

Effect of Norepinephrine on Swelling-induced Potassium Transport in Duck Red Cells

Evidence against a Volume-regulatory Decrease under Physiological Conditions

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ABSTRACT Duck red cells exhibit specific volume-sensitive ion transport processes that are inhibited by furosemide, but not by ouabain. Swelling cells in a hypotonic synthetic medium activates a chloride-dependent, but sodium-independent, potassium transport. Shrinking cells in a hypertonic synthetic medium stimulates an electrically neutral co-transport of $[\text{Na} + \text{K} + 2\text{Cl}]$ with an associated 1:1 K/K (or K/Rb) exchange. These shrinkage-induced modes can also be activated in both hypo- and hypertonic solutions by β -adrenergic catecholamines (e.g., norepinephrine). Freshly drawn cells spontaneously shrink ~4–5% when removed from the influence of endogenous plasma catecholamines, either by incubation in a catecholamine-free, plasma-like synthetic medium, or in plasma to which a β -receptor blocking dose of propranolol has been added. This spontaneous shrinkage resembles the response of hypotonically swollen cells in that it is due to a net loss of KCl with no change in cell sodium. Norepinephrine abolishes the net potassium transport seen in both fresh and hypotonically swollen cells. Moreover, cells swollen in diluted plasma, at physiological pH and extracellular potassium, show no net loss of KCl and water (“volume-regulatory decrease”) unless propranolol is added. Examination of the individual cation fluxes in the presence of catecholamines demonstrates that activation of $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport with its associated K/Rb exchange prevents, or overrides, swelling-induced $[\text{K} + \text{Cl}]$ co-transport. These results, therefore, cast doubt on whether the swelling-induced $[\text{K} + \text{Cl}]$ system can serve a volume-regulatory function under in vivo conditions.

INTRODUCTION

It has been suggested that duck red cells have a “volume-controlling mechanism” sensitive to osmotic perturbations (Kregenow, 1971*a, b*, 1981). When these cells

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are allowed to swell or shrink in synthetic, anisotonic media, they manifest two apparently distinct modes of ion transport, both of which are insensitive to ouabain (Kregenow, 1971*b*, 1974; Schmidt and McManus, 1977*a*), completely inhibited by 1 mM furosemide (Schmidt and McManus, 1974; McManus and Haas, 1981), and require the presence of chloride or bromide (Kregenow and Caryk, 1979; McManus and Haas, 1981; Haas et al., 1982). There are, however, serious difficulties with the notion that these volume-sensitive pathways serve a regulatory function under physiological conditions. Before addressing this question, it will be helpful to summarize what is known about volume-sensitive ion transport in avian red cells.

In hypotonic synthetic media, swollen duck red cells develop an increased flux of potassium, but not sodium. If the external concentration of potassium is in the physiological range, net loss of KCl and water, driven by the outwardly directed potassium gradient, results in the shrinkage of cells toward their isotonic volume (Kregenow, 1971*a*). We have shown that this process is independent of both internal and external sodium (McManus and Haas, 1981), and appears to involve an electrically neutral co-transport of $[K + Cl]$ (McManus et al., 1985).

In hypertonic synthetic media, shrunken cells also show an increased flux of cations, involving in this case both sodium and potassium. This effect has been shown to be due to activation of $[Na + K + 2Cl]$ co-transport (Schmidt and McManus, 1974, 1977*a*; Haas et al., 1982), a pathway that allows a net transfer of salt, with water following osmotically, in either direction across the cell membrane according to the direction and magnitude of the sum of the chemical potential gradients of the respective ions (Haas et al., 1982). If a hypertonic medium has plasma-like levels of these ions, the calculated driving force is zero (Schmidt and McManus, 1977*a*). Therefore, once initial osmotic shrinkage has taken place, the cells neither reswell nor shrink further. It is only when extracellular potassium is raised above the physiological level that the cells take up salt and water and reswell toward their isotonic volume (Ørskov, 1954; Kregenow, 1971*b*; Schmidt and McManus, 1977*a*). On the other hand, if external potassium is lowered below the level normally found in plasma, the cells continue to shrink, but at a rate slower than the initial osmotic response to hypertonicity (Schmidt and McManus, 1977*a*).

$[Na + K + 2Cl]$ co-transport can also be activated over a wide range of cell volumes by exposure to β -adrenergic catecholamines (Schmidt and McManus, 1977*b*, *c*) or cyclic AMP (or one of its analogues) (Riddick et al., 1971; Gardner et al., 1974; Palfrey et al., 1980). Moreover, duck red cells incubated in their own plasma appear to be under the influence of endogenous catecholamines. Although they maintain a constant volume when incubated in plasma, they lose KCl and water after addition of the β -adrenergic antagonist propranolol. Under these circumstances, shrinkage to a new, constant volume, termed the "lower steady state" (LSS), occurs (Riddick et al., 1971). Cells from freshly drawn blood incubated in a synthetic, isotonic medium similar to plasma, but free of catecholamines, also lose KCl and shrink. This spontaneous change—which we will call here the "fresh-LSS transition"—does not occur if norepinephrine is present in the synthetic medium (Riddick et al., 1971).

Cells swollen in a hypotonic medium containing increased potassium (20 mM) also shrink, losing KCl and water, although more slowly than in a similar medium with a physiological level of potassium (Schmidt and McManus, 1977c). The addition of norepinephrine to this high-potassium hypotonic medium not only prevents shrinkage but promotes further swelling. Thus, in hypotonic as well as in hypertonic or isotonic media, catecholamines promote salt and water uptake, provided that external potassium is elevated and external sodium is maintained near the physiological range. This catecholamine-stimulated response also appears to take precedence over the net potassium and water loss usually seen when cells are swollen.

In this paper, we present results relating the fresh-LSS transition to known ion transport processes in duck red cells, and examine the mechanism by which catecholamines prevent this phenomenon. We also explore further the relationship between catecholamine-stimulated [Na + K + 2Cl] co-transport and swelling-induced potassium transport, examining cation movements in swollen cells under different conditions in the presence and absence of norepinephrine. Finally, we examine the cells' response to swelling in their own plasma, and the effect of propranolol on this response.

Preliminary reports of these experiments have been presented at a meeting of the Red Cell Club at the Duke University Medical Center (December, 1980) and to the American Physiological Society (Haas and McManus, 1984).

