Ouabain-Insensitive Salt and Water Movements in Duck Red Cells

II. *Norepinephrine Stimulation of Sodium plus Potassium Cotransport*

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ABSTRACT Catecholamines induce net salt and water movements in duck red cells incubated in isotonic solutions. The rate of this response is approximately three times greater than a comparable effect observed in 400 mosmol hypertonic solutions in the absence of hormone (W. F. Schmidt and T. J. McManus. 1977 *a. J. Gen. Physiol.* 70:59–79. Otherwise, these two systems share a great many similarities. In both cases, net water and salt movements have a marked dependence on external cation concentrations, are sensitive to furosemide and insensitive to ouabain, and allow the substitution of rubidium for external potassium. In the presence of ouabain, but the absence of external potassium (or rubidium), a furosemide-sensitive net extrusion of sodium against a large electrochemical gradient can be demonstrated. When norepinephrine-treated cells are incubated with ouabain and sufficient external sodium, the furosemide-sensitive, unidirectional influxes of both sodium and rubidium are half-maximally saturated at similar rubidium concentrations; with saturating external rubidium, the same fluxes are half-maximal at comparable levels of external sodium. In the absence of sodium, a catecholamine-stimulated, furosemide-sensitive influx of rubidium persists. In the absence of rubidium, a similar but smaller component of sodium influx can be seen. We interpret these results in terms of a cotransport model for sodium plus potassium which is activated by hypertonicity or norepinephrine. When either ion is absent from the incubation medium, the system promotes an exchange-diffusion type of movement of the co-ion into the cells. In the absence of external potassium, net movement of potassium out of the cell leads to a coupled extrusion of sodium against its electrochemical gradient.

INTRODUCTION

A catecholamine effect on ion and water movements in bird red cells was first observed by Ørskov in 1956. He found that norepinephrine and, to a lesser extent, epinephrine stimulated net potassium uptake in pigeon red cells in vitro. Riddick et al. (1971) suggested that norepinephrine is actually required for Muscovy duck red cells to maintain steady states of ions and water at normal levels in artificial, buffered salt solutions. Omission of the hormone from their incubations resulted in a spontaneous decline in cell potassium and water until a

new "lower steady state" was reached after 90 min. If $[K]_o$[1] was then elevated above plasma levels, and norepinephrine added, cell volume increased toward its original level. This water gain was accompanied by a net uptake of potassium against its electrochemical gradient. Thus, norepinephrine appeared to stimulate an active uptake of potassium, with water following passively.

The notion that catecholamines acted primarily on the Na-K pump was questioned by Kregenow (1973) who suggested, instead, that the volume response of duck red cells to norepinephrine is concerned primarily with total salt and water taken up rather than with the specific composition of the accumulated solution. He found that addition of ouabain merely shifted the cation composition of the fluid taken up from mainly potassium to mainly sodium, with the total amount of salt and water accumulated remaining the same. Some evidence suggests that the actual mechanism responsible for net salt uptake may be at least one step removed from the stimulatory effect of catecholamines. Thus, solute and water accumulation can also be triggered by placing bird cells in hypertonic solutions, as originally reported by Ørskov (1954). This reswelling phenomenon is affected neither by various agents which interfere with catecholamine binding, nor by ouabain concentrations sufficient to inhibit the Na-K pump (Kregenow, 1971).

Our results (Schmidt and McManus, 1977a) with Pekin duck red cells confirm that the volume response in hypertonic solutions containing ouabain is secondary to net cation movements. We have also presented evidence that simple diffusion cannot account for the observed ion movements. Instead, net cation accumulation with ouabain present appears to be mediated by a cotransport of sodium plus potassium into the cells. This mechanism is highly sensitive to both $[Na]_o$ and $[K]_o$, has a relatively high temperature coefficient, and is inhibited by the diuretic, furosemide. Rubidium can serve as an effective substitute for $[K]_o$.

This paper reports a study of the ouabain-insensitive net salt and water movements induced in Pekin duck red cells by norepinephrine and compares the changes with those observed in hypertonic solutions. For all conditions studied, these two mechanisms of salt transport are remarkably similar. We suggest that a final common pathway may be involved, and propose that norepinephrine also stimulates a cotransport of sodium plus potassium. A net uptake or loss by this mechanism can occur only when external cation concentrations are appropriately adjusted.

A preliminary report of some of these results was presented to the Biophysical Society in April, 1974 (Schmidt and McManus, 1974).

