Ouabain-Insensitive Salt and Water Movements in Duck Red Cells

III. *The Role of Chloride in the Volume Response*

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ABSTRACT This paper describes the effect of external chloride on the typical swelling response induced in duck red cells by hypertonicity or norepinephrine. Lowering chloride inhibits swelling and produces concomitant changes in net movements of sodium and potassium in ouabain-treated cells, which resemble the effect of lowering external sodium or potassium. Inhibition is the same whether chloride is replaced with gluconate or with an osmotic equivalent of sucrose. Since changes in external chloride also cause predictable changes in cell chloride, pH, and water, these variables were systematically investigated by varying external pH along with chloride. Lowering pH to 6.60 does not abolish the response if external chloride levels are normal, although the cells are initially swollen due to the increased acidity. Cells deliberately preswollen in hypotonic solutions with appropriate ionic composition can also respond to norepinephrine by further swelling. These results rule out initial values of cell water, chloride, and pH as significant variables affecting the response. Initial values of the chloride equilibrium potential do have a marked effect on the direction and rate of net water movement. If chloride is lowered by replacement with the permeant anion, acetate, E_{Cl} is unchanged and a normal response to norepinephrine, which is inhibited by furosemide, is observed. Increasing internal sodium by the nystatin technique also inhibits the response. A theory is developed which predicts that the cotransport carrier proposed in the previous paper (W. F. Schmidt and T. J. McManus. 1977*b*. *J. Gen. Physiol.* **70**:81–97) moves in response to the net electrochemical potential difference driving sodium and potassium across the membrane. Predictions of this theory fit the data for both cations and anions.

INTRODUCTION

This is the third in a series of papers dealing with ouabain-insensitive salt and water movements in duck red cells. The previous two papers (Schmidt and McManus, 1977*a*, *b*) presented studies of the response of ouabain-treated cells to a hypertonic environment or to norepinephrine in an isotonic medium. It was concluded that the net accumulation of salt and water is mediated in both cases

by a cotransport of sodium plus potassium into the cell. Influx of either cation is dependent on the presence of the other, and is inhibited by furosemide.

This paper examines the role of chloride in both the hypertonic and norepinephrine systems. Results demonstrate a marked anion dependence of the overall response. Factors which determine the extent of cell swelling once the cotransport mechanism is activated are also considered. Finally, a concept of the volume response is presented which integrates the anion data reported here with the evidence on cations from the previous papers. We propose that once the system is activated, the directions and extent of net salt (and water) movement can be related directly to the sum of the electrochemical potentials driving sodium and potassium across the membrane. Thus, the energy for co-ion transport resides in existing ion gradients.

MATERIALS AND METHODS

Fresh blood from an adult white Pekin duck was obtained on the day of experimentation by the technique previously described (Schmidt and McManus, 1977 *a*). After removal of plasma and buffy coat, cells were washed four times in 5 vol of ice-cold 170 mM NaCl, then preincubated at 41°C for 90 min in a buffered solution isotonic with duck plasma (323 mosmol). Composition of the preincubation solution has also been given in the previous paper. After preincubation, cells were washed once with 323 mosmol $MgCl_2$, then added to hypertonic or isotonic test solutions at a final hematocrit of 2%. The general experimental procedure used in these incubations, as well as methods of preparation of ouabain, norepinephrine, and furosemide, is also outlined in the two previous papers.

Chloride substitution was made by isosmotic replacement of NaCl and KCl by sucrose or by sodium and potassium gluconate. The sodium salt of D-gluconic acid (Sigma Chemical Co., St. Louis, Mo.) was yellow in solution. After repeated extraction with activated charcoal, the color disappeared with no change in the osmolality. Aqueous solutions of the potassium salt of D-gluconic acid were uncolored and therefore were not treated further.

Cells added to isotonic test solutions containing varying $[Cl]_o$[1] were allowed to equilibrate for several minutes before the experimental period was initiated by addition of norepinephrine. When cells were added to hypertonic solutions, the initial samples were taken as rapidly as possible (see Schmidt and McManus, 1977*a*). Both hypertonic and isotonic incubation solutions always contained (mM): ouabain, 0.1; glucose, 10; inorganic phosphate, 2.0–5.0; magnesium, 1.0. Specific concentrations of $[Na]_o$, $[K]_o$, and $[Cl]_o$ are noted in legends of individual tables and figures. Osmolality was always adjusted by suitable addition of choline chloride.

The technique for adjusting cell sodium levels with nystatin (E. R. Squibb & Sons, New York), based on the work of Cass and Dalmark (1973) with human red cells, is presented in detail in the legend to Table V.

RESULTS

Lowering $[Cl]_o$ and $[Na]_o$ under Hypertonic Conditions

Fig. 1 compares the effect of lowering either $[Cl]_o$ or $[Na]_o$, or both, on the

[1] As in the previous papers (Schmidt and McManus, 1977 *a*, b). $[Cl]_o$, $[Na]_o$, $[K]_o$, etc. refer to the concentration (mM) of the ion in the external solution bathing the cells. $[Cl]_c$, $[Na]_c$, $[K]_c$, etc. are the concentration in the cells (millimoles/liter cell water), while Cl_c, Na_c, K_c, etc. are the amount of substance in the cells (millimoles/kg dry cell solids).

reswelling of duck red cells in hypertonic solutions. Approximately 50% of external NaCl was replaced by an isosmotic equivalent of tetramethylammonium (TMA) chloride, [B], Na-gluconate, [C], or sucrose, [D]. $[K]_o$ was maintained at 13 mM so that under normal circumstances the cells would reswell in the hypertonic environment (Schmidt and McManus, 1977*a*). Decreasing $[Na]_o$ by 84 mM by replacement with a nonpenetrating cation (TMA) reduces net water

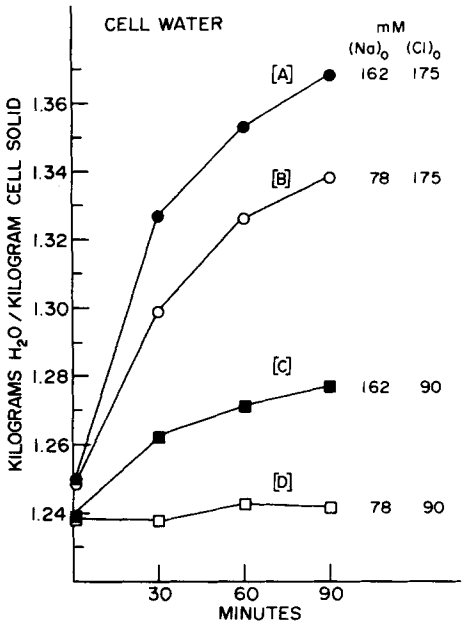

FIGURE 1. Effects of lowering $[Cl]_o$ and $[Na]_o$ on the hypertonic volume response. Ouabain was not present in these incubations. All incubation solutions contain (mM): $[K]_o$ = 13; Mg-TES = 20. pH_o = 7.38, 41°C. 400 mosmol. [A] represents the control where the initial chloride distribution ratio r_{Cl} is 0.62. In [B], tetramethylammonium chloride + NaCl = 162 mM and initial r_{Cl} = 0.66. In [C], Na-gluconate + NaCl = 161 mM, and initial r_{Cl} = 1.21. In [D], 168 mM sucrose substitutes for 84 mM NaCl and initial r_{Cl} = 1.06. Initial internal cation concentrations (millimoles/liter cell water): $[Na]_c = 6.0 \pm 0.2$; $[K]_c = 180 \pm 1$. Actual decreases in water uptake due to removal of either $[Cl]_o$ or $[Na]_o$ in this experiment are given in Table I.

uptake slightly over a 90-min incubation (curve [B]). The degree of this reduction is consistent with that observed in previous experiments (see Fig. 4, Schmidt and McManus, 1977*a*) where choline instead of TMA was substituted for $[Na]_o$. Lowering $[Cl]_o$ without altering $[Na]_o$ also inhibits the volume response (curve [C]), but to a greater extent than the equivalent $[Na]_o$ reduction of curve [B]. When sucrose is used as an osmotic replacement, both $[Na]_o$ and $[Cl]_o$ are lowered, and no water is accumulated (curve [D]). Similar inhibition of water movement by lowering $[Cl]_o$ in hypertonic solutions was seen with ouabain present.