MATERIALS AND METHODS

Preparation and Preincubation of Cells

On the day of the experiment, heparinized blood was drawn by syringe from a large vein in the anterior thorax of the White Pekin duck. Preparation of buffers and incubation media, as well as determination of ions and cell water, has been described in detail in previous publications (Schmidt and McManus, 1977a; Haas et al., 1982).

For incubations carried out in synthetic media, the blood was first centrifuged and then the plasma and buffy coat were removed. The cells were washed three times in 5 vol of ice-cold, isotonic (323 mosmol) sodium chloride or tetramethylammonium (TMA) chloride. They were then either used immediately (fresh cells) or preincubated until they shrank to a lower steady volume (LSS cells). This process took 90 min at 41°C, 10% hematocrit, in a medium containing 3.5 mM KCl, 1.0 mM KH₂PO₄, 120.5 mM NaCl, 10 mM glucose, 20 mM TMA-*TES* (*N*-Tris [hydroxymethyl] methyl-2-aminoethane-sulfonic acid [Sigma Chemical Co., St. Louis, MO], titrated to pH 7.4 at 41°C with TMA hydroxide), and sufficient TMA chloride to maintain osmolality at 323 mosmol. While shrinking to the LSS, the cells lost 8–20 mmol K/kg cell solid and 0.040–0.100 kg H₂O/kg cell solid. The initial water contents of fresh cells obtained from different animals varied within the range of 1.52–1.59 kg H₂O/kg cell solid. After preincubation, the water content of LSS cells was 1.46–1.52 kg H₂O/kg cell solid.

Alteration of Cell Ionic Composition

In one set of experiments reported in the next section (Fig. 3), a zero-*trans* technique was employed. Net effluxes of sodium and potassium into sodium- and potassium-free TMA chloride solutions were measured. We have previously used this method to demonstrate the stoichiometric relationship between co-transported ions (Haas et al., 1982). The best

results were obtained when net efflux via $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport was maximized by raising the sodium and lowering the potassium in the cells, using the following modification of the nystatin technique of Cass and Dalmark (1973). Cells were incubated for 1 h at 0°C , 2% hematocrit, in a solution containing 50 mM NaCl, 100 mM KCl, 50 mM sucrose, and 5 mM glucose in the presence of 20 $\mu\text{g}/\text{ml}$ nystatin (Squibb, New York, NY), which was added from a stock solution of 20 mg/ml in dimethylsulfoxide. This procedure was then repeated by centrifuging and resuspending the cells in the same loading solution without nystatin. To restore the membrane to its normal state of low cation permeability, nystatin was eluted by washing the cells six times at 25°C in 50 vol of loading solution containing 0.1 mM ouabain (Sigma Chemical Co.) and 0.25% bovine serum albumin (fraction V, Sigma Chemical Co.). The albumin, which promotes elution of nystatin from the cell membrane, was first prepared as a 10% aqueous solution and treated with cation exchange resin (AG 50W-X, Bio-Rad Laboratories, Richmond, CA) to remove any residual sodium contamination. The resulting acid solution was then back-titrated to pH 7.4 at 25°C with TMA hydroxide. After the albumin washes, the cells were resuspended in a solution of the same ionic composition as that used for loading and elution and stored at 4°C to await test incubation. Immediately before the test incubation, they were washed three times with ice-cold, isotonic TMA chloride. TMA chloride (Aldrich Chemical Co., Milwaukee, WI) was routinely recrystallized from absolute ethanol and stored in a closed container at -20°C to minimize water uptake.

Test Incubation Technique

Unless otherwise noted, test incubations were carried out at 41°C , 3% hematocrit. Where indicated, ouabain was added from a 10-mM stock solution in distilled water to give a final concentration of 0.1 mM, and furosemide (Hoechst-Roussel Pharmaceuticals, Somerville, NJ) was added from a 50-mM stock solution in 40 mM TMA- TES to yield a final concentration of 1 mM in the incubation medium. Fresh aqueous solutions of 10^{-3} M norepinephrine (levarterenol bitartrate; Winthrop Laboratories, New York) and 10^{-2} M *dl*-propranolol (Sigma Chemical Co.) were prepared immediately before each set of test incubations in which they were to be used. Initial samples were withdrawn from the incubation flasks before the addition of either agent. Within 0.2 min of withdrawal of the initial sample, the test incubation was started by adding norepinephrine (final concentration: 10^{-6} M) or *dl*-propranolol (final concentration: 10^{-4} M). For the flasks that did not receive either agent, zero time was taken to be the time of initial sampling.

Test incubations in plasma were performed only on fresh cells immediately after the initial centrifugation and removal of buffy coat, without further washing. An atmosphere of 95% air, 5% CO_2 was used to maintain plasma pH. Immediately before the start of the test incubation, the plasma was rewarmed to 41°C and the cells were resuspended in it at a final hematocrit of 20%. When hypotonic plasma was needed, distilled water containing 3.5 mM KHCO_3 and 20.5 mM NaHCO_3 was added to fresh plasma until the desired level of osmolality was achieved. When equilibrated with 95% air, 5% CO_2 , this diluted plasma showed no significant change in pH or potassium concentration.

Presentation of Data

Because the cells were changing volume during most of the incubations reported in this paper, cation levels have been expressed in millimoles per kilogram cell solid, and water in kilograms of H_2O per kilogram cell solid. Since the absolute rates of the transport processes studied tend to vary in red cells from different ducks, data have not been pooled from replicate experiments. Instead, representative findings have been presented in the tables and figures. In every case, however, one or more repeat experiments with cells from different animals have yielded qualitatively consistent results.

RESULTS

The effect of norepinephrine on the water content of duck red cells incubated in high-sodium, low-potassium media is presented in Fig. 1. In the experiment depicted in the left panel, fresh cells were suspended in a synthetic isotonic solution without prior treatment. In the center and right panels, they were preincubated for 90 min—long enough for their volume to shrink spontaneously to the LSS. They were then resuspended in either an isotonic (center panel) or hypotonic (right panel) medium.