MATERIALS AND METHODS

Fresh heparinized blood from the adult white Pekin duck was obtained on the day of experimentation as previously described (Schmidt and McManus, 1977a). After centrifugation and removal of plasma and buffy coat, cells were washed four times in 5 vol of iced 170 mM NaCl, then preincubated at 41°C (normal body temperature for ducks) for 90 min in an artificial buffer. This solution, which was adjusted to a final pH of 7.4 (41°C)

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[1] As in the previous paper (Schmidt and McManus, 1977a), $[K]_o$, $[Na]_o$, etc. refer to the concentration (mM) of the ion in the external solution bathing the cells. $[K]_c$, $[Na]_c$, etc. represent the *concentration* in the cells (millimoles/liter cell water), while K_c, Na_c, W_c, etc. designate the *amount* in the cells (millimoles/kilogram cell solids).

with 10 mM Na-TES (*N*-Tris (hydroxy methyl) methyl-2-aminoethane sulfonic acid) (Sigma Chemical Co., St. Louis, Mo.) contained 2.5 mM KCl and sufficient NaCl to adjust the osmolality to 323 mosmol, which is normal for duck plasma. The exact composition of this solution, as well as the method of preparation of the TES buffers and test incubation media, has also been described in the previous paper.

A fresh aqueous solution of 10 mM norepinephrine (levarterenol bitartrate, Winthrop Laboratories, Evanston, Ill.) was prepared for each experiment. A sufficient amount was added to test incubation solutions to yield a final concentration of 10^{-6} M. In general, preincubated cells were washed once with isotonic (323 mosmol) $MgCl_2$ and introduced directly into the test incubation at a final hematocrit of 2%. After the first sample had been withdrawn (representing zero time), norepinephrine was added to initiate the response. Total elapsed time between initial sampling and addition of the hormone never exceeded 2 min. Cell separation was accomplished with the aid of specially prepared nylon tubes. The general experimental protocol, as well as the technique of cell separation and assay of ions and water using these tubes was described in the previous paper (Schmidt and McManus, 1977a). Incubation solutions generally contained (mM): ouabain, 0.1; glucose, 10; inorganic phosphate, 2.0–5.0; and magnesium, 1.0. Deviations from this protocol are noted in legends of individual tables and figures, as are specific concentrations of $[Na]_o$, $[K]_o$, $[Rb]_o$, and $[Cl]_o$. Osmolality was always adjusted by suitable addition of choline chloride.

Because of the extremely high rates of ion flux encountered in duck red cells stimulated by catecholamines, unidirectional sodium and rubidium influxes were carried out at 25°C to slow the reaction enough to approximate a quasi-steady state. For these experiments, cells were incubated at a 30% hematocrit in 3.0 ml test solutions initially containing all components save norepinephrine. Buffered ^{22}Na (1.0 μCi) was allowed to equilibrate with cells for several minutes before the first sample was taken. Norepinephrine was then immediately added, marking the start of the flux period. Subsequent samples were withdrawn at 10-min intervals. The high hematocrit was chosen so that the cells could be introduced directly into the precooled nylon tubes and centrifuged without the preliminary step of cell concentration required for the usual low (2%) hematocrit suspensions used in the other experiments reported here. This approach also provided for rapid cooling of the suspension by the chilled aluminum block supporting the nylon tubes. Even at 25°C, the rate of isotope movement was impressive compared to that of the unstimulated cells. For experiments where rubidium influx was measured simultaneously with sodium influx, cells were exposed to $[Rb]_o$ during the preliminary ^{22}Na equilibration. Although ouabain was always present, some rubidium had already entered the cells by the time of initial sampling. However, such uptake before addition of norepinephrine was minimal, and did not affect interpretation of the results. Sodium influx was calculated according to the nonsteady-state method of Tosteson and Robertson (1956). Rubidium influx was estimated as net uptake in 10 min for cells initially free of that cation. Net uptake was linear with respect to time over this initial 10-min interval. All data depicted are representative of several other experiments of similar design.