In order to compare volume changes due to lowering $[Cl]_o$ with those due to lowering $[Na]_o$, differences between appropriate curves of Fig. 1 were com-

puted. For example, $[Na]_o$-dependent water uptake was computed by subtracting curve [B] from [A], and [D] from [C] at each time point. Similarly, $[Cl]_o$-dependent water uptake was computed by subtracting curve [C] from [A], and [D] from [B]. Results are shown in Table I. $[Na]_o$-dependent water uptake is similar whether TMA or sucrose is the substituting species. Also, $[Cl]_o$-dependent water uptake is equivalent whether 85 mM $[Cl]_o$ is replaced by gluconate or sucrose. Thus, inhibition of water uptake appears directly related to a lowering of either $[Na]_o$ or $[Cl]_o$, rather than some unspecified effect of the substituting species. Water uptake appears to be about three times more sensitive to $[Cl]_o$ than to $[Na]_o$.

TABLE I

DECREASE IN WATER UPTAKE DUE TO REMOVAL OF EITHER $[Cl]_o$ OR $[Na]_o$

	A. $[Na]_o$-dependent water uptake	
	ΔTMA $[Cl]_o$=175 mM (A) − (B)	ΔSucrose $[Cl]_o$=90 mM (C) − (D)
min	*g H_2O/kg cell solids*	
30	28	24
60	27	28
90	30	35
	B. $[Cl]_o$-dependent water uptake	
	ΔGluconate $[Na]_o$ = 162 mM (A) − (C)	ΔSucrose $[Na]_o$=78 mM (B) − (D)
min	*g H_2O/kg cell solids*	
30	65	61
60	82	83
90	91	96

All values reported here are computed from curves A, B, C, and D of Fig. 1. For further details see text.

Lowering $[Cl]_o$ in Isotonic Solutions Containing Norepinephrine

Fig. 2 shows the effect of substituting gluconate for $[Cl]_o$ in isotonic solutions containing norepinephrine. Here, $[Na]_o$ was maintained at 54 mM, and the cation deficit was balanced with choline. This enabled comparison with experiments where $[Cl]_o$ was lowered by replacement of choline chloride with an osmotic equivalent of sucrose, leaving $[Na]_o$ and $[K]_o$ unaltered. Lowering of $[Cl]_o$ under these conditions results in an inhibition of the volume response similar to that observed with $[Cl]_o$ lowering under hypertonic conditions (Fig. 1). As $[Cl]_o$ is lowered, sodium uptake decreases, potassium loss increases, and cells accumulate less water. These changes are qualitatively similar to those produced by reduction of either $[Na]_o$ or $[K]_o$ at constant $[Cl]_o$ (see Figs. 2 and 3, Schmidt and McManus, 1977*b*). Measured values of Cl_c and $[Cl]_c$, as well as computed values of r_{Cl} and E_{Cl} for the same experiment are given in Table II. The amount of chloride that accumulates in the cells is diminished by lowering $[Cl]_o$. However, once the chloride concentration $[Cl]_c$ is set, there is little change through-

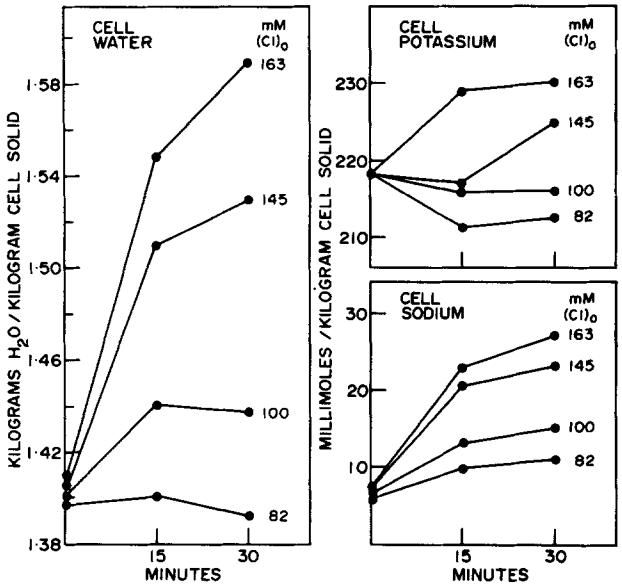

FIGURE 2. Effect of replacing $[Cl]_o$ with gluconate on the norepinephrine-induced volume response. All incubation solutions contain (mM); ouabain = 0.1; $[Na]_o$ = 54; $[K]_o$ = 20; Mg-TES = 10. pH_o = 7.42, 41°C. 323 mosmol. $[Cl]_o$ was varied by isosmotic replacement with Na-gluconate and K-gluconate so that the $[Cl]_o$ plus gluconate was 163 mM. Norepinephrine (10^{-6} M) was added after initial (t = 0) samples were taken. Initial cell cation concentrations (millimoles/liter cell water): $[Na]_c$ = 5.8 ± 0.2 (SEM); $[K]_c$ = 149 ± 2. Cell chloride values for the same experiment are given in Table II. When sucrose was used as an osmotic replacement for $[Cl]_o$ identical results were obtained.

TABLE II

EFFECT ON THE RESPONSE OF REPLACING $[Cl]_o$ WITH GLUCONATE

$[Cl]_o$	Min	Cl_c	$[Cl]_c$	r_{Cl}	E_{Cl}
mM					
163	0	151	107	0.66	−11.2
	15	165	107	0.66	−11.2
	30	173	109	0.67	−10.8
145	0	146	104	0.72	−8.9
	15	159	105	0.73	−8.5
	30	164	107	0.74	−8.1
100	0	134	95	0.95	−1.4
	30	134	93	0.93	−2.0
82	0	118	87	1.09	+1.6
	15	118	87	1.06	+1.6
	30	114	84	1.03	+0.8

These results are from the same experiment as illustrated in Fig. 2. Cl_c = mmol/kg cell solids. $[Cl]_c$ = mmol/liter cell water. $r_{Cl} = [Cl]_c/[Cl]_o$. $E_{Cl} = \frac{RT}{F} \ln \frac{[Cl]_c}{[Cl]_o}$.

out the incubation, regardless of $[Cl]_o$. Thus, computed values of r_{Cl} and E_{Cl} do not change with time.

Identical results were obtained when $[Cl]_o$ was lowered by replacement of choline chloride with sucrose. Data from the sucrose incubations are incorporated into Fig. 3 which is a plot of cell water and cation changes during the first 15 min of incubation as a function of initial $[Cl]_o$ levels. The linear relationship between cell swelling, net cation uptake, and external chloride concentration over the range of 80–160 mM $[Cl]_o$ is in contrast to the nonlinear curves produced by varying $[Na]_o$ or $[K]_o$ (compare Figs. 2 and 3, Schmidt and McManus, 1977*b*).

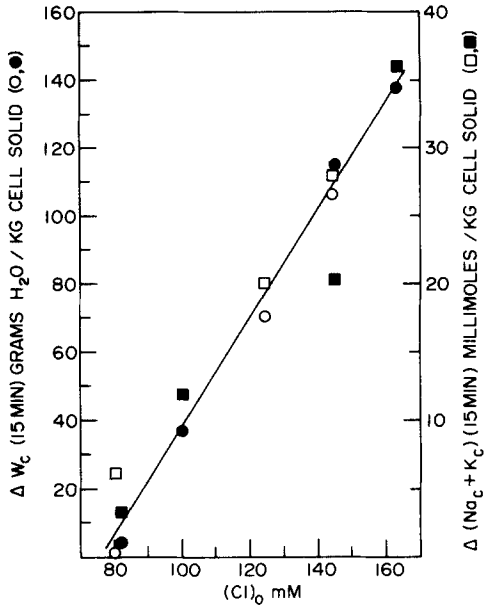

FIGURE 3. Changes in cell water (○, ●) and cations (□, ■) as a function of $[Cl]_o$ during the first 15 min after addition of norepinephrine (10^{-6} M) to isotonic solutions containing ouabain (10^{-4} M). Open symbols represent changes observed when gluconate substitutes for $[Cl]_o$, as in Fig. 2. Closed symbols depict changes observed when sucrose substitutes for $[Cl]_o$. All incubations contain (mM): $[Na]_o$ = 54; $[K]_o$ = 20; Mg-TES = 10. pH_o = 7.42, 41°C. 323 mosmol.

