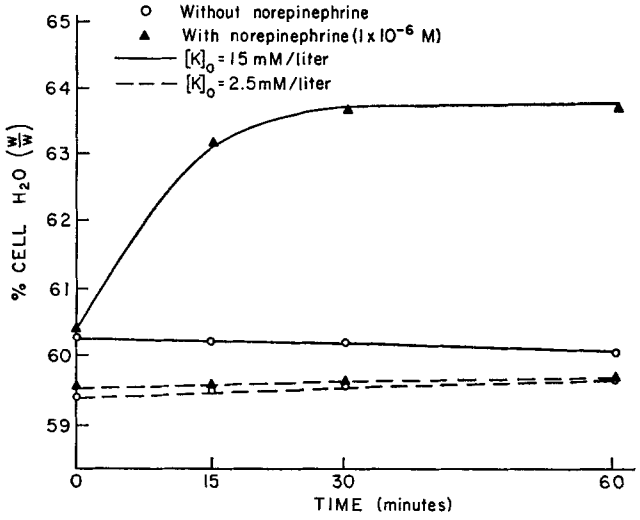

FIGURE 1. Volume changes of duck erythrocytes (LSS) in response to variations in the extracellular potassium concentration and the addition of norepinephrine to the bathing medium. Lower steady-state cells were obtained by preincubating the cells for 90 min in the standard synthetic medium ($[K]_o$ = either 2.5 or 15 mM/liter). Norepinephrine (10^{-6} M) was added 30 sec before time 0.

the presence of norepinephrine or in medium containing 15 mM K without epinephrine.

Changes in cell volume under these circumstances are illustrated in Fig. 1. The net changes in electrolyte composition and water content during similar and other manipulations are summarized in Table IV. When cells swell in the presence of norepinephrine and an elevated concentration of K (15

TABLE IV

NOREPINEPHRINE-DEPENDENT CHANGES IN THE K, Na, AND H_2O CONTENT OF LSS DUCK ERYTHROCYTES

Standard synthetic medium		ΔK_c		ΔNa_c		Δ% cell H_2O (w/w)	
		Time, min		Time, min		Time, min	
$[K]_o$	Additions	30	60	30	60	30	60
		mM/liter onc		mM/liter onc			
Experiment 1							
2.5 mM/liter	None	0	0	+0.2	+0.3	0	+0.2
	10^{-6} M norepinephrine	0	+1	0	0	0	+0.1
15 mM/liter	None	0	0	0	0	0	−0.1
	$+10^{-4}$ M propranolol	0	0	−0.1	0	0	−0.1
	$+10^{-6}$ M norepinephrine	+12	+20	+2.0	+1.1	+3.0	+3.3
	$+10^{-6}$ M norepinephrine $+10^{-4}$ M propranolol	0	0	−0.1	0	−0.2	−0.1
Experiment 2							
2.5 mM/liter	None	0	−2	0	+0.1	−0.1	−0.2
	10^{-6} M norepinephrine	+1	0	+0.2	+0.2	0	+0.1
15 mM/liter	None	0	0	0	0	0	0
	$+10^{-4}$ M propranolol	0	0	0	0	0	0
	$+10^{-6}$ M norepinephrine	+11	+14	+2.1	+1.0	+2.6	+2.4
	$+10^{-6}$ M norepinephrine $+10^{-4}$ M propranolol	−1	0	−0.1	0	−0.1	−0.1

Lower steady-state cells were obtained by preincubating cells for 90 min in the standard synthetic medium ($[K]_o$ = either 2.5 mM/liter or 15 mM/liter). Following this period norepinephrine and propranolol were added. 30 sec later the 0 time sample was obtained. Δ cellular changes were obtained by calculating the difference in the values at 30 and 60 min from the value at 0 time.

millimolar), the gain in cell water is accompanied primarily by an uptake of potassium. Simultaneous with these changes there is at first a slight increase in cell sodium content followed by a fall though not to the initial level. No changes were noted in lower steady-state cells incubated in the standard synthetic medium with or without norepinephrine. It is pertinent that propranolol alone had no effect on the lower steady-state composition of duck erythrocytes, but did inhibit the action of norepinephrine.

Changes in net chloride content of lower steady-state cells incubated in synthetic medium containing 15 millimolar K plus norepinephrine are compared with simultaneous changes in net cation accumulation in Table IV A. It is apparent that virtually all the accumulated cation is accompanied by chloride. The explanation for the small, though statistically significant anion deficit ($p < 0.05$, $N = 5$) is not clear. However, this result would occur if bicarbonate, the other major anion in the medium were distributed passively, as is generally assumed.