RESULTS

Effect of Ouabain

We have previously demonstrated that 10^{-4} M ouabain has little effect on cell volume in isotonic incubation, although it promotes rapid sodium uptake at the expense of potassium loss (see Table III, Schmidt and McManus, 1977a). Fig. 1 shows the effect of ouabain on water and ion shifts after addition of norepinephrine to an isotonic incubation. $[K]_o$ is elevated from the normal duck plasma level

of 2.5 mM to 21 mM, a maneuver necessary to demonstrate net water uptake under these conditions (Riddick et al., 1971). Although ouabain significantly alters the pattern of cation accumulation, converting it from mainly potassium to mainly sodium, there is no change in the total amount of salt entering the cell. With or without ouabain, Na_c plus K_c increases by approximately 40 mmol/kg cell solids after 30 min. A similar increase occurs in Cl_c. Comparable results with
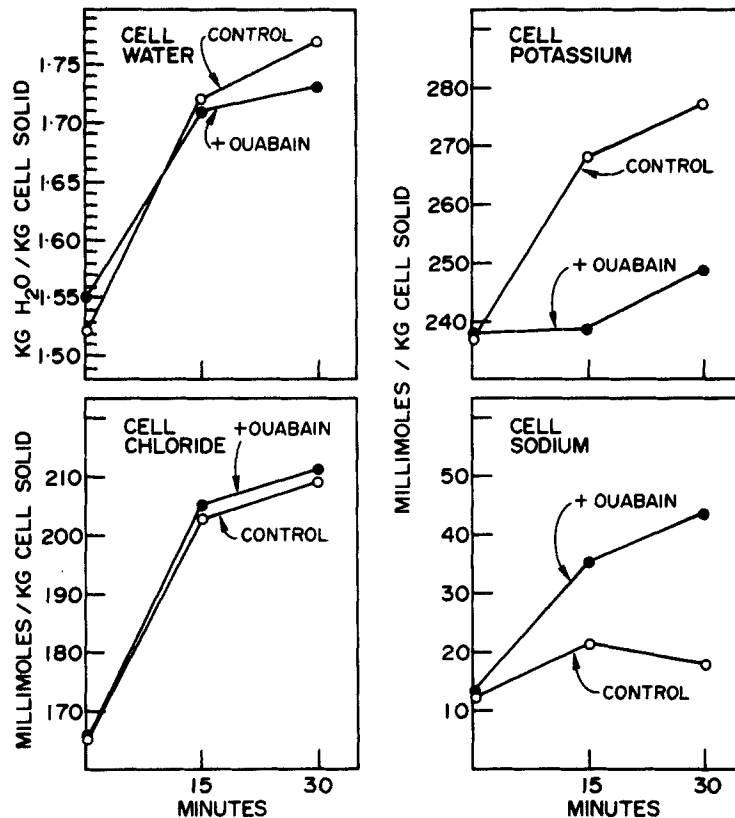

FIGURE 1. Effect of ouabain (10^{-4} M) on the response to norepinephrine. Incubation solutions contain (mM): $[Na]_o$ = 92; $[K]_o$ $\doteq$ 21; $[Cl]_o$ = 163; Mg-TES = 10. pH_0 = 7.4, 41°C, 323 mosmol. At zero time, cells contain (millimoles/liter cell water): $[Na]_c$ = 8.4; $[K]_c$ = 154; $[Cl]_c$ = 108.

ouabain were obtained for duck red cells in hypertonic solutions (Kregenow, 1971; Schmidt and McManus, 1977a).

Effects of Altering External Sodium and Potassium

One significant finding in the study of cell swelling in hypertonic solutions containing ouabain is the marked dependence of the volume response on $[Na]_o$ and $[K]_o$ (Schmidt and McManus, 1977a). To define and compare further the norepinephrine-induced volume changes, this response was also studied by varying $[Na]_o$ and $[K]_o$ in the presence of ouabain. Results are depicted in the next two figures.