When changes in $[Cl]_o$ are made, it is inevitable that many other parameters (e.g., Cl_c, pH_c, W_c) change. Any or all of these could play a critical role in determining the results shown in Figs. 1 and 2. According to the theory of chloride equilibrium developed for mammalian red cells (Jacobs and Stewart, 1947; Davson, 1970), a lowering of $[Cl]_o$ at constant pH_o will cause: (*a*) decreased $[Cl]_c$; (*b*) increased r_{Cl}; (*c*) decreased E_{Cl}; (*d*) increased pH_c; and (*e*) loss of cell water. An approach to sorting out these variables can be made by changing pH_o along with $[Cl]_o$. Acidification of the external medium at constant $[Cl]_o$ will cause increases in r_{Cl}, $[Cl]_c$, and cell water while decreasing pH_c; alkalinization at constant $[Cl]_o$ will cause reciprocal effects on these parameters.

Preliminary experiments were carried out to determine if this general model of chloride, OH^-, and H^+ equilibria applies as well to the nucleated red cells of ducks. Cell water was found to be a linear function of pH_o over the range of 6.5–8.0. The cells swell as the solution becomes more acid. Varying $[Cl]_o$ displaces the curve of W_c vs. pH_o up or down, but does not alter the slope. Cell chloride concentration is also a linear function of pH_o regardless of $[Cl]_o$. $[Cl]_c$ increases as the solution becomes more acid.

Fig. 4 shows the time course of volume changes in an experiment designed to

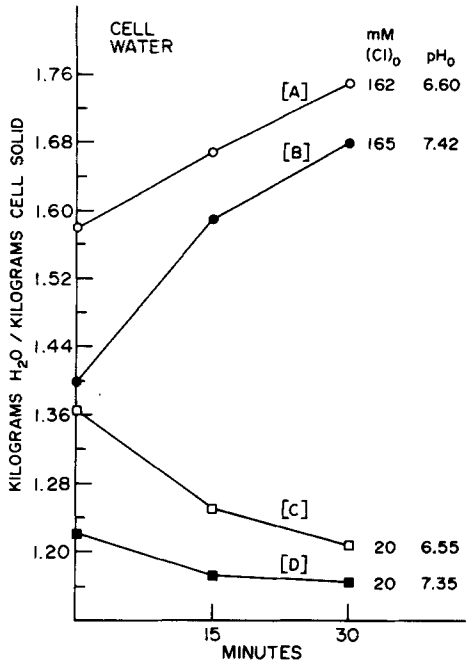

FIGURE 4. Effect of decreased $[Cl]_o$ and pH_o on the norepinephrine-induced volume response. All incubation solutions contain (mM): ouabain = 0.1; $[Na]_o$ = 145; $[K]_o$ = 19; Mg-TES = 10. 41°C, 323 mosmol. Mg-TES was titrated to the desired pH as described previously (Schmidt and McManus, 1977*a*). Na and K salts of gluconic acid isosmotically replaced NaCl and KCl so that $[Cl]_o$ plus gluconate = 165 mM. Cells were allowed to equilibrate in the various solutions for 5 min before the first sample (t = 0) was taken. Immediately afterward 10^{-6} M norepinephrine was added to each incubation. Initial cell cation concentrations (millimoles/liter cell water) were: $[Na]_c$ = 6.7 ± 0.1 (SEM); $[K]_c$ = 163 ± 2. Cell sodium and chloride, as well as various calculated parameters from this experiment, are given in Table III.

test the importance of initial values of $[Cl]_c$, W_c, and pH_c on the response to norepinephrine. Values of Na_c, Cl_c, and various calculated parameters from the same experiment are given in Table III. An inhibition of the response is seen in 20 mM $[Cl]_o$, pH 7.35 solution (curve [D]). As long as $[Cl]_o$ is maintained high enough, lowering pH_o to 6.60 (curve [A]) does not abolish the response, although the cells start off in a swollen state secondary to the increased acidity. The effect of cell swelling per se will be considered further below (see Fig. 7). Cells in 20 mM $[Cl]_o$, pH_o 6.55 (curve [C]) have initial values of W_c, $[Cl]_c$, and pH_c

which are approximately the same as those in 165 mM $[Cl]_o$, pH_o 7.42 (curve [B]) as expected from the reciprocal changes in external chloride and hydrogen ion concentrations. Although $[Cl]_o$ was reduced ninefold in the medium bathing the cells represented by curve [C], the hydrogen ion concentration was increased exactly ninefold. However, addition of norepinephrine causes strikingly divergent water movements under these two conditions. While the control cells swell as expected, the cells depicted by curve [C] lose salt and shrink. It appears that initial values of W_c, $[Cl]_c$ and pH_c by themselves are not critical determinants of the direction of salt and water movements initiated by norepinephrine. The total amount of external chloride available cannot be rate limiting because only 2% of the suspension is occupied by cells. Furthermore, if the amount of chloride was

TABLE III

EFFECT OF $[Cl]_o$ AND pH_o ON THE NOREPINEPHRINE RESPONSE

Curve	$[Cl]_o$	pH_o	Min	Na_c	Cl_c	$[Cl]_c$	r_{Cl}	E_{Cl}	pH_c
	mM								
[B]	165	7.42	0	8.9	145	104	0.63	−12.4	7.23
			15	39.2	184	116	0.70	−9.4	7.23
			30	43.1	196	116	0.71	−9.4	7.26
[A]	162	6.60	0	9.4	223	141	0.87	−3.7	6.51
			15	16.0	245	147	0.91	−2.6	6.55
			30	24.9	240	139	0.86	−4.2	6.54
[D]	20	7.35	0	9.4	66	54	2.72	+27.0	7.78
			15	11.0	60	51	2.56	+25.4	7.77
			30	16.8	56	48	2.39	+23.5	7.74
[C]	20	6.55	0	9.4	133	98	4.89	+42.8	7.19
			15	8.5	108	87	4.33	+39.6	7.19
			30	7.5	100	83	4.14	+38.3	7.17

Results are from the same experiment as shown in Fig. 4. E_{Cl} and r_{Cl} were computed as stated in the legend to Table II. pH_c was computed, assuming Cl^- and H^+ equilibrium, according to the expression: $pH_c = pH_o - \log r_{Cl}$.

limiting, one would expect a fall in $[Cl]_o$ during the experiment. In 20 mM $[Cl]_o$, external chloride was unchanged after 30 min. Note also from Table III that cells in the 20 mM $[Cl]_o$, pH_o 6.55 solution actually lose sodium during the incubation. This resembles the net sodium extrusion against an electrochemical gradient seen in zero $[K]_o$ media (Schmidt and McManus, 1977*b*).

Two variables which do appear related to cell volume changes when $[Cl]_o$ is altered are r_{Cl} and E_{Cl}. As seen in Figs. 2 and 4, and Tables II and III, the response to norepinephrine is diminished as r_{Cl} increases, or as E_{Cl} becomes less negative. To illustrate this point, cell water changes during the first 15 min of incubation with norepinephrine are plotted in Fig. 5 as a function of the initial value of E_{Cl}. Results from three separate experiments are included. Solid symbols represent data from experiments with 145 mM $[Na]_o$ where $[Cl]_o$ was varied from 20 to 165 mM and pH_o was varied from 6.5 to 8.0. Maximum

swelling under these conditions occurs at $E_{Cl} = -14.7$ mV and is completely inhibited at $E_{Cl} = +12.1$ mV. As E_{Cl} becomes more positive, there is actually net water loss after addition of norepinephrine.