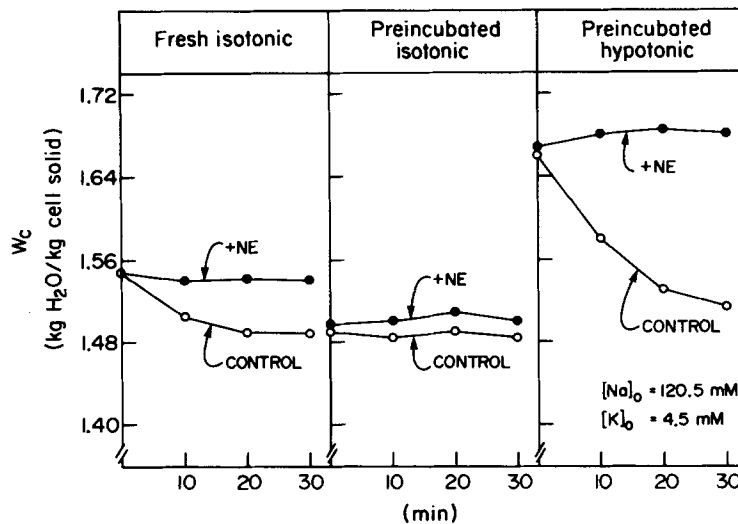


FIGURE 1. Changes in water content of duck red cells incubated in high-sodium, low-potassium media. The cells used in the experiment shown in the left panel were fresh, whereas those in the center and right panels were preincubated for 90 min to reach the LSS, as described in Materials and Methods. All test incubation media contained 3.5 mM KCl, 1.0 mM KH₂PO₄, 120.5 mM NaCl, 10 mM glucose, and 20 mM TMA-*TES* (pH 7.4 at 41 °C). TMA chloride was used to adjust the osmolality to either 323 (isotonic) or 280 mosmol (hypotonic). Where indicated, norepinephrine (NE) was present at 10⁻⁶ M. The initial cell cation contents in this experiment and that shown in Fig. 2 were (mmol/kg cell solid): for fresh cells, Na_c = 10.0 ± 0.5 (SEM for four incubations) and K_c = 244.6 ± 1.1; for preincubated cells, Na_c = 9.9 ± 0.6 (SEM for eight incubations) and K_c = 228.5 ± 1.2. The SEM of initial cell water contents (done in quadruplicate for each panel) was ±0.004 kg H₂O/kg cell solid.

The left panel shows the time course of shrinkage from the initial fresh cell volume to the LSS. This decrease in water content was accompanied by a net loss of KCl but no change in cell sodium (data not shown). The addition of norepinephrine prevented the fresh-LSS transition. On the other hand, introducing the catecholamine after the cells reached the LSS (center panel) did not cause reswelling, which confirms the findings of Riddick et al. (1971).

When LSS cells were incubated in a hypotonic medium with the same levels of sodium and potassium as in the isotonic medium (right panel, Fig. 1), their

behavior was similar to that of fresh cells; i.e., after the initial swelling response, they slowly shrank back toward their isotonic volume. This effect was also prevented by norepinephrine. As was the case with fresh cells, this shrinkage was accompanied by net KCl loss, with no significant change in cell sodium either in the presence or absence of norepinephrine (data not shown).

The resemblance between the fresh-LSS transition (left panel) and the response to hypotonic swelling (right panel) was investigated further under conditions where net influx via $[Na + K + 2Cl]$ co-transport was prevented by removing sodium from the medium (Fig. 2). In addition, external potassium was raised to

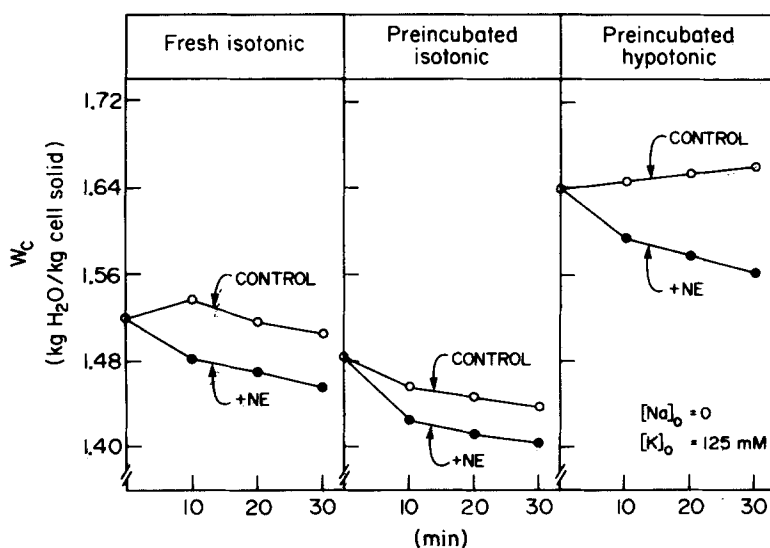


FIGURE 2. Volume shifts in fresh (left panel), LSS (center panel), and hypotonically swollen cells (right panel) in high-potassium, sodium-free media. Before test incubations, cells were preincubated, as in Fig. 1. Test incubation media contained 124 mM KCl, 1.0 mM KH_2PO_4 , 10 mM glucose, and 20 mM TMA- TES (pH 7.4 at $41^\circ C$). TMA chloride was used to adjust the osmolality to either 323 (isotonic) or 280 mosmol (hypotonic). Where indicated, norepinephrine (NE) was present at 10^{-6} M. The SEM of initial cell water content determinations (done in quadruplicate for each panel) was ± 0.004 kg H_2O/kg cell solid. The amount of water taken up during the 0–10-min period in the left panel was 0.017 kg H_2O/kg cell solid. Initial cell cation contents are listed in the legend to Fig. 1.

the point where net influx via $[K + Cl]$ co-transport was energetically favorable. Under these conditions, activation of $[Na + K + 2Cl]$ co-transport should promote a net outflow of salt and water, whereas stimulation of the swelling-induced $[K + Cl]$ pathway should result in a net uptake of KCl and water. In the isotonic medium (left panel), fresh cells lost volume in the presence of norepinephrine, but showed a transient swelling in its absence. Although this swelling response was small and limited to the first 10 min of incubation, it was observed in each of three replicate experiments. The increase in cell water in each case was 0.010, 0.019, and 0.017 kg H_2O/kg cell solid, respectively. Furthermore,

no evidence of even transient swelling was seen in cells that were first preincubated in a plasma-like isotonic medium to reach the LSS (center panel). The response of LSS cells after hypotonic swelling (right panel) again resembled that of fresh cells in the isotonic medium (left panel), i.e., the cell water content increased in the absence of the catecholamine and decreased in its presence.