Fig. 2 shows the results of an experiment where $[K]_o$ was maintained at 20 mM, while $[Na]_o$ was varied from 2 to 92 mM by isosmotic replacement with choline chloride. Upon elevation of $[Na]_o$, cells gain more Na_c, lose less K_c, and accumu-
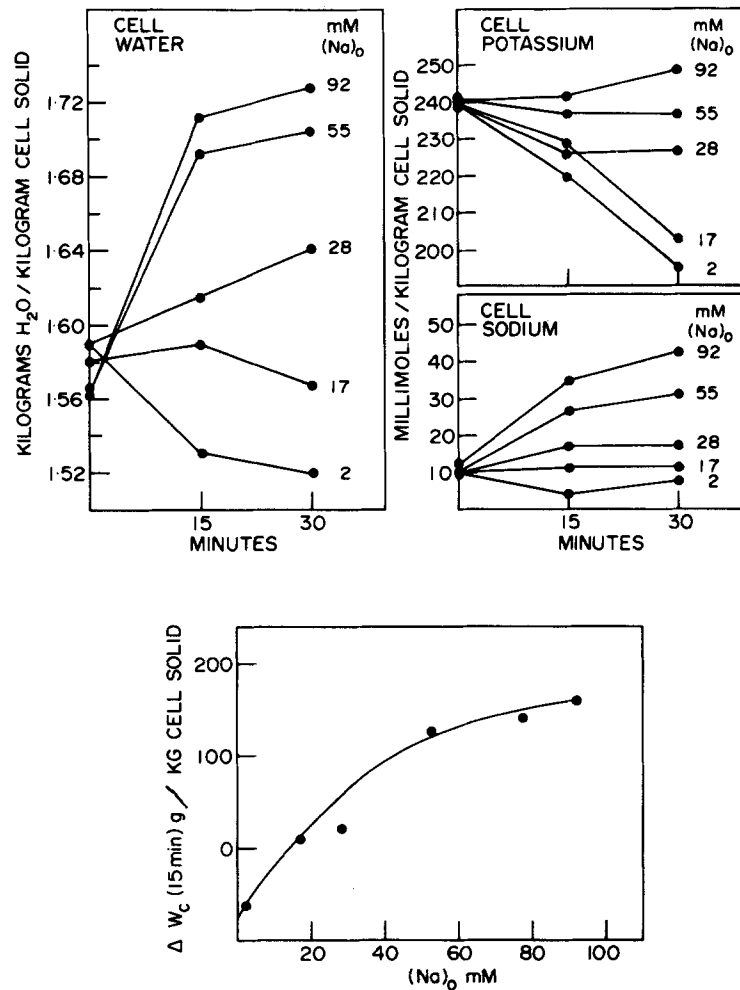

FIGURE 2.   Effect of $[Na]_o$ on the response to norepinephrine (10^{-6} M). Incubation solutions contain (mM): ouabain = 0.1; $[K]_o$ = 20; $[Cl]_o$ = 160; Mg-TES = 10. pH_0 = 7.4, 41°C, 323 mosmol. Choline was substituted for $[Na]_o$. At zero time, cells contain (millimoles/liter cell water): $[Na]_c$ = 7.2 ± 0.3 (SEM); $[K]_c$ = 153 ± 1; $[Cl]_c$ = 108 ± 1. Net change in cell water (ΔW_c) in the first 15 min of incubation is replotted in the lower panel as a function of $[Na]_o$. Note that the ordinate is changed from kilograms to grams H_2O/kg cell solids.

late water whenever net salt gain exceeds net salt loss. Note in particular the slight but significant increase in K_c at 30 min for cells incubated in 92 mM $[Na]_o$. Such a transient net increase in K_c is observed consistently in the presence of norepinephrine and ouabain in solutions containing 15-20 mM $[K]_o$ and approximately 100 mM $[Na]_o$. Cell sodium is constant at an external concentration of 17

mM, even though the sodium electrochemical gradient still favors net uptake. The lower panel is a plot of changes in cell water (ΔW_c) as a function of $[Na]_o$ during the first 15 min after addition of norepinephrine. About 20 mM $[Na]_o$ is required to prevent volume changes. Higher $[Na]_o$ causes swelling, and lower $[Na]_o$ causes the cells to shrink.

Net cation and water movements in the presence of norepinephrine and ouabain also exhibit a dependence on $[K]_o$, as illustrated in Fig. 3. In addition to diminishing the loss of potassium from the cells, elevating $[K]_o$ promotes sodium gain. Similar results have been reported for Muscovy red cells incubated in the absence of ouabain (Riddick et al., 1971). The mechanism mediating net salt and water uptake in response to norepinephrine appears to be at least five times more sensitive to $[K]_o$ than to $[Na]_o$ (compare lower panels of Figs. 2 and 3).

Potassium is not the only cation which norepinephrine stimulates to move against its electrochemical gradient in the presence of ouabain. Net sodium extrusion can also occur (Fig. 3). In the absence of $[K]_o$, cells lose more than half of their original sodium content in 30 min. This occurs even though $[Na]_o$ is almost 20 times greater than $[Na]_c$ (97 mM vs. 5.3 mM), and the chloride distribution ratio ($r_{Cl} = 0.68$) predicts a negative membrane potential favoring sodium entry. Raising $[K]_o$ to only 1.3 mM without altering the sodium electrochemical gradient abolishes net sodium loss and produces instead a small sodium gain.