TABLE IV A

CHANGES IN CELL CHLORIDE ASSOCIATED WITH THE RESPONSE OF DUCK ERYTHROCYTES (LSS) TO 10^{-6} M NOREPINEPHRINE AND AN ELEVATED $[K]_o$

	Δ Na + K		Δ Cl		$\frac{[Cl]_o}{[Cl]_i}$		
	Time, min		Time, min		Time, min		
	30	60	30	60	0	30	60
	mM/liter$_{onc}$		*mM/liter$_{onc}$*				
Experiment 1	+16	+16	+15	+13	1.49	1.40	1.45
Experiment 2	+18	+20	+16	+16	1.51	1.38	1.40
Experiment 3	+19	+20	+13	+14	1.59	1.47	1.46

Lower steady-state cells were obtained by preincubating cells for 90 min in the standard synthetic medium ($[K]_o$ = 15 mM/liter). Following this period norepinephrine was added. 30 sec later the 0 time sample was obtained. Control cells, incubated without norepinephrine, remained in the steady state with respect to chloride during the experimental period. Δ cellular changes were obtained by calculating the difference in the value for sodium plus potassium, or chloride at 30 and 60 min from the corresponding value at 0 time.

TABLE V

A COMPARISON OF THE CELLULAR CHANGES PRODUCED BY NOREPINEPHRINE AND DIBUTYRYL CYCLIC-AMP IN DUCK ERYTHROCYTES (LSS)

Addition	30 min			60 min		
	ΔNa_c	ΔK_c	Δ % cell H_2O (w/w)	ΔNa_c	ΔK_c	Δ % cell H_2O (w/w)
	mM/liter$_{onc}$			*mM/liter$_{onc}$*		
Norepinephrine (10^{-6} M)	2.4	12	3.4	1.5	18	4.0
Norepinephrine (10^{-6} M)	2.5	14	3.7	1.1	19	4.0
DC-AMP (10^{-2} M)	2.2	14	4.0	1.5	17	3.7
DC-AMP (10^{-2} M)	2.0	14	3.5	0.9	18	3.5

Lower steady-state cells were obtained by preincubating cells for 90 min in the standard synthetic medium ($[K]_o$ = 15 mM/liter). Either 10^{-6} M norepinephrine or 10^{-2} M dibutyryl cyclic-AMP was added 30 sec before time 0 and after the 90 min preincubation period. Δ cellular changes were obtained by calculating the difference in the values at 30 and 60 min from the value at 0 time.

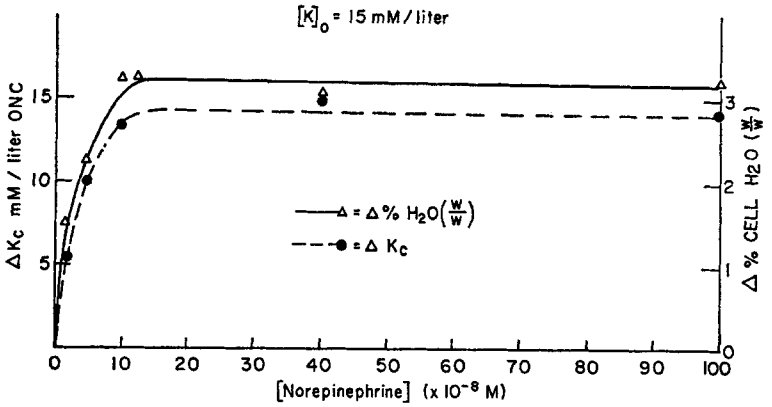

FIGURE 2. The increase in the uptake of K and H_2O in duck erythrocytes (LSS) in response to increasing the norepinephrine concentration of the bathing medium ($[K]_o$ = 15 mM/liter). Lower steady-state cells were obtained by preincubating cells for 90 min in the standard synthetic medium ($[K]_o$ = 15 mM/liter). Norepinephrine was added at the end of the preincubation period. Each point represents the net accumulation during 30 min of exposure to the appropriate dose of the catecholamine. Control cells, incubated without norepinephrine, remained in the LSS during the experimental period. Δ cellular changes were obtained by calculating the difference in the values for norepinephrine-treated cells at 30 min from the LSS value of control cells.