In order to display volume-dependent changes in stoichiometry and in the magnitude of ion movements, a "zero-trans" technique was developed. Since efflux through the diuretic-sensitive pathway does not have a *trans* requirement

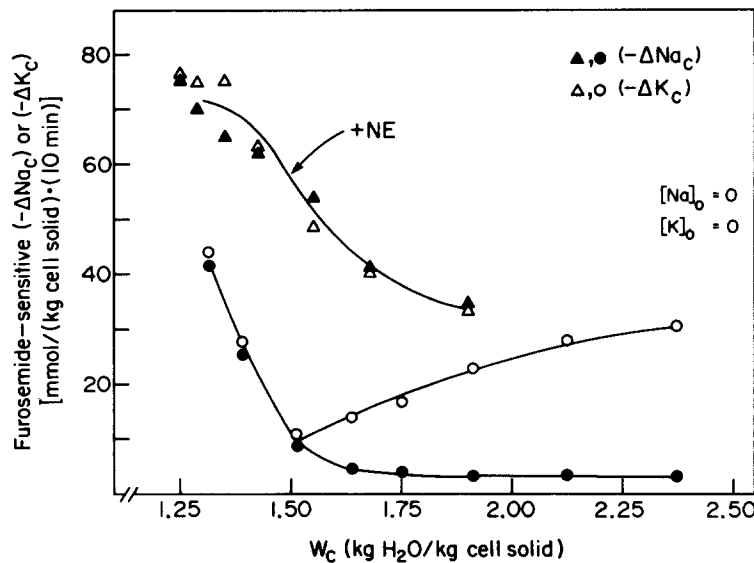


FIGURE 3. Effects of cell volume and norepinephrine (NE) on net sodium and potassium effluxes into TMA chloride media free of sodium and potassium. Cells were prepared by the nystatin method to contain (mmol/kg cell solid): $\text{Na}_c = 103.2 \pm 0.5$ (SEM for 30 incubations) and $\text{K}_c = 203.9 \pm 0.9$. They were then test-incubated at 41°C in media containing 30 mM TMA-TES (pH 7.4 at 41°C), 10 mM glucose, 0.1 mM ouabain, and an amount of TMA chloride sufficient to adjust osmolality to between 200 and 410 mosmol in increments of 30 mosmol, in the presence or absence of 10^{-6} M norepinephrine and 1 mM furosemide. All test incubation media were free of sodium and potassium. Open symbols represent furosemide-sensitive net potassium efflux ($-\Delta\text{K}_c$); closed symbols represent furosemide-sensitive net sodium efflux ($-\Delta\text{Na}_c$). Circular symbols represent results obtained in the absence of norepinephrine; triangular symbols show results obtained in its presence. Qualitatively similar results were obtained using cells containing normal physiological levels of sodium. See text for further details.

(Kregenow, 1971a; Haas et al., 1982), the system can be studied by measuring net effluxes into media free of sodium and potassium. Incubation solutions were designed in which these ions were replaced by the impermeable cation TMA. Since all sodium and potassium movements were unidirectional and outwardly directed, cells incubated in these solutions lost salt and water at all osmolalities.

Fig. 3 illustrates the effect of cell volume on net sodium and potassium effluxes

into sodium- and potassium-free media in the presence and absence of norepinephrine. Cells were pretreated with nystatin to raise their sodium content and then test-incubated in TMA chloride solutions of different osmolalities. Furose-mide-sensitive net effluxes of sodium and potassium are plotted as a function of average cell water content over the 10-min test incubation period. In the absence of norepinephrine, net potassium efflux, without a concomitant efflux of sodium, was seen at all cell waters of >1.5 kg H₂O/kg cell solid. Below this level, a net loss of sodium plus potassium was observed with a Na/K stoichiometry of 1:1. It is noteworthy that the value of 1.5 kg H₂O/kg cell solid, where the transport process changes from the shrinkage-induced mode to the swelling-induced mode, is similar to the water content of LSS cells (Fig. 1).

This experiment reveals the presence of two volume-sensitive ion transport processes: sodium-independent net potassium (or [K + Cl]) transport in swollen

TABLE I
Effect of Norepinephrine on Net Cation Movements in Hypotonic Media

10 ⁻⁶ M norepi- nephrine	<i>t</i>	W _c	Na _c	K _c	Rb _c	[K _c + Rb _c]
	<i>min</i>		<i>kg H₂O or mmol ion/kg cell solid</i>			
0	0	1.738	8.6	220.2	0.8	221.0
	15	1.570	8.3	189.6	7.7	197.3
	30	1.485	7.6	174.0	12.9	186.9
+	0	1.743	8.6	221.0	1.0	222.0
	15	1.748	10.9	190.6	29.4	220.0
	30	1.749	9.4	176.6	43.0	219.6

Cells were preincubated for 90 min to reach the LSS, as described in Materials and Methods, and then reincubated at 41°C, 3% hematocrit, in a hypotonic medium (267 mosmol) containing 4.3 mM Rb, 120 mM Na, 10 mM TMA-*TES* (pH 7.4 at 41°C), and 10 mM glucose. The standard error of the mean for the determinations of initial water content (four total, two for each condition) was ± 0.002 kg H₂O/kg cell solid.

cells, and [Na + K + 2Cl] co-transport in shrunken cells. In the presence of norepinephrine, however, a 1:1 loss of [Na + K] occurred at all osmolalities, even at cell volumes well above 1.5 kg H₂O/kg cell solid. The failure to find a sodium-independent component of net potassium efflux in norepinephrine-stimulated cells suggests that swelling-induced potassium transport has been suppressed or abolished. A similar result was found when the cells were stimulated by 8-bromo cyclic AMP (1 mM) instead of norepinephrine (data not shown).