To explore further the effect of norepinephrine on net sodium loss, furosemide was added to zero $[K]_o$ solutions. Results of a typical experiment are shown in Fig. 4. In the absence of norepinephrine (control), the cells suffer a loss of potassium and water which is not affected by furosemide. Norepinephrine markedly increases the water loss by producing an increase in the net loss of *both* potassium and sodium, the latter against its electrochemical gradient. After 30 min, enough potassium has left the cells to cause $[K]_o$ to increase from zero to 0.3 mM. Furosemide blocks the norepinephrine-stimulated loss of both ions and thus returns the rate of cell shrinkage to the control level.

Rubidium Substitution for Potassium

Rubidium can successfully fill the role of $[K]_o$ in cell swelling produced by hypertonic solutions. Salt and water uptake activated by this ion is also inhibited by furosemide (Schmidt and McManus, 1977 a). A similar situation is observed in isotonic solutions containing ouabain and norepinephrine. Identical changes in cell water, Cl_c, and Na_c occur with high $[Rb]_o$ as with high $[K]_o$ (Fig. 5). This resemblance between the effect of the two ions is apparent in both the presence and the absence of norepinephrine (control). Furosemide also blocks norepinephrine-induced net uptakes of sodium, chloride, and water.

Fig. 6 presents a detailed study of K_c and Rb_c changes during the same incubation as depicted in Fig. 5. The left panel shows K_c levels in the presence of 19 mM $[K]_o$. The slight net loss of K_c which occurs in the absence of norepinephrine (control) is reversed by addition of catecholamine, so that by 30 min, a transient net accumulation of K_c can be demonstrated. Furosemide blocks this norepinephrine-induced, ouabain-insensitive net potassium uptake. The middle panel illustrates changes in K_c when $[Rb]_o$ is substituted for $[K]_o$. Net losses at 30
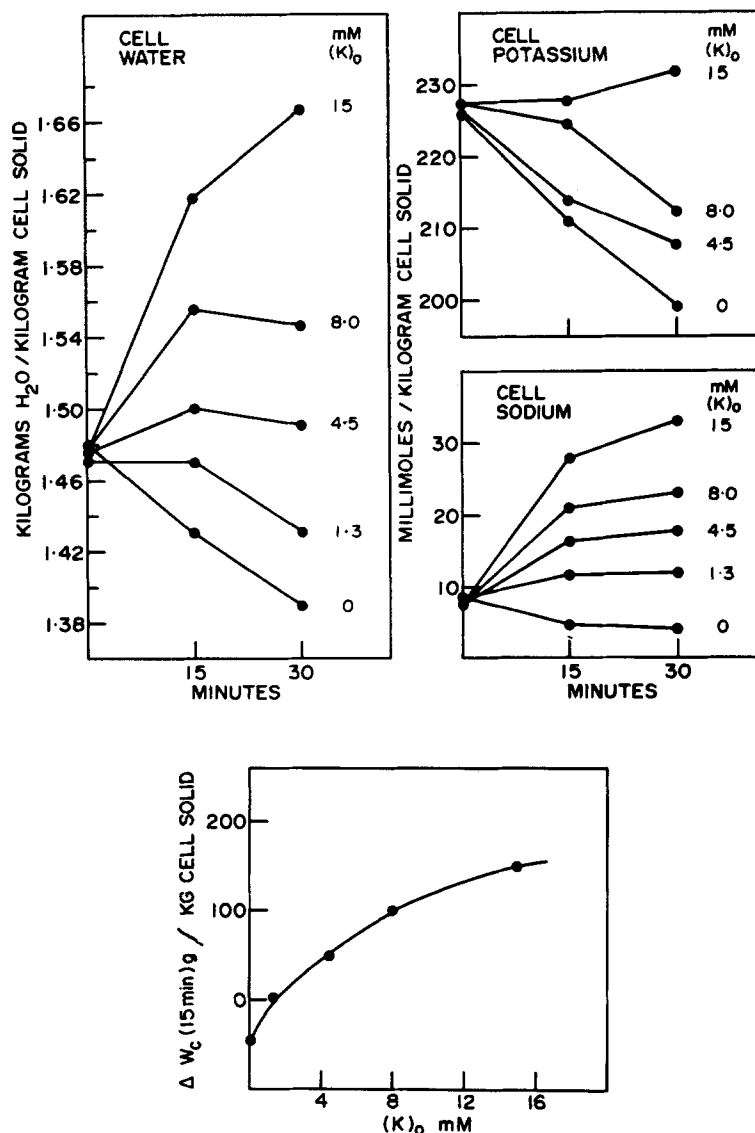
FIGURE 3.   Effect of $[K]_o$ on the response to norepinephrine (10^{-6} M). Incubation solutions contain (mM): ouabain = 0.1; $[Na]_o$ = 97; $[Cl]_o$ = 152; Mg-TES = 10. pH_o = 7.4, 41°C, 323 mosmol. Choline was substituted for $[K]_o$. At zero time, cells contain (millimoles/liter cell water): $[Na]_c$ = 5.3 ± 0.2 (SEM); $[K]_c$ = 156 ± 1; $[Cl]_c$ = 104 ± 2. Net change in cell water (ΔW_c) during the first 15 min of incubation is replotted in the lower panel as a function of $[K]_o$. As before, the ordinate is changed from kilograms to grams H_2O/cell solids.