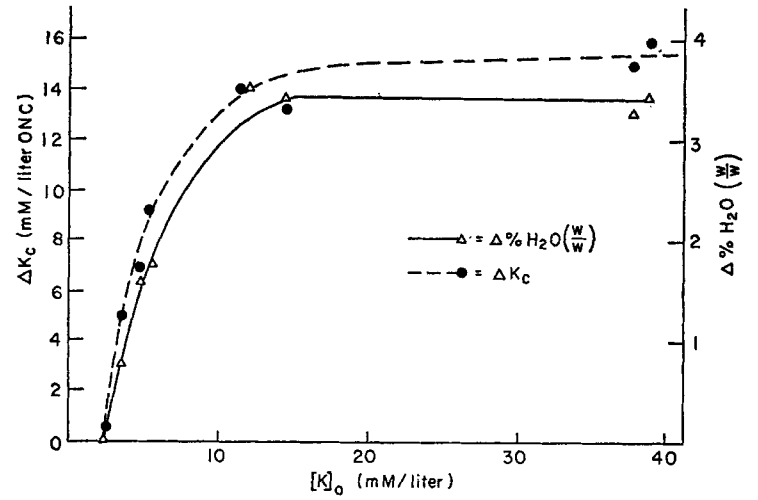

FIGURE 3. The uptake of K and H_2O by duck erythrocytes (LSS) as a function of the external potassium concentration. Lower steady-state cells were obtained by preincubation for 90 min in standard synthetic media in which the $[K]_o$ varied from 2.5 to 40 mM/liter. All solutions were isosmolal. Duplicate flasks were employed for each $[K]_o$. 10^{-6} M norepinephrine was added to one of the two duplicate flasks. The other served as a control with the cells remaining in the lower steady state during the experimental period. Δ cellular values for each $[K]_o$ were obtained by calculating the difference between the 30 min content of norepinephrine-treated cells and the steady-state content of the appropriate control cells.

In contrast to its variable effect in the upper steady state, dibutyryl cyclic-AMP (10^{-2} M) when added to lower steady-state cells bathed in synthetic medium containing 15 mM K produced an uptake of electrolyte and water in all studies ($N = 7$) similar to that due to norepinephrine (Table V).

The characteristics of the norepinephrine- and potassium-dependent uptake of electrolytes and H_2O of lower steady-state cells were examined in the following studies. Fig. 2 is a norepinephrine dose-response curve. Net accumulation was measured for a 30 min period only. The maximum response with respect to K and water uptake required approximately 10^{-7} M norepinephrine. It should be noted that synthetic medium with 15 mM K served as the bathing solution since this concentration of K is also necessary to evoke a maximal response (see below). Not only is the extent of K and water uptake dependent upon the concentration of norepinephrine but so also is that of sodium. At 30 min, cells incubated in 10^{-8} M norepinephrine gain one-half to one-third the quantity of sodium accumulated by cells incubated with 10^{-6} M norepinephrine, a maximally effective concentration.

TABLE VI

THE EFFECT OF REPLACING $[Na]_o$ ON THE CELLULAR RESPONSE OF DUCK ERYTHROCYTES (LSS) TO NOREPINEPHRINE AND AN ELEVATED $[K]_o$

Experiment	Medium	10^{-6} M Norepinephrine	ΔK_c Time, min		ΔNa_c Time, min		Δ % cell H_2O (w/w) Time, min	
			30	60	30	60	30	60
			mM/liter onc		mM/liter onc			
A	Na-free choline	−	−2	−1	0	+0.3	−1.2	−1.2
	$[K]_o$ = 30 mM/liter	+	−2	−1	0.6	+0.6	−1.9	−1.8
	Synthetic	−	+1	+2	+1.0	+1.6	0	0
	$[K]_o$ = 30 mM/liter	+	+12	+16	+5.7	+4.7	+3.5	+3.3
B	Na-free Mg	−	0	−1	−0.2	−0.2	−0.2	0
	$[K]_o$ = 20 mM/liter	+	−1	−3	−2.1	−2.0	−0.9	−0.7
	Synthetic	−	0	−1	0	+0.6	0.0	−0.2
	$[K]_o$ = 20 mM/liter	+	+13	+18	+5.0	+4.7	+2.8	+3.6

Lower steady-state cells were obtained by preincubating cells in the standard synthetic medium ($[K]_o$ = 2.5 mM/liter). At the end of the 90 min preincubation period, the cells were reisolated without washing, introduced into appropriate flasks containing standard synthetic medium ($[K]_o$ = 20 or 30 mM/liter), or isotonic Na-free choline medium ($[K]_o$ = 30 mM/liter), or isotonic Na-free Mg^{++} medium ($[K]_o$ = 20 mM/liter). Immediately after adding the cells and prior to time 0, 10^{-6} M norepinephrine was added to one of two duplicate flasks containing the same experimental medium. Δ cellular changes were obtained by calculating the difference in the values at 30 and 60 min from the value at time 0. The final Na^+ concentrations of the choline and Mg media were always less than 7 mM. The residual Na^+ represented that transferred with the cells from the preincubation solutions.