Table I shows another approach to the study of this phenomenon. Cells preincubated to reach the LSS were resuspended in a high-sodium hypotonic medium similar to that used in Fig. 1 (right panel), except that rubidium replaced potassium. In this way, unidirectional movements of potassium out of the cells, or of its congener rubidium into the cells, could be followed. In the absence of catecholamine, cells showed a net loss of [K_c + Rb_c] and shrank back to isotonic volume in 30 min (Table I). With norepinephrine present, no net loss of [K_c +

Rb_c] or water occurred, although a substantial exchange of internal potassium for external rubidium occurred. Under these conditions, several parallel transport processes contributed to potassium and rubidium movements, including the Na/K pump (ouabain was not present), as well as simple diffusion down electrochemical gradients. It is apparent, nevertheless, that the process causing net loss

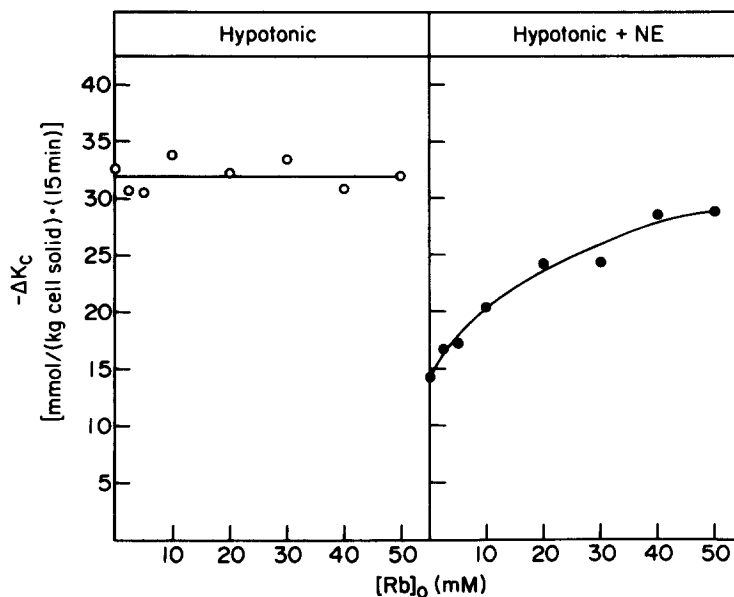


FIGURE 4. Effect of external rubidium on potassium efflux from cells swollen in hypotonic sodium-free media in the absence (left panel) or presence (right panel) of 10^{-6} M norepinephrine. Fresh cells were incubated at 41°C , 3% hematocrit in media containing 30 mM TMA- TES (pH 7.4 at 41°C), 10 mM glucose, 0.1 mM ouabain, rubidium chloride as indicated on the abscissa, and an amount of TMA chloride sufficient to adjust osmolality to 270 mosmol. Initial cell cation contents were (mmol/kg cell solid): $\text{Na}_c = 13.5 \pm 0.1$ (SEM for 16 incubations) and $\text{K}_c = 266.7 \pm 0.8$. Initial cell water was 1.764 ± 0.003 kg $\text{H}_2\text{O}/\text{kg}$ cell solid. Cells incubated in the presence of norepinephrine at $[\text{Rb}]_o = 0$ lost sodium at the rate of 9.6 mmol/kg cell solid over the 15-min incubation period. As $[\text{Rb}]_o$ was increased, sodium loss decreased. At $[\text{Rb}]_o = 50$ mM, sodium loss was 5.8 mmol/kg cell solid. In the absence of norepinephrine, cell sodium loss did not exceed 2.0 mmol/kg cell solid in any incubation. Raising $[\text{Rb}]_o$ also decreased the rate of shrinkage in the catecholamine-free incubations (left panel) because of an influx of rubidium that partially counterbalanced the swelling-induced loss of potassium. Therefore, cells remained slightly more swollen during high $[\text{Rb}]_o$ incubations. The average water content over the incubation period (average of zero-time and 15-min values) was 0.073 kg $\text{H}_2\text{O}/\text{kg}$ cell solid greater at $[\text{Rb}]_o = 50$ than at zero $[\text{Rb}]_o$. Examination of the slope of $-\Delta\text{K}_c$ vs. volume for swollen cells in the absence of norepinephrine (Fig. 3) shows that the increase in potassium efflux expected from this difference in water content would be less than the scatter of points around the line (left panel).

of potassium and water in the absence of norepinephrine was not operative in its presence.

The [K/Rb] exchange shown in Table I is a distinctive feature of catecholamine-stimulated cation transport in duck red cells (Schmidt and McManus, 1977*b*). A further study of this mode, this time in the presence of ouabain, is

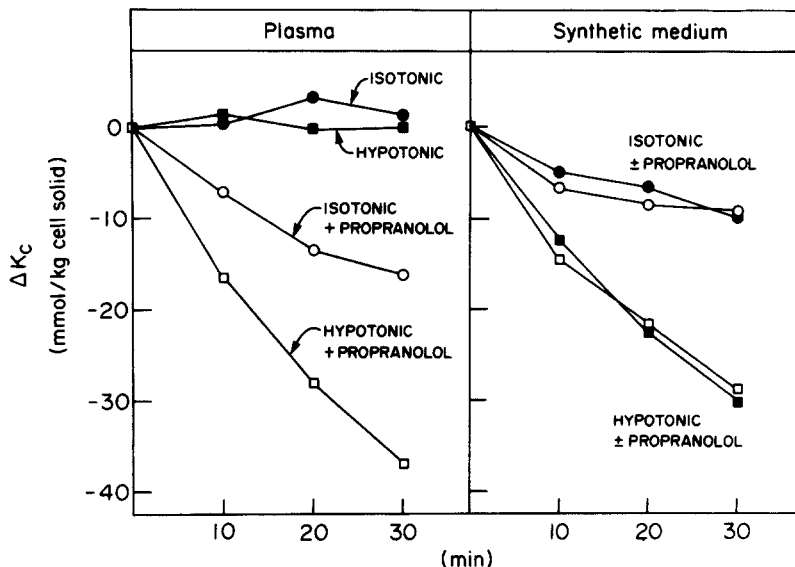


FIGURE 5. Effect of propranolol (10^{-4} M) on net potassium movements in plasma (left panel) and synthetic high-sodium, low-potassium media (right panel). Plasma was either used whole (298 mosmol) or diluted to 253 mosmol with 3.5 mM KHCO_3 plus 20.5 mM NaHCO_3 , as described in the text. Incubations in plasma were done at 20% hematocrit, 41°C, under an atmosphere of 95% air, 5% CO_2 . Ion contents of plasma were: for whole plasma, $(\text{Na})_o = 141.3$ mM and $(\text{K})_o = 3.09$ mM; for diluted plasma, $(\text{Na})_o = 116.9$ mM and $(\text{K})_o = 3.18$ mM. Synthetic media contained 3.5 mM KCl, 1 mM KH_2PO_4 , 120.5 mM NaCl, 10 mM glucose, 20 mM TMA- TES (pH 7.4 at 41°C), and an amount of TMA chloride sufficient to adjust osmolality to either 323 (isotonic) or 265 mosmol (hypotonic). Incubations in synthetic media were performed under the same conditions as in plasma, except that 100% air was used in the gas phase. All cells were fresh and contained (mmol/kg cell solid): $\text{Na}_c = 15.4 \pm 0.4$ (SEM for eight incubations) and $\text{K}_c = 266.3 \pm 1.5$. Cell sodium content did not change by more than ± 2.0 mmol/kg cell solid in any incubation. The cell water contents for this experiment are listed in Table II.