min may be considered fair estimates of unidirectional potassium efflux since the cells are incubated at a 2% hematocrit, and very little $[K]_o$ is available in the K-free medium for back flux into the cells. For example, after 30 and 60 min, $[K]_o$ was measured and found to be, respectively, 0.6 and 0.8 mM. Potassium

efflux in 30 min is increased threefold (from 27 to 93 mmol/kg cell solids) after addition of norepinephrine. Furosemide reduces this to a level indistinguishable from that observed in the 19 mM $[K]_o$ solution containing hormone and inhibitor (left panel).

Since one effect of norepinephrine is actually to produce a transient net increase in K_c (left panel, Fig. 6), it is reasonable to expect that the large
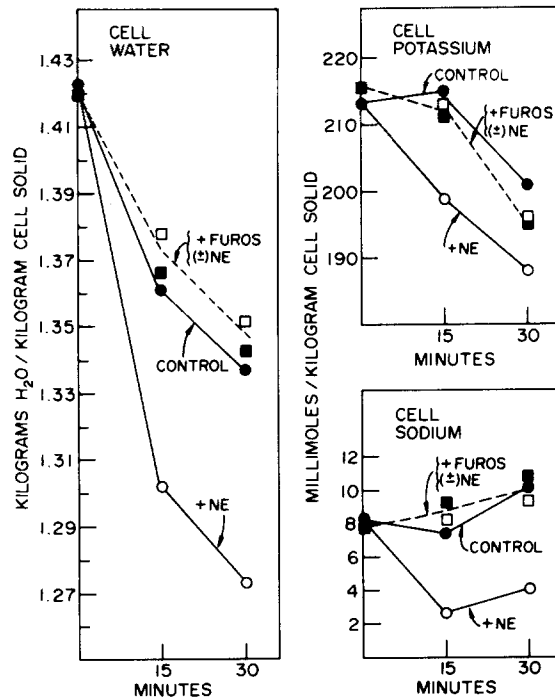

FIGURE 4. Effects of norepinephrine (10^{-6} M) and furosemide (10^{-3} M) on cell water and ion contents in $[K]_o$-free isotonic solutions. Incubation solutions contain (mM): ouabain = 0.1; $[Na]_o$ = 153; $[Cl]_o$ = 163; Mg-TES = 10. pH_o = 7.4; 41°C; 325 mosmol. At zero time cells contain (millimoles/liter cell water): $[Na]_c$ = 5.6 ± 0.2 (SEM); $[K]_c$ = 151 ± 2; $[Cl]_c$ = 100 ± 1.

potassium efflux promoted by catecholamine in the presence of 21 mM $[Rb]_o$ (middle panel) must be associated with an even greater net rubidium influx. The data support this expectation. Rubidium contents, measured at 0, 30, and 60 min for the cells in the middle panel are given in Table I. After 30 min in the norepinephrine-containing $[Rb]_o$ solution, cell rubidium has increased to 117 mmol/kg cell solids, which is equivalent to 68 mmol/liter cell water, even though $[Rb]_o$ is only 21 mM. Thus this cation can also be accumulated against its electrochemical gradient. Furosemide causes reduction of rubidium uptake in both norepinephrine-stimulated and control cells. In fact, in the presence of furosemide we have never observed any statistically significant difference with or without catecholamine, in salt and water movements. By use of data from the $[Rb]_o$ incubation, total amounts of K_c plus Rb_c were calculated. The results are

plotted in the right panel of Fig. 6. For all conditions tested, the curves are closely similar to those plotted in the left panel.