Open symbols of Fig. 5 represent cell water changes after addition of norepinephrine when $[Na]_o$ was lowered to 54 mM. For this case, E_{Cl} was varied only by

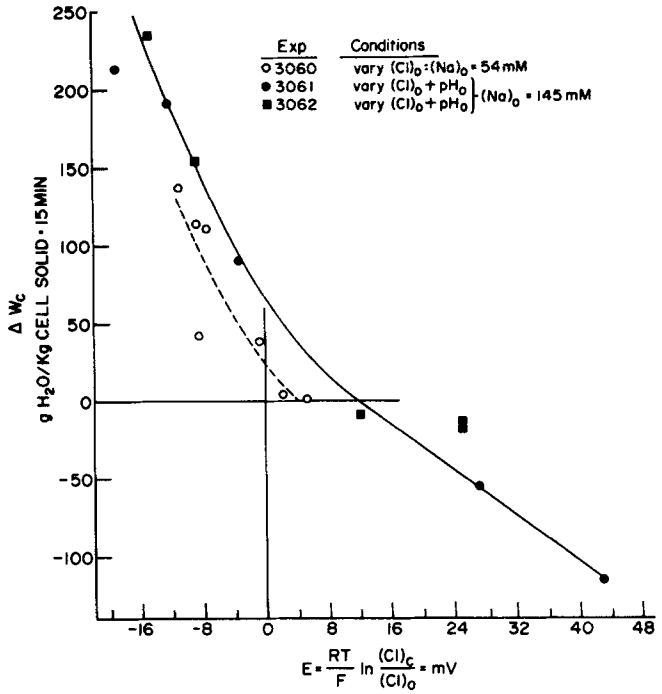

FIGURE 5. Relationship between the rate of water movement in response to norepinephrine and the initial chloride equilibrium potential. Before starting the experiment, cells were preincubated for 5 min in solutions containing varying $[Cl]_o$ and pH_o (see Fig. 4 legend). All solutions contain (mM): ouabain = 0.1; $[K]_o$ = 19; gluconate plus $[Cl]_o$ = 165; Mg-TES = 10. 41°C, 323 mosmol. After equilibration, samples were taken for determination of initial cell chloride and water content. E_{Cl} was computed from these values. Norepinephrine (10^{-6} M) was then added and the change in water (ΔW_c) over the next 15 min was determined. Solid symbols represent results from two experiments (one of which is shown in Fig. 4 and Table III) where E_{Cl} was varied with external Na at 145 mM. Open symbols represent results from two experiments (one of which is shown in Fig. 2 and Table II) where E_{Cl} was varied with external Na at 54 mM $[Na]_o$. For further details, see text.

altering $[Cl]_o$. A marked dependence of cell swelling on E_{Cl} is also evident at this lower $[Na]_o$. However, the curve has been shifted to the left so that a lower value of initial E_{Cl} (+2 to +5 mV) is sufficient to prevent swelling. Such interaction between $[Na]_o$ and E_{Cl} on norepinephrine-induced water movements agrees with results observed in hypertonic solutions (see Fig. 1) where decreasing $[Na]_o$ inhibits water uptake at different values of $[Cl]_o$.

Effect of Substituting Acetate for $[Cl]_o$

Acetate (Ac) is an organic anion that rapidly penetrates red cell membranes, probably by nonionic diffusion of the undissociated form (Deuticke, 1973). If net salt and water uptake with norepinephrine is determined by E_{Cl} when $[Cl]_o$ is replaced by impermeant anions,then little inhibition of the usual response might be expected when E_{Cl} is left unchanged by replacing $[Cl]_o$ with the permeant acetate. To test this prediction, cells were incubated in low $[Cl]_o$, high $[Ac]_o$ solutions. Furosemide, which inhibits cation movements in high $[Cl]_o$ solutions (Schmidt and McManus, 1977*b*), was included to test the effect of this agent on a system which allows anion movement via a pathway separate from that of chloride. In spite of the low level of $[Cl]_o$, the cells in $[Ac]_o$ still accumulate water in response to norepinephrine (Fig. 6). Furosemide inhibits swelling in both instances.

Cell ion contents from the same experiment are given in Table IV. Cells in $[Ac]_o$ gain little Cl_c in spite of marked increases in Na_c and K_c. It can be deduced, therefore, that there must have been a net uptake of acetate to balance the cations. The inhibition of cation uptake by furosemide in the acetate system as well as the chloride system suggests that the mode of anion penetration is not important for the effect of this agent on net salt and water movements in duck red cells. Computed values of E_{Na}, E_K, and E_{Cl} are listed in Table IV. As before, there is little change in E_{Cl} (and presumably E_{Ac}) throughout the incubation. E_K is also relatively constant. However, E_{Na} markedly decreases with time, particularly in the absence of furosemide. This characteristic is examined in further detail below.

Effect of Increasing Cell Sodium

As noted in Fig. 5 as well as in our previous paper (Schmidt and McManus, 1977*b*), lowering $[Na]_o$ inhibits net salt and water movements induced by norepinephrine. In addition, when cells swell with ouabain present, $[Na]_c$ increases or E_{Na} becomes less positive (Tables III and IV). This suggests that $[Na]_c$ or E_{Na} might also be important determinants of both the initiation and cessation of cell swelling. To test this hypothesis, internal cell concentrations were manipulated by preincubating cells in solutions containing a fixed $[K]_o$, varying $[Na]_o$, and nystatin, a polyene fungicidal antibiotic known to render human red cell membranes extremely permeable to cations (Cass and Dalmark, 1973). Choline was substituted for $[Na]_o$ in these solutions. After the cells were loaded to desired levels of $[Na]_c$, they were washed free of nystatin then added to standard incubation media containing ouabain. Typical results of the effect of norepinephrine on cell water and sodium contents of these cells are presented in Table V. It is apparent that increasing $[Na]_c$ or making E_{Na} less positive results in diminished cell sodium gain and swelling after norepinephrine addition. Elevation of $[Na]_c$ from 11.4 to 27 mM lowers E_{Na} by 22 mV. This is associated with a reduction of Na_c uptake of 70% and water uptake of 90% over 30 min. Again, as in Table IV, E_{Na} becomes less positive as cells swell. Throughout the incubation, E_K and E_{Cl} remained relatively constant at -60 and -12 mV, respectively.

Effect of Norepinephrine in Hypotonic Solutions

Results shown in Fig. 4 demonstrate that the cells will respond to norepinephrine even though they are already swollen by exposure to low pH_o. To explore further the importance of cell volume in the catecholamine response, cells exposed to hypotonic solutions were compared with those incubated in isotonic media. A hypotonic osmolality was selected such that initial cell volumes were

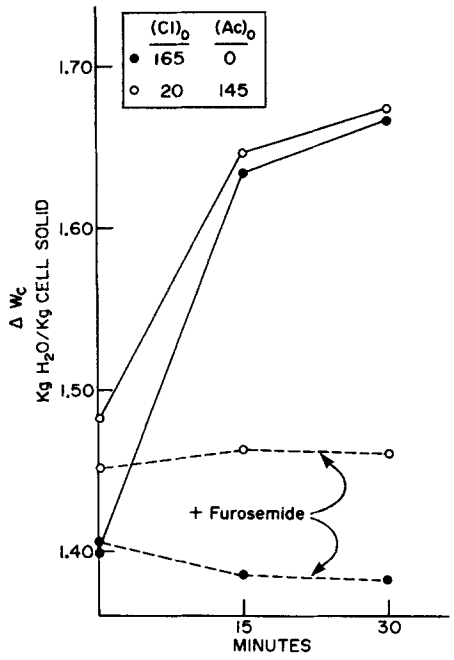

FIGURE 6. Effect of substituting acetate for $[Cl]_o$ on the norepinephrine-induced volume response. Cells were preincubated for 5 min in solutions containing the same ionic constituents used for the final incubation. After centrifugation, they were separated from their supernates and resuspended in the test solutions. 5 min more were allowed for complete equilibration before the first sample was taken. Immediately afterward, 10^{-6} M norepinephrine was added to each flask. All solutions contain (mM): ouabain = 0.1; $[Na]_o$ = 134, $[K]_o$ = 18; Mg-TES = 10. 41°C, 323 mosmol. Cell sodium, potassium, and chloride, as well as the equilibrium potentials for these ions are given in Table IV.

greater than the maximum volumes attained by cells in isotonic solutions after the swelling induced by catecholamine had ceased. If cell volume is not a factor in either initiating or terminating the response, preswollen cells should swell even more upon addition of the hormone. Fig. 7 illustrates such an experiment. Decreased osmolality causes little change in initial Na_c and K_c but increases initial water content by approximately 400 g/kg cell solids. Cells in the hypotonic solution without norepinephrine rapidly lose KCl and shrink, an effect which has been thoroughly studied and elucidated by Kregenow (1971*a*, 1974). Addi-

tion of norepinephrine reverses this shrinkage and initiates even greater swelling over the next 15 min. The amount of Na_c, K_c, and water taken up is similar to the isotonic case, even though initial cell volume in the hypotonic solution is much greater.