presented in Fig. 4. Fresh cells in hypotonic sodium-free solutions were exposed to various concentrations of external rubidium. In the absence of catecholamine, net potassium loss was not affected by increasing $[\text{Rb}]_o$ (left panel). In the presence of norepinephrine, two major changes were seen (right panel). First, at zero $[\text{Rb}]_o$, net loss of potassium was reduced by half. Second, potassium loss became dependent on $[\text{Rb}]_o$ (right panel). As will be discussed, these results are further

confirmation that [K + Cl] co-transport in swollen cells is replaced by [Na + K + 2Cl] co-transport in the presence of norepinephrine.

Fig. 5 and Table II illustrate the effect of endogenous plasma catecholamines on the swelling response. After the initial centrifugation, half the cells were resuspended in fresh isotonic plasma, and the other half in plasma made hypotonic by diluting 13 vol of isotonic plasma with 3 vol of 3.5 mM KHCO₃ plus 20.5 mM NaHCO₃. pH was maintained by gassing with 95% air, 5% CO₂. The cells maintained a steady potassium content in isotonic plasma, but lost potassium and water in the presence of propranolol (Fig. 5, left panel, and Table II), an

TABLE II
*Effect of Propranolol on the Water Content
of Cells Incubated in Plasma or Synthetic Media*

Medium	10 ⁻⁴ M propranolol	<i>t</i> <i>min</i>	Plasma		Synthetic	
			W _c	ΔW _c	W _c	ΔW _c
Isotonic	0	0	1.569		1.532	
		30	1.573	+0.004	1.470	-0.062
	+	0	1.574		1.535	
		30	1.475	-0.099	1.478	-0.057
Hypotonic	0	0	1.833		1.790	
		30	1.827	-0.006	1.571	-0.219
	+	0	1.842		1.772	
		30	1.550	-0.292	1.573	-0.199

Cell water contents (W_c) are given in kilograms H₂O per kilogram cell solid. A positive value for ΔW_c indicates cell swelling; a negative value indicates cell shrinkage. Results are from the experiment shown in Fig. 5.

effect resembling the typical fresh-LSS transition. Similar results were obtained in hypotonic plasma. Propranolol in synthetic media free of catecholamines did not affect either the fresh-LSS transition or swelling-induced net potassium loss (Fig. 5, right panel). Table II confirms that the net potassium movements shown in Fig. 5 are associated in all instances with appropriate changes in cell water content.

DISCUSSION

These data demonstrate that the sodium-independent net potassium transport induced by swelling duck red cells in hypotonic solutions does not appear when β-adrenergic catecholamines are present. A similar effect is observed when comparing cells incubated in fresh plasma with those in an isotonic synthetic

medium. In plasma, they maintain a constant volume, whereas in synthetic media they lose KCl and shrink (Fig. 5). The addition of propranolol causes cells in plasma to behave like those in the synthetic medium. Since this drug had no effect on potassium movements in the absence of catecholamines (Fig. 5, right panel), its action in plasma is probably due to β -adrenergic blockade rather than to activation of a calcium-dependent potassium channel ("Gardos effect"), which occurs when human red cells are exposed to this agent (Manninen, 1970; Porzig, 1975). Indeed, we have been unable to demonstrate an effect of calcium on potassium permeability in duck red cells even by exposing them to the ionophore A23187 in the presence of calcium (McManus et al., 1985).

Although $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport is activated by norepinephrine, the fact that cell volume in the LSS does not change (Fig. 1, center panel) shows that under the conditions of this experiment there was no net driving force capable of moving salt and water by this mechanism; i.e., the transmembrane chemical gradients of the three ions were balanced. This interpretation of the LSS data suggests that prevention of the spontaneous shrinkage of fresh cells (fresh-LSS transition) by norepinephrine (Fig. 1, left panel) is not the result of a concurrent stimulation of cell swelling. The net driving force for $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport does not differ significantly between fresh and LSS cells, since they have similar ionic concentrations (see the legend to Fig. 1), a consequence of the fact that only a small amount of fluid is lost from the cells during the fresh-LSS transition and it is isotonic (Kregenow, 1971a).

In the experiment shown in Fig. 2, norepinephrine caused the cells to shrink in all three instances. This results from the absence of external sodium, thereby eliminating influx via $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport. The exposure of cells to catecholamine when influx is not possible results in a net outward movement of salt through this pathway (Schmidt and McManus, 1977b; Haas et al., 1982). The continued shrinkage of LSS cells in the absence of norepinephrine (Fig. 2, center panel), as well as shrinkage of fresh cells after transient swelling (Fig. 2, left panel), can also be explained by $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport, which still shows significant activity in cells washed free of catecholamine (Schmidt and McManus, 1977b).

In summary, Figs. 1 and 2 show that all net water (and therefore ion) movements in norepinephrine-treated cells can be accounted for by $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport, which strongly suggests that stimulation of this system overrides, or abolishes, swelling-induced $[\text{K} + \text{Cl}]$ co-transport. This conclusion is further supported by the remarkable change in stoichiometry of net sodium and potassium effluxes from swollen cells when norepinephrine is present in the zero-trans experiment (Fig. 3).