To test for a possible contribution of ouabain to the inhibitory effect of furosemide on the norepinephrine response, cells were incubated in ouabain-free isotonic solutions with and without furosemide. The results are presented in Table II. Because ouabain is not present, norepinephrine stimulates a salt uptake composed mainly of KCl (compare Fig. 1). However, water data (W_c) show that furosemide is just as effective in the absence of ouabain as in its presence in preventing norepinephrine-induced volume increases.
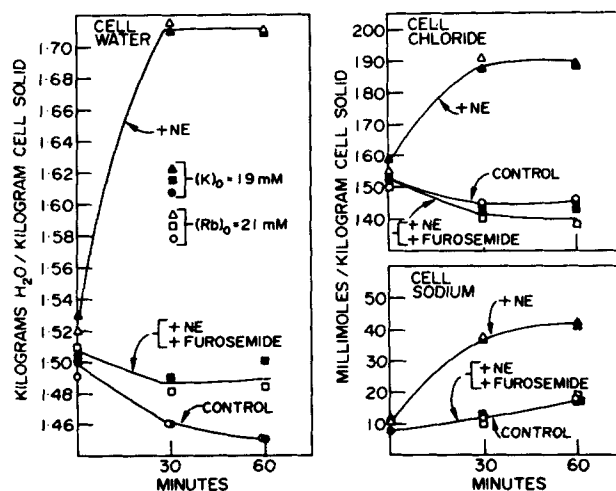

Figure 5. Effect of substituting $[Rb]_o$ for $[K]_o$ in the presence and absence of norepinephrine (10^{-6} M) and furosemide (10^{-3} M). "Control" denotes the condition where both norepinephrine and furosemide were omitted. Incubation solutions contain (mM): ouabain = 0.1; $[Na]_0 = 91$; $[K]_0$ or $[Rb]_0 = 21$; $[Cl]_0 = 152$; Na-TES = 10. $pH_o = 7.4$; 41°C; 323 mosmol. At zero time cells contain (millimoles/liter cell water): $[Na]_c = 6.0 \pm 0.4$ (SEM); $[K]_c = 171 \pm 2$; $[Cl]_c = 103 \pm 1$.

Unidirectional Influxes

Table III shows the effects of norepinephrine and furosemide on sodium and rubidium influxes in the presence of ouabain. As noted above, temperature was lowered from 41°C to 25°C to slow the rate of net salt uptake. Although these net fluxes were slower than those observed at 41°C, a net uptake of rubidium against its electrochemical gradient similar to that shown in Table I can be demonstrated.

Norepinephrine markedly increases both sodium and rubidium influxes (Table III). With furosemide present, stimulation by the catecholamine is blocked. Since both $[Na]_0$ and $[K]_0$ (or $[Rb]_0$) are required for *net* cation uptake (Figs. 2 and 3), these results suggest that the norepinephrine-induced, furosemide-sensitive influxes of both sodium and potassium (or rubidium) are coupled. To test this idea further, sodium and rubidium influxes were measured in the presence of ouabain and norepinephrine with varying $[Na]_0$ at saturating $[Rb]_0$

and with varying $[Rb]_o$ at saturating $[Na]_o$. Results are presented in Figs. 7 and 8.

The left panel of Fig. 7 shows rubidium influx with varying $[Na]_o$ during the first 10 min after addition of norepinephrine. The furosemide-sensitive component was computed by subtracting the influx measured in the presence of the
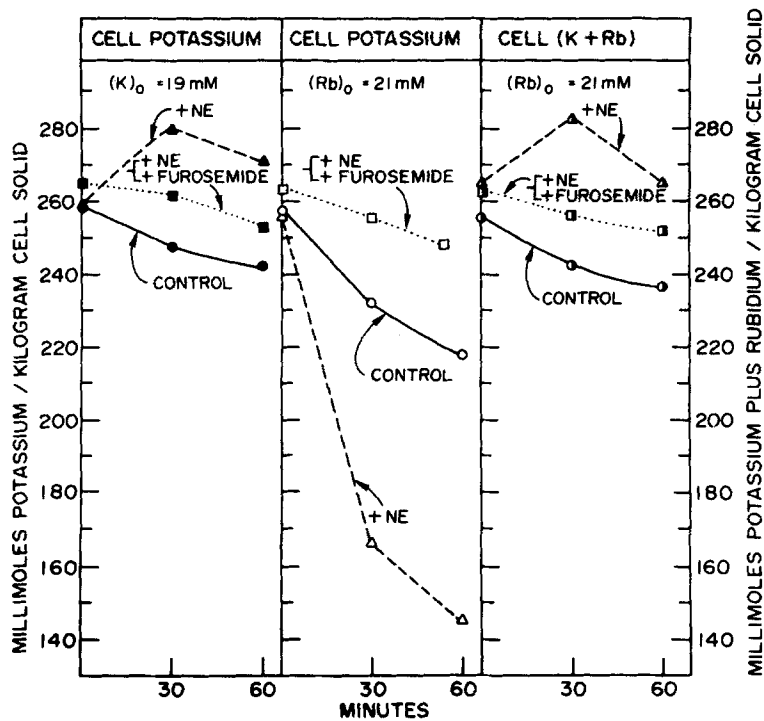