DISCUSSION

Since chloride appears to be at thermodynamic equilibrium in duck red cells, manipulating the external concentration probably affects net salt and water movements induced by hypertonicity or norepinephrine by its effect on the

TABLE IV

EFFECT ON THE NOREPINEPHRINE RESPONSE OF SUBSTITUTING ACETATE FOR $[Cl]_o$

$[Cl]_o$	$[Ac]_o$	10^{-3} M furosemide	Min	Na_c	K_c	Cl_c	E_{Na}	E_K	E_{Cl}
mM	*mM*						*mV*	*mV*	*mV*
165	0	0	0	9.0	239	137	+84	−61	−14.7
			15	34.7	255	181	+52	−59	−11.4
			30	40.7	259	187	+48	−59	−11.1
20	145	0	0	12.5	232	22	+77	−59	−9.7
			15	26.3	244	25	+60	−59	−8.7
			30	32.4	249	27	+55	−58	−8.3
165	0	+	0	8.7	237	142	+78	−62	−13.3
			15	11.4	233	131	+70	−62	−14.9
			30	15.1	223	131	+63	−61	−14.9
20	145	+	0	9.0	225	25	+83	−59	−4.3
			15	12.2	220	23	+75	−58	−6.9
			30	14.8	224	23	+70	−59	−8.2

Results are from the same experiment shown in Fig. 6. E_{Cl} was calculated as indicated in the legend to Table II. E_{Na} and E_K were calculated from a similar equation: $E_B = \frac{RT}{F} \ln \frac{[B]_o}{[B]_c}$, where $[B]_o$ and $[B]_c$ represent, respectively, extracellular and intracellular concentrations (millimoles/liter cell water) of Na or K.

electrochemical driving forces moving sodium and potassium. As we have already pointed out, it is unlikely that the total amount of chloride is a limiting factor in net salt movement. Furthermore, if it is, then one would predict that a further increase in cation permeability might have no effect on the response. This was tested in several experiments (data not reported here) in which 10^{-7} M valinomycin was added to the norepinephrine system under conditions where the cells would be expected to swell and take up salt. With valinomycin, they lost large amounts of KCl and water even with norepinephrine present. Since chloride conductance is the limiting factor in salt loss induced by valinomycin (Tosteson et al., 1973) it appears certain that chloride permeability of the cells studied in this investigation is still far greater than the hormone-stimulated cation permeability. Consequently, solute uptake in low $[Cl]_o$ solutions must be

reduced because net cation uptake is reduced. Again, the fact that acetate can substitute for $[Cl]_o$ (Fig. 6) suggests that the role of chloride is passively to follow net cation movements and not to interact in some specific fashion with a cation carrier.

Inhibition of the response by manipulation of external anions appears to result from changes in the chloride ratio (and therefore of E_{Cl}), rather than the actual

TABLE V

EFFECT ON THE NOREPINEPHRINE RESPONSE OF INCREASING CELL SODIUM

Min	Na_c	ΔNa_c	$[Na]_c$	E_{Na}	W_c	ΔW_c
				mV	*kg/kg*	*g/kg*
0	17.2		11.4	+61	1.510	
10	28.2	+11.0	17.5	+59	1.615	+105
20	33.8	+16.6	20.7	+45	1.623	+122
0	20.6		15.0	+54	1.379	
10	32.2	+11.6	22.1	+43	1.456	+77
20	36.8	+16.2	24.9	+39	1.478	+99
0	27.8		19.4	+47	1.435	
10	36.6	+8.8	25.1	+40	1.458	+23
20	38.8	+11.0	26.4	+39	1.471	+36
0	40.1		27.0	+39	1.484	
10	44.5	+4.4	30.0	+36	1.496	+12
20	45.0	+4.9	30.1	+35	1.497	+13

Cell sodium was varied by suspending cells at a 1% hematocrit in loading solutions containing (mM): KCl=130; choline chloride+NaCl=30; sucrose=68; Mg-TES=10. pH_o=7.4, 41°C. To each 100 ml of whole suspension, 200 μl of nystatin (20 mg/ml) dissolved in dimethylsulfoxide were added, and the cells were incubated 5 min at 0°C. This loading procedure was repeated a total of four times, by alternate centrifugation and resuspension. Nystatin was eluted from the cells by incubation at 41°C for 5 min in solutions identical to those used to load them except for the absence of the drug. This procedure was also performed a total of four times by alternate centrifugation and resuspension. Cells were then added to test solutions which contained (mM): ouabain=0.1; $[Na]_o$=111; $[K]_o$=17; $[Cl]_o$=146; Mg-TES=10. pH_o=7.4, 41°C, 323 mosmol. After the initial sample (t=0), 10^{-6} M norepinephrine was added, and further samples were withdrawn at 10 and 20 min. At zero time, cells contain (millimoles/liter cell water): $[K]_c$=155; $[Cl]_c$=93. $[Na]_c$ varied as indicated above. E_{Na} was computed as indicated in the legend to Table IV. These results are typical of several experiments of this type.

concentrations of internal (Fig. 4 and Table III) or external (Fig. 6 and Table IV) chloride. Shifting E_{Cl} in a positive direction from its normal resting level of about −11 mV decreases the rate of water uptake in response to norepinephrine and may even cause the cells to shrink (Fig. 5). It is important to note, however, that these values of E_{Cl} do not change to any large extent during the response itself (Tables II–IV). Therefore, cessation of swelling cannot be attributed to a decrease in E_{Cl}. It is interesting that less shift of E_{Cl} is required to obtain zero volume change (ΔW_c = 0) when $[Na]_o$ is 55 mM than when it is 145 mM (Fig. 5). Thus, there appears to be an interaction between $[Na]_o$ and E_{Cl}, as well as

between $[Na]_o$ and $[K]_o$. The following discussion is an attempt to explain these interactions by consideration of the thermodynamic driving forces acting on the putative carrier of the cotransport pathway.

In the previous paper (Schmidt and McManus, 1977*b*), we presented a scheme which was helpful in explaining many of the findings reported there. Since the

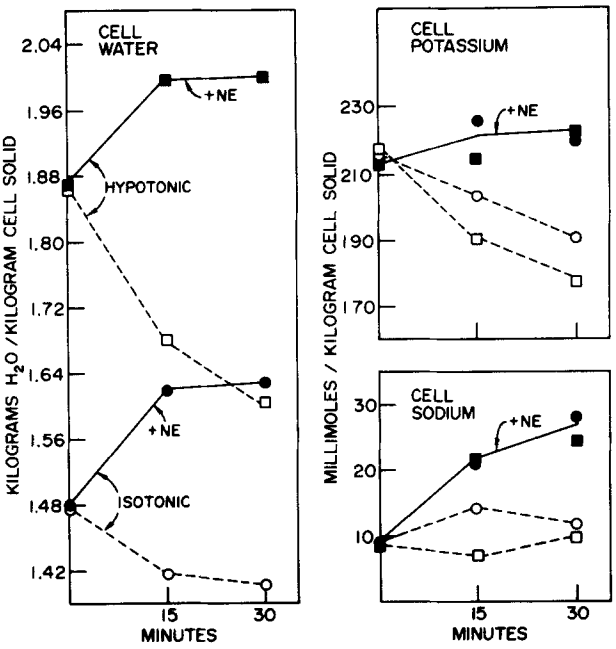

FIGURE 7. A comparison of the effect of norepinephrine in isotonic and hypotonic solutions. All solutions contain (mM): ouabain = 0.1; $[K]_o$ = 20; Mg-TES = 10. pH_o = 7.42, 41°C. Isotonic (323 mosmol) solutions contain in addition (mM): $[Na]_o$ = 145; $[Cl]_o$ = 165. Hypotonic (270 mosmol) solutions contain in addition (mM): $[Na]_o$ = 108; $[Cl]_o$ = 128. After initial samples were taken (t = 0), cells were incubated in the presence (solid symbols, solid lines) and absence (open symbols, dashed lines) of norepinephrine (10^{-6} M). At zero time, cells contained (millimoles/liter cell water): $[Na]_c$ = 6.6; $[K]_c$ = 153; $[Cl]_c$ = 125 in the isotonic incubations, and $[Na]_c$ = 5.2; $[K]_c$ = 117; $[Cl]_c$ = 97 in the hypotonic incubations. Initial values computed for the various equilibrium potentials (mV) were: E_{Na} = +84; E_K = −55; E_{Cl} = −7.5 in the isotonic incubations, and E_{Na} = +82; E_K = −48; E_{Cl} = −7.5 in the hypotonic incubations.