Increasing the cell volume from the shrunken to the swollen state in the absence of catecholamine inhibits $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport and stimulates $[\text{K} + \text{Cl}]$ co-transport (Fig. 3). This observation, together with the effect of norepinephrine in overriding the $[\text{K} + \text{Cl}]$ system in swollen cells, suggests that these two pathways may be related, perhaps as alternative modes of the same transport process. Although they show significant kinetic differences, each of these modes is chloride-dependent and inhibited by furosemide or bumetanide (McManus

and Haas, 1981; Haas and McManus, 1982, 1983). An alternative interpretation is that those intracellular events which activate $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport concurrently inactivate a separate and distinct $[\text{K} + \text{Cl}]$ pathway. Although we think this explanation is unlikely, the results presented here cannot rule out this model. An analogous situation has been investigated in *Amphiuma* red cells, where Na/H exchange induced by shrinkage appears to be replaced by K/H exchange as cell volume is increased. It has been suggested that these two volume-sensitive modes may also represent alternative forms of the same system (Cala, 1983a).

The water content of cells after 90 min preincubation to bring them to the LSS is 1.50 ± 0.01 (SEM for 59 incubations; see Table I, Schmidt and McManus, 1977a). At this level, a point of inflection is seen in the lower curves in Fig. 3. Above this point, only the sodium-independent, swelling-induced $[\text{K} + \text{Cl}]$ pathway is manifest. Below this point, only $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport is seen. The LSS volume, therefore, appears to be a reference volume for these cells in synthetic media without catecholamines. No such point of inflection is seen in the presence of norepinephrine (Fig. 3).

The curve for the norepinephrine-stimulated cells in Fig. 3 appears to be shifted to the left in that the highest efflux values on the curve are plotted at lower values of cell water than those on the lower curves. This reflects the fact that the initial and final water contents taken over the 10-min incubation period were averaged to obtain the numbers plotted on the abscissa. Because the cells were losing salt and shrinking at all osmolalities in these sodium- and potassium-free solutions, the data were plotted against the average, rather than the initial, water values. Since cells treated with norepinephrine lost more salt (Fig. 3), their time-averaged water contents were necessarily lower. The magnitude of catecholamine-stimulated $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport also decreases as cell volume increases (Fig. 3), which could reflect a decrease in driving force caused by dilution of internal sodium and potassium by water taken up from the hypotonic medium. However, net uptake of salt and water by this system is also inhibited as cells swell (Schmidt and McManus, 1977c). Perhaps one or more of the reactions activating co-transport after the formation of cyclic AMP (Rudolph and Greengard, 1974; Alper et al., 1980) is affected by increasing cell volume. However, human red cells, which are not sensitive to cyclic AMP, also show swelling-induced inhibition of furosemide-sensitive net sodium efflux into a zero-trans medium (Adragna and Tosteson, 1984). It is therefore possible that cell swelling itself directly inhibits $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport.

The chloride-dependent $[\text{K}/\text{Rb}]$ exchange, which appears after catecholamine stimulation of duck red cells (Schmidt and McManus, 1977b), closely resembles $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport with respect to both kinetics and diuretic sensitivity (McManus and Haas, 1981; Haas and McManus, 1982, 1983). This phenomenon also appears in swollen cells treated with norepinephrine (Table I and Fig. 4). Although sodium is not transported by this mode, it (or lithium) must be present, either in the cells or in the medium, for $[\text{K}/\text{Rb}]$ exchange to operate maximally (Haas et al., 1983). $[\text{K}/\text{Rb}]$ exchange may therefore represent an incomplete or abortive form of $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport. This interpretation is based on

the assumption that only the fully loaded (or completely empty) form of the carrier can cross the membrane. For example, the sodium, potassium, and chloride sites must all be occupied for the complex to move in the outward direction. At the outer surface, however, instead of unloading and taking on fresh ions for the return or returning empty, the carrier exchanges potassium for rubidium. With the fully loaded, permeable form of the carrier thus re-established, the complex can now move back across the membrane to its inward-facing conformation, where the process is reversed, resulting in [K/Rb] exchange even in the complete absence of external sodium. Obviously, some carriers must unload completely and return empty to account for net salt efflux, but as external rubidium is raised, the probability of [K/Rb] exchange increases. If we make the usual assumption that the total number of carriers is finite, this model predicts that an increase in [K/Rb] exchange must lead to a decrease in net outward [Na + K + 2Cl] co-transport. Indeed, that is what we have observed. For example, catecholamine-stimulated net sodium loss from cells into a sodium-free medium decreases as $[Rb]_o$ increases (see the legend to Fig. 4 of this paper and Table II of Haas et al., 1982). We have not found any circumstances in which this obligatory exchange mode of [Na + K + 2Cl] co-transport contributes to net salt movement, which excludes it from playing a role in cell volume regulation.

The marked decrease in net potassium loss from cells in a zero $[Rb]_o$ solution when norepinephrine is added in Fig. 4 (right panel) contrasts with the results shown in Fig. 3, where the addition of catecholamine increased net potassium loss even in swollen cells. The cells used in Fig. 3, however, were preincubated to raise internal sodium in order to maximize outward [Na + K + 2Cl] co-transport. In the experiment depicted in Fig. 4, cell sodium was low enough to limit the amount of outward co-transport that could occur in a sodium-free medium before internal sodium was depleted (see the legend to Fig. 4). We have shown in isotonic media that furosemide-sensitive potassium efflux does not take place under *zero-trans* conditions in cells depleted of sodium, either in the presence or absence of norepinephrine (Haas et al., 1982). The observation that net potassium efflux from norepinephrine-treated swollen cells is limited by internal sodium is further evidence that [Na + K + 2Cl] co-transport overrides the swelling-induced, sodium-independent [K + Cl] pathway.

The increase in potassium efflux as $[Rb]_o$ increases (Fig. 4, right panel) cannot be attributed to *trans*-stimulation of [Na + K + 2Cl] co-transport, since net efflux via this mode, as noted above, actually decreases with increasing $[Rb]_o$ (see the legend to Fig. 4 of this paper and Table II of Haas et al., 1982). This increase in potassium loss is a manifestation of the [K/Rb] exchange mode of [Na + K + 2Cl] co-transport, which is not apparent in the absence of catecholamine (Fig. 4, left panel).