The effect of different concentrations of K in the bathing medium on the reaccumulation phenomenon of lower steady-state cells is illustrated in Fig. 3. A maximally effective concentration of norepinephrine (10^{-6} M) was present in all experimental flasks. Net accumulation of potassium and water in a 30 min time period reaches an apparent maximum when the concentration of potassium in the bathing medium approximates 15 millimolar. At this juncture it should be reemphasized that negligible reaccumulation if any, of electrolyte and water by lower steady-state cells occurs when standard synthetic medium ([K] = 2.5 mM) is employed, though this concentration is sufficient to maintain cells in the upper steady state.

The results of the experiment summarized in Table VI demonstrate that norepinephrine (10^{-6} M) dependent reaccumulation of water and electrolyte is sodium-dependent as well. Substitution of either choline or magnesium for sodium in the bathing medium eliminates the norepinephrine-dependent reaccumulation. The concentrations of K employed in the control standard synthetic media and in the experimental media (20 and 30 millimolar) were within the maximally effective range noted earlier (Fig. 3).

DISCUSSION

A catecholamine-dependent adenyl cyclase system has been reported to be present in the plasma membrane of red cells from a variety of vertebrates including certain mammals (4, 15, 16). Its functional significance is unknown and the role of catecholamines in erythrocytes has not been clearly defined. Orskov's original studies (1) did implicate the catecholamines in shifts of K and H_2O in pigeon red cells. On the basis of the present studies it is reasonable to assume that the electrolyte content and volume of avian erythrocytes, at least, may be regulated in part by catecholamines via the intermediacy of cyclic-AMP. Lower steady-state duck red cells respond similarly to norepinephrine and dibutyryl cyclic-AMP in vitro under appropriate circumstances. Though a similar relationship has not been established with certainty in the upper steady state, it is not unreasonable to conclude that maintenance of the intracellular composition in the upper steady state requires norepinephrine and by inference participation of the adenyl cyclase system.

Whether similar changes occur in vivo and contribute (*a*) to the regulation of extracellular K concentration by providing a sink for the dissipation of K extruded into the extracellular fluid from other tissues, or (*b*) to the regulation of red cell volume under isotonic conditions has not yet been established. The inhibitory effect of propranolol a β-adrenergic blocking agent, on the response to norepinephrine is analogous to that observed with respect to other cyclic-AMP–mediated changes in other cell types. In these, norepinephrine stimulation of adenyl cyclase activity is presumed to involve interaction of the hor-

mone with a putative adrenergic receptor site associated with the enzyme (17).

Maintenance of the upper steady state in vitro by norepinephrine occurs when the concentration of K in the bathing medium is within "normal" limits (approximately 2.5 mM per liter). In contrast, catecholamine-dependent reaccumulation of water and K by the erythrocyte in the lower steady state is maximal only when the outside K concentration is elevated to approximately 15 millimolar. Net uptake of K under these circumstances also requires the presence of sodium in the external medium. If both hormone-dependent phenomena, maintenance of the upper steady state and uptake of electrolyte and water by cells in the lower steady state, reflect an effect of the catecholamine on the same rate-limiting step, the difference in their requirement for extracellular K is not readily explicable.

It is generally assumed that chloride is distributed passively in duck erythrocytes (12, 18) as in the human erythrocyte whereas neither Na nor K is. With an electrical potential of less than 13 mv (inside negative), as calculated from the chloride ratio, the net accumulation of K induced by the hormone in lower steady-state cells is clearly against an electrochemical gradient and by definition is an active transport process. The hormone conceivably could induce net uptake of K by (*a*) stimulating the active inward flux of K, (*b*) by decreasing passive efflux of K from the cell, or (*c*) by an appropriate combination of the two effects. On the basis of preliminary studies the second possibility has been excluded. The efflux of K in the lower steady state (medium [K] 15 mM) without hormone is approximately 9–12 mM per liter per hour. Were this to represent the maximal passive flux from the cell, total elimination of this component of the flux by the hormone would only permit the cells to gain a maximum of 6 mM per liter in 30 min. Since, as is seen in Table V, the net uptake of K following administration of norepinephrine exceeds this figure and approximates 12 mM per liter in 30 min, active inward flux of K must necessarily have been activated by the hormone. An associated change in efflux cannot be excluded. Although the normal concentration of norepinephrine in duck plasma is not known, it is conceivable that the lower concentrations found to be effective in this study fall within the physiological range. It should be pointed out therefore that the above response of lower steady-state duck erythrocytes to the catecholamine may be an example of stimulation of active transport induced by a physiological concentration of hormone.

A preliminary report of some of these findings was presented to the Federation of American Societies for Experimental Biology in April, 1968.

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