basic emphasis of this whole investigation has been on the study of net ion and water shifts rather than unidirectional fluxes, a detailed kinetic analysis of the model cannot be generated from these results. On the other hand, a phenomenological approach has proved to be quite useful, although it can give no insights into the actual mechanism of transport. If we know the driving forces which move the carrier, it should be possible to predict the direction of net transport as well as the point at which the system comes to rest for either water or ion movements. We will call that the balance point.

The first basic assumption of the theory is that net movements of sodium and potassium in the presence of ouabain may occur significantly only via two pathways: simple electrodiffusion and sodium-potassium cotransport. We have shown that cells incubated with ouabain in the absence of norepinephrine or hypertonicity maintain relatively stable volume by a simple exchange of internal potassium for incoming sodium (Schmidt and McManus, 1977*a*). The addition of furosemide to catecholamine-stimulated or hypertonically activated cells results in a stabilization of cell volume by a similar mechanism (Schmidt and McManus, 1977*a*, *b*). We may conclude, therefore, that the simple cationic diffusion pathway, which appears to predominate in unstimulated ouabain-treated cells in isotonic solutions, is not responsible for the net salt and water movements observed in these experiments. Ion movements which result in cell volume changes must be attributed to the sodium-potassium cotransport system. Whether the cells swell or shrink should be explicable entirely in terms of the net electrochemical driving forces urging sodium and potassium through that pathway. On the other hand, net movements of the ions themselves can only be accounted for by considering both the cotransport system and the parallel diffusion pathway.

Several other simplifying assumptions must be made to facilitate the further development of this approach. For example, the activity coefficients for sodium, potassium, and chloride in the cell are considered the same as in the external medium and do not change during the course of the reaction. This is admittedly an over-simplification, particularly in the hypertonic case. Nevertheless, we suspect that a more complex theory would not alter the final conclusions to any significant degree.

The internal pools of these ions are also assumed to form a single homogeneous compartment. Since duck red cells are nucleated and possess mitochondria, they have morphological compartments. Hoffman (personal communication) has evidence that sodium is highly concentrated in the nucleus of the salamandroid amphibian *Amphiuma means*. Nevertheless, he finds that the uptake of radioactive sodium by the intact cells yields a single exponential on semilogarithmic plots. We have similar results with the duck red cell. This would argue against any significant pools of bound sodium. Furthermore, the nucleus of the bird cell forms a smaller fraction of the cell volume than is the case with amphibian red cells. It is difficult to understand how the nucleus can concentrate sodium without binding when one considers the large pores seen in electron micrographs of the nuclear membrane of avian red cells (Davies, 1961). For these reasons, it is probably not too far wrong to use total cell sodium values in the computation of E_{Na}.

Finally, we propose that E_{Cl} approximates the true membrane potential. There is now good evidence that this is reasonable for nucleated red cells (Lassen, 1972). Even if it is not correct in detail, it is unlikely that the discrepancy would be very great. Again, we doubt that such a discrepancy would drastically alter our conclusions.

Given these assumptions, the difference in electrochemical potential for sodium and potassium across the cell membrane can be written:

$$\Delta\tilde{\mu}_{Na} = F(E_{Na} - E_m) = RT \ln \frac{[Na]_o}{[Na]_c} - FE_{Cl}, \quad (1)$$

$$\Delta\tilde{\mu}_{K} = F(E_{K} - E_m) = RT \ln \frac{[K]_o}{[K]_c} - FE_{Cl}, \quad (2)$$

Where E_m represents the membrane potential and F, R, and T, have their usual meaning. We have presented evidence that a single rubidium (or potassium) ion is transported into the cell with each sodium ion (Schmidt and McManus, 1977*b*). Therefore, the net driving force moving the cotransport system is simply the sum of these two gradients. Eq. (1) and (2) can be combined and rearranged to give:

$$\Delta\tilde{\mu}_{net} = \Delta\tilde{\mu}_{Na} + \Delta\tilde{\mu}_{K} = RT \ln \left\{ \frac{[Na]_o}{[Na]_c} \cdot \frac{[K]_o}{[K]_c} \cdot \left[\frac{[Cl]_o}{[Cl]_c} \right]^2 \right\}. \quad (3)$$

The units of this net force are joules·mole^{-1}. The convention adopted here is that a positive value of $\Delta\tilde{\mu}_{net}$ favors net inward movement, whereas a negative value would tend to promote a net loss of salt (and water) by the cells. When $\Delta\tilde{\mu}_{net} = 0$, there is no net force operating on the cotransport system, and the cells should remain in balance with respect to volume, although net exchange of potassium for sodium may still take place via the parallel diffusion pathway mentioned above.

It is apparent from Eq. (3) that a reduction externally or an increase internally of the concentrations of sodium, potassium, or chloride would make $\Delta\tilde{\mu}_{net}$ less positive. Furthermore, because of the squared term, the system should be more sensitive to an alteration in chloride than in the other ions. An experimental demonstration of this latter characteristic was presented in Fig. 1.

The initial rate of cell swelling, which is assumed to be proportional to the net driving force, should increase as $\Delta\tilde{\mu}_{net}$ is made more positive by experimental manipulation of the ion gradients. Finally, given the other five terms in Eq. (3), it should be possible to predict the concentration in either phase of the remaining ion which will balance the driving forces so that the cells neither swell nor shrink in response to norepinephrine or hypertonicity.

An experimental test of this theory is feasible since cell sodium, potassium, chloride, and water were measured on every sample. Therefore, the equilibrium potentials of all these inorganic ions can readily be calculated. The results from several experiments were pooled and $\Delta\tilde{\mu}_{net}$ was computed for each condition. This included results from both hypertonic and norepinephrine studies where $[Na]_o$, $[K]_o$, $[Cl]_o$, pH_o, and $[Na]_c$ as well as tonicity were varied. The amount of water gained or lost by the cells in the first 15 min (norepinephrine) or the first 30 min (hypertonic) is plotted in Fig. 8 against the initial value of $\Delta\tilde{\mu}_{net}$. It is apparent that the relationship is linear and passes through the origin. Thus, one prediction has been confirmed, i.e. the rate of swelling appears to be a function of the driving force. When $\Delta\tilde{\mu}_{net}$ is negative, the relationship is no longer linear, at least in the norepinephrine case. However, there must be a finite point below which the cells can no longer shrink. In most of the cases giving shrinkage rather than swelling in norepinephrine, the cells actually begin the experiment with less

water content than those which have been deliberately dehydrated in hypertonic solutions. For example, replacing $[Cl]_o$ with gluconate will cause an immediate loss of water (curve [D], Fig. 4) because of the Donnan equilibrium enjoyed by chloride. Consequently, these cells have little water left to give.