Although these results show that swelling-induced [K + Cl] co-transport, which mediates the so-called "volume-regulatory decrease" in swollen duck red cells (Kregenow 1971a, 1981), cannot be found in cells incubated in hypotonic plasma, two important questions must be answered before it can be concluded that it does not occur *in vivo*. First, are plasma catecholamines always high enough to ensure the override effect shown in Fig. 5? It seems certain that catecholamines

were elevated in the plasma used in these experiments because of the circumstances under which the blood was obtained. During bleeding, our animals necessarily had to be restrained, although they did not struggle during the procedure. Jurani et al. (1980) report that immobilization of turkeys for 30 min caused plasma catecholamines (epinephrine plus norepinephrine) to increase as much as sixfold. They also measured resting levels in five species of domestic birds, and found them to exceed 10^{-8} M in every case, with three specimens showing levels $>5 \times 10^{-8}$ M. In preliminary experiments, we found that net potassium and water loss from swollen cells incubated in a solution like that used in the experiment shown in Fig. 1 (right panel) is inhibited $\sim 80\%$ at 5×10^{-9} M and completely blocked at 5×10^{-8} M norepinephrine.

The second question to be answered is why fresh duck red cells seem to behave as though they were slightly swollen, as shown by their spontaneous loss of KCl and shrinkage when removed from the influence of plasma catecholamines (Fig. 5 and Table II). The resemblance between the volume response of fresh cells in catecholamine-free isotonic media and their reaction to hypotonic swelling (Figs. 1 and 2) suggests a common mechanism, i.e., swelling-induced [K + Cl] co-transport. Thus, fresh cells that have been washed and resuspended in an isotonic synthetic medium spontaneously shrink (the fresh-LSS transition), even though they initially have the same volume as in their own plasma. Indeed, they even behave this way in plasma, if propranolol is added (Fig. 5). This phenomenon cannot be ascribed to stress-related elevation of catecholamines during bleeding, since the addition of norepinephrine to LSS cells in a plasma-like synthetic medium does not cause them to reswell (Fig. 1, center panel; see also Riddick et al., 1971). Reswelling from the LSS can occur only if external potassium is increased. We have found no evidence for increased plasma potassium in our blood samples, although it has been reported that blood obtained from rainbow trout by cardiac puncture has plasma potassium increased above resting levels (Nikinmaa, 1982). Somehow, in the physiological steady state, ducks maintain the volume of their red cells above the LSS. Elucidation of this effect will require further studies of water and electrolyte metabolism of these cells in the circulation.

A fresh-LSS transition sensitive to β -adrenergic catecholamines has been noted in red cells from other avian species. We have found it in turkey red cells (Haas, M., and T. J. McManus, unpublished experiments), and Leskovac et al. (1984) report inhibition of net potassium loss from fresh pigeon red cells by isoproterenol and cyclic AMP, an effect that can be readily explained by this same mechanism. In addition, several recent reports suggest that this phenomenon may not be limited to avian red cells. DeVries and Ellory (1982) found that forced activity and stress before bleeding had a profoundly stimulatory effect on the influx of potassium into fish red cells, and Nikinmaa (1982) observed that either stress or injection of epinephrine in vivo caused an increase in red cell volume in rainbow trout that did not occur in animals treated with propranolol. Bourne and Cossins (1982) reported that washed red cells from the rainbow trout progressively lost volume, accompanied by a decrease in potassium influx during incubation in a synthetic medium without added hormones. If catechol-

amines were present, or if cells were incubated in their own plasma, flux rates remained high and stable over a 6-h period. They also presented preliminary data that cell volume had only a slight effect on furosemide-sensitive potassium influx in trout red cells in plasma, but much larger effects in cells washed and incubated in synthetic media. Thus, those who study volume regulation by osmotically perturbing cells and tissues *in vitro* should carefully consider the possible influence of endogenous hormonal agents such as catecholamines on volume-sensitive ion transport.

The physiological role of catecholamine-stimulated $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport in the duck red cell is unknown. A contribution to extrarenal potassium regulation has been previously suggested (McManus and Schmidt, 1978; Rudolph and Lefkowitz, 1978; Cala, 1983*b*). Schmidt and McManus (1977*a*) found that the system responds linearly in the physiological range of extracellular potassium. Therefore, during transient hypokalemia, $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport might help mobilize potassium from red cells to supplement extracellular stores. Conversely, during hyperkalemia, excess potassium could be taken up by red cells, remaining there until renal or other mechanisms adjust plasma levels back to normal. Of course, such an uptake would cause swelling, but the excess potassium would not leak back out of the cells, since plasma catecholamines prevent the swelling-induced loss. As plasma potassium returned to normal, the driving force for co-transport would favor net loss of the excess cell potassium, leading to a restoration of cell volume.

There are reports that β -adrenergic catecholamines influence extrarenal potassium disposal in humans (Rosa et al., 1980; Wang and Clausen, 1976; Brown et al., 1983). In this case, however, the effector organs appear to be skeletal muscle and liver (for reviews, see Bia and DeFronzo, 1981; Epstein and Rosa, 1983). Human red cells lack the fully coupled adrenergic system found in some avian species (Rasmussen et al., 1975). Therefore, it is unlikely that they contribute acutely to potassium regulation, although a long-term role cannot be ruled out. The $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport system is present in the human red cell (Wiley and Cooper, 1974), but accounts for only a small fraction of total cation flux and is not affected by catecholamines (McManus and Schmidt, 1978). Recently, Duhm and Göbel (1984) have suggested that this system may function in human red cells to promote potassium extrusion during the steady state *in vivo*. In duck red cells, which have somewhat lower chloride than human red cells, the gradients of sodium, potassium, and chloride under physiological conditions are closer to the balance point. Extrarenal regulation of potassium by red cells may therefore be of significance in those avian species whose red cells possess the complete β -adrenergic system (Davoren and Sutherland, 1963; Bilezikian and Aurbach, 1973) plus a coupled transport of $[\text{Na} + \text{K} + 2\text{Cl}]$.

Since most cells in the body contain less chloride than red cells, the driving force for $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport would favor net salt and water uptake and could thus promote cell swelling. It is now becoming clear that this effect may be of importance in the regulation of salt and water movements across many secretory and reabsorptive epithelia. On the other hand, if duck red cells experience a loss of volume *in vivo*, $[\text{Na} + \text{K} + 2\text{Cl}]$ co-transport cannot help

them recover unless extracellular potassium is elevated, and if they become swollen while in the circulation, plasma catecholamines would effectively prevent a "volume-regulatory decrease."

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