To compute the concentrations of individual ions necessary to maintain a

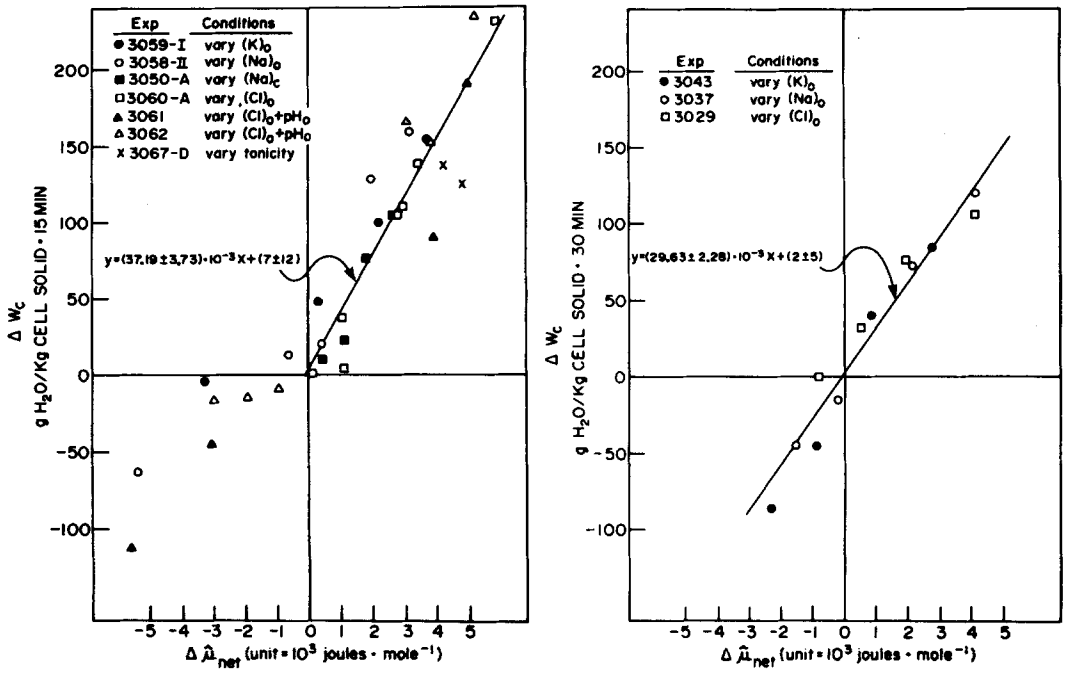

FIGURE 8. Effect of net ionic electrochemical potential on initial cell water changes induced by norepinephrine or hypertonicity. The left panel shows water uptake during 15 min after addition of norepinephrine (10^{-6} M) plotted as a function of $\Delta\tilde{\mu}_{net}$ which was computed from the ionic concentrations at time zero, just before hormone addition. The right panel shows water uptake in 30 min in hypertonic solutions plotted as a function of $\Delta\tilde{\mu}_{net}$ at time zero. Eq. (4) was used to compute $\Delta\tilde{\mu}_{net}$. Ouabain (10^{-4} M) was always present, except for Exp. 3029 (varying $[Cl]_o$) which is also plotted in Fig. 1.

constant volume in cells stimulated by norepinephrine or hypertonicity, $\Delta\tilde{\mu}_{net}$ was set equal to zero, and Eq. (3) rearranged to give.

$$\left[\frac{[Cl]_c}{[Cl]_o}\right]^2 = \frac{[Na]_o}{[Na]_c}\cdot\frac{[K]_o}{[K]_c} = r_{Cl}^2. \tag{4}$$

This expression was then solved for the unknown variable. For example, in experiments where r_{Cl} was varied (Fig. 5, Table III), initial values of $[Na]_o$, $[K]_o$, $[Na]_c$, and $[K]_c$ were measured, and the value of r_{Cl} required for zero cell water change was computed. Similar computations were made for conditions where

$[Na]_c$, $[Na]_o$, and $[K]_o$ were the experimental variables. Results of these computations are compared in Table VI (under the column $\Delta W_c = 0$) with values obtained experimentally for minimal volume changes. Regardless of the method of ion manipulation, there is a reasonable agreement between calculated and observed values, reinforcing the concept that the net electrochemical driving force for both cations is the major determinant of the direction of ion and water movements after stimulation of cotransport in ouabain-treated cells.

Eq. (4) cannot by itself be used to predict the balance point for sodium and potassium movement. As noted above, consideration must also be taken of net movements of these ions through a simple diffusion pathway running in parallel with the cotransport system. Therefore, sodium or potassium levels will be

TABLE VI

CONDITIONS FOR STEADY-STATE CELL WATER AND SODIUM WITH NOREPINEPHRINE AND HYPERTONICITY

Source	Variable	$\Delta W_c=0$		$\Delta Na_c=0$	
		Calculated	Observed	Calculated	Observed
A. Isotonic plus norepinephrine					
Fig. 5*	r_{Cl}	$r_{Cl}=1.12$	$r_{Cl}\sim1.09$	$r_{Cl}=2.27$	–
Table III*	r_{Cl}	$r_{Cl}=1.53$	$r_{Cl}\sim1.55$	$r_{Cl}=3.79$	$r_{Cl}\sim4.0$
Table V*	$[Na]_c$	$[Na]_c=29$	$[Na]_c\sim27$	$[Na]_c=72$	–
Fig. 2‡	$[Na]_o$	$[Na]_o=20$	$[Na]_o\sim17$	$[Na]_o=17$	$[Na]_o\sim17$
Fig. 3‡	$[K]_o$	$[K]_o=4.0$	$[K]_o\sim3$	$[K]_o=0.2$	$[K]_o\sim1$
B. Hypertonic					
Fig. 1*	r_{Cl}	$r_{Cl}=0.97$	$r_{Cl}\sim1.06$	$r_{Cl}=2.30$	–
Fig. 4§	$[Na]_o$	$[Na]_o=25$	$[Na]_o\sim23$	$[Na]_o=12.5$	$[Na]_o\sim12$
Fig. 2§	$[K]_o$	$[K]_o=5.2$	$[K]_o\sim7$	$[K]_o=0.2$	$[K]_o\sim0$

The column labeled "source" lists the Figures and Tables from which the data were extracted. Initial values of ion concentrations used in the computations can be found in the legends to these Figures and Tables. References are: (*) this paper; (‡) Schmidt and McManus, 1977 *b*; (§) Schmidt and McManus, 1977 *a*. The balance point for cell water ($\Delta W_c=0$) was computed by solving Eq. (5) for the unknown variable. The balance point for sodium ($\Delta Na_c=0$) was calculated by solving Eq. 6 for the unknown variable.

constant only when the force acting on the cotransport carrier exactly balances the force promoting simple diffusion of the cation in question. For example, cells incubated with ouabain will extrude sodium against its electrochemical gradient only when the force urging it out of the cell by its cotransport with potassium ($\Delta\tilde{\mu}_{net}$) is greater than the force driving it back by simple diffusion ($\Delta\tilde{\mu}_{Na}$). When sodium levels are constant, the sum of $\Delta\tilde{\mu}_{net}$ and $\Delta\tilde{\mu}_{Na}$ must equal zero. This may be represented conveniently by setting the sum of Eq. (1) and (3) equal to zero and rearranging:

$$\left[\frac{[Cl]_c}{[Cl]_o}\right]^3 = \frac{[K]_o}{[K]_c}\cdot\left[\frac{[Na]_o}{[Na]_c}\right]^2 = r_{Cl}\,. \tag{5}$$

Given a set of fixed parameters, we can readily predict values of r_{Cl}, $[Na]_o$, or $[K]_o$ at which cell sodium will not change in response to norepinephrine or

hypertonicity. These calculated values are also compared in Table VI (under the heading, $\Delta Na_c = 0$) with estimates from the experimental data where net sodium changes were minimal. Again, there is a good agreement between predicted and observed values. When internal and external cation concentrations of the experiment shown in Fig. 4 and Table III are inserted into Eq. (5), an r_{Cl} value of 3.79 (E_{Cl} = +36.0 mV) is predicted as necessary to prevent net sodium change. The initial r_{Cl} of 4.89 (E_{Cl} = +42.8 mV) given by 20 mM $[Cl]_o$ and pH_o 6.55 led to a modest but measurable net loss of cell sodium (Table III) as predicted by the theory. The calculated value of $[K]_o$ = 0.2 mM necessary for constant sodium also affords insight into how these cells may extrude that ion in the absence of $[K]_o$ yet show a net gain with merely 1.5 mM $[K]_o$ (Schmidt and McManus, 1977*b*). Net sodium is transported against its electrochemical gradient simply because the total forces acting on that ion favor extrusion. There appears to be no need to invoke concepts of residual pump activity, or a second pump not inhibited by ouabain, to explain the experimental findings.

Considerations similar to those used in deriving Eq. (5) can also be applied to potassium. Since the total force acting on potassium includes $\Delta\tilde{\mu}_{net}$ and $\Delta\tilde{\mu}_K$, the potassium rather than the sodium ratio of Eq. (5) becomes the squared term. When typical values of internal cation concentrations (Table I, Schmidt and McManus, 1977*a*) in the presence of 145 mM $[Na]_o$ and 20 mM $[K]_o$ are entered into an equation of this type, constant potassium levels are predicted to occur at r_{Cl} = 0.66–0.69 (E_{Cl} = −11 to −10 mV). Since this represents the usual chloride ratio for the experiments where external cations were varied (Schmidt and McManus, 1977*a*, *b*), it may explain the variability of net potassium uptake observed.

Since red cell membranes are freely permeable to water, cell volume changes are a necessary consequence of net salt movement. Are the hypertonic and norepinephrine responses primarily concerned with volume control, or are the observed volume shifts merely secondary to the more important regulation of cell cations? If water content is of primary importance, it might be expected that some parameter associated with cell volume finally determines the extent of swelling or shrinking. One logical site for such a control mechanism is the membrane itself. Reversible conformational changes of a membrane receptor have been suggested for dog (Romualdez et al., 1972), human (Poznansky and Solomon, 1972*a*, *b*), and duck (Kregenow, 1971*b*) shrunken red cells. Data presented here suggest that cell volume is not a controlling factor in either the norepinephrine or the hypertonic response. Cells initially swollen in hypotonic solutions (Fig. 7) can still accumulate salt and water after addition of norepinephrine. Cells initially shrunken in 400 mosmol solutions accumulate varying amounts of salt and water depending on levels of external cations (Schmidt and McManus, 1977*a*) and anions (Fig. 1). For all cases, the initial amount of net salt (and hence water) entering or leaving the cell is dependent, as described above, on the net electrochemical force acting on both sodium and potassium (Figs. 8 and 9). Thus, it appears that in both the norepinephrine and hypertonic responses salt is moved rapidly across the cell membrane in response to electrochemical forces rather than specifically to control cell volume.

Eq. (3) predicts that stimulated cells will swell or shrink until the changing ionic gradients lead to a dissipation of the driving force at the point where $\Delta\tilde{\mu}_{net}$ becomes zero. It is apparent from Fig. 9 that the rate of cell swelling in ouabain-treated cells does indeed decrease as the ionic composition of the cells changes and the net electrochemical potential is dissipated. Here, results were pooled from several experiments where external ions were adjusted to yield a maximum response. The rate of swelling over each time interval, as indicated in the legend, was compared with the driving force computed from the beginning of the interval. The decrease of $\Delta\tilde{\mu}_{net}$ over time occurs in these cells because $[Na]_c$

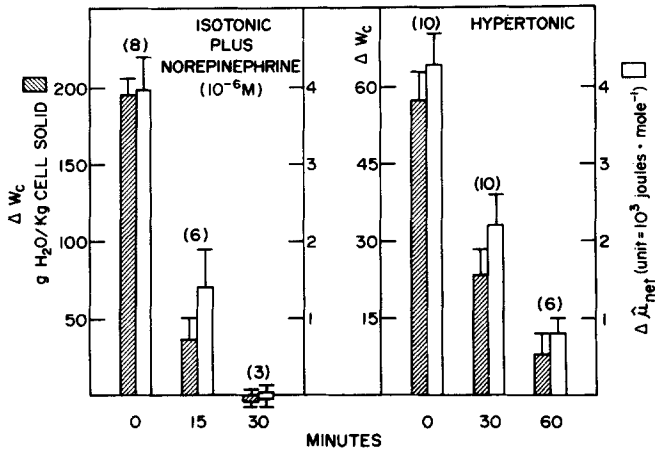

FIGURE 9. Net water movements and electrochemical driving forces during the volume response in the presence of ouabain (10^{-4} M). $\Delta\tilde{\mu}_{net}$ (open bars) was computed at 0, 15, and 30 min for the isotonic plus norepinephrine conditions and at 0, 30, and 60 min for the hypertonic condition. Changes in water content, ΔW_c (hatched bars) were determined over the invervals 0–15, 15–30, and 30–45 min for the norepinephrine experiments, and 0–30, 30–60, and 60–90 min for the hypertonic experiments. Only results included are those where external ion concentrations were set to yield a maximal response. Parentheses indicate the number of observations at each time interval. Eq. (4) was used to compute $\Delta\tilde{\mu}_{net}$.

increases, causing E_{Na} to become less positive, while E_K and E_{Cl} remain relatively stable (for example, Table IV).

When ouabain is omitted from the incubation, and the Na-K pump permitted to operate, the response becomes more complex. If parallel pathways exist for the pump and cotransport, one might expect stimulated cells to accumulate potassium until E_K changed sufficiently to dissipate $\Delta\tilde{\mu}_{net}$. However, with the pump in operation, the net driving forces do not change significantly over the incubation periods employed in these experiments. Cell sodium, and therefore E_{Na}, remains constant. Even though these cells accumulate potassium, they already have such high levels that additional uptake produces little change in E_K. Thus, cells not inhibited by ouabain are able to maintain their electrochemical gradients, yet the curves describing the change in volume are superimposable over those seen with ouabain-treated cells.

The possibility of a permeability change during the reaction has been studied by Kregenow. He reports estimates of tracer influxes over several intervals during the responses to hypertonicity (Kregenow, 1971*b*), or norepinephrine (Kregenow, 1973). Both these stimuli promoted a marked elevation of potassium influx over the controls. This was not further enhanced by raising $[K]_o$ from 2.6–2.8 mM to 17–19 mM. At the lower $[K]_o$, where volume changes are minimal, the fluxes remained elevated throughout the entire period of observation. At the higher $[K]_o$, where the cells are increasing in volume due to the net uptake of salt, the influxes decreased progressively with time toward the control levels. In the norepinephrine system, this effect was shown for both sodium and potassium influxes. These interesting observations are difficult to relate directly to the net water movements plotted in Fig. 9. Kregenow's fluxes were not analyzed with regard to kinetic components. In Pekin duck red cells, we have found that norepinephrine-stimulated sodium and rubidium influxes can be separated into exchange and co-ion-dependent fractions (Schmidt and McManus, 1977*b*). Raising $[Rb]_o$ in the presence of the catecholamine produces a marked increase in rubidium influx in the presence of ouabain. Gardner et al. (1975) have observed a similar effect of $[K]_o$ on potassium influx in isoproterenol-stimulated turkey red cells. When ouabain was present in Kregenow's experiments, it was added only at the beginning of each successive 15-min flux period. Nevertheless, a decrease in cation permeability with time due to changing cell volume may also be involved in the cessation of swelling, as Kregenow's results suggest.

The problem of what metabolic events are responsible for termination of the response is still unsolved. Gardner et al. (1973) have shown that cyclic-AMP continues to accumulate in turkey red cells stimulated by isoproterenol after the increase in sodium influx has returned to control levels. They propose that the response to catecholamines is limited by the accumulation of some unspecified inhibitor, or the depletion of a necessary intermediate, during the course of the reaction. On the other hand, Kregenow et al. (1976) have found that hypertonicity (427 mosmol) does not induce an increase in cyclic-AMP in Muscovy duck red cells, although the increase in K influx is the same under their conditions as that seen in response to norepinephrine (2.5×10^{-8} M). If these studies on turkey and Muscovy duck red cells can be applied to our experiments on the Pekin duck cells, then it appears unlikely that the termination of the response is related to the adenyl cyclase system since the hypertonic response is also self-limiting without any detectable change in cyclic-AMP from the unstimulated controls. Although dissipation of the driving force appears to be the determining factor in the experiments shown in Fig. 9, this does not occur in the absence of ouabain. The resolution of this problem must await further investigation.

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