FIGURE 6. Effect on K_c and $[K_c + Rb_c]$ of substituting $[Rb]_o$ for $[K]_o$ in the presence and absence of norepinephrine (10^{-6} M) and furosemide (10^{-3} M). Results are from the same experiment as in Fig. 5. Actual changes in Rb_c during the incubation with $[Rb]_o$ are given in Table I.

TABLE I

EFFECTS OF NOREPINEPHRINE (10^{-6} M) AND FUROSEMIDE (10^{-3} M) ON NET RUBIDIUM UPTAKE IN ISOTONIC SOLUTIONS CONTAINING OUABAIN (10^{-4} M)

Min	Control	+Norepinephrine	Control + furosemide	+ Norepinephrine + furosemide
		mmol/kg cell solids		
0	2.4	11.5	0	0
30	9.6	117.0	1.8	1.4
60	15.3	123.0	4.0	4.4

Results are from the same experiment as illustrated by Figs. 5 and 6. $[Rb]_o$ = 21 mM.

drug from the control. The half-maximum saturation of this component was calculated to occur at 17.1 mM $[Na]_o$. The right panel shows rubidium influx simultaneously measured on the same cells. Two components of rubidium influx are inhibited by furosemide: one that is insensitive to $[Na]_o$, and another that

depends significantly on $[Na]_0$. With furosemide present, rubidium influx is unaffected by $[Na]_0$ and persists at a rate of approximately 1 mmol/kg cell solids·10 min. Even in the absence of $[Na]_0$, rubidium influx occurs at a faster rate with norepinephrine present than in incubations containing $[Na]_0$ without the hormone (compare control cells, Table III). The furosemide-sensitive com-

TABLE II

INHIBITION OF THE NOREPINEPHRINE (10^{-6} M) RESPONSE BY
FUROSEMIDE (10^{-3} M) IN THE ABSENCE OF OUABAIN

	Min	W_c	Na_c	K_c	Cl_c
		kg/kg		*mmol/kg cell solids*	
Control	0	1.498	13.7	213	145
	30	1.456	13.5	205	138
	90	1.439	12.7	200	—
+Norepinephrine	0	1.487	15.8	210	144
	30	1.604	18.0	223	171
	90	1.624	14.7	236	182
+Norepinephrine	0	1.485	15.0	212	146
+ Furosemide	30	1.463	13.0	190	146
	90	1.472	13.4	202	150

Composition of incubation solutions (mM): $[Na]_0 = 91$; $[K]_0 = 11$; $[Cl]_0 = 150$; Na-TES = 30, $pH_0 = 7.0$; 41°C, 323 mosmol.

TABLE III

EFFECTS OF NOREPINEPHRINE (10^{-6} M) AND FUROSEMIDE (10^{-3}
M) ON UNIDIRECTIONAL INFLUXES IN THE PRESENCE OF
OUABAIN (10^{-4} M)

	Furosemide	Na influx	Rb influx
		mmol/kg cell solids·10 min	
Control	0	2.8	1.6
	+	1.3	0.9
+ Norepinephrine	0	10.3	14.0
	+	2.0	1.2

Composition of incubation solutions (mM): $[Na]_0 = 120$; $[Rb]_0 = 13$; $[Cl]_0 = 163$; Mg-TES = 10; $pH_0 = 7.4$; 25°C; 323 mosmol.

ponent at zero $[Na]_0$ may represent an exchange of $[Rb]_0$ for $[K]_c$ induced by norepinephrine. To compare the components of rubidium and sodium influxes which depend on $[Na]_0$, the amount of rubidium influx at zero $[Na]_0$ (6 mmol/kg cell solid·10 min) was subtracted from each subsequent value of rubidium influx as $[Na]_0$ increases. This makes the curve pass through the origin at zero $[Na]_0$, and at the same time permits kinetic assessment of the furosemide-sensitive, $[Na]_0$-dependent component. Half-maximal saturation was computed to occur at 22 mM $[Na]_0$. This is to be compared with the half-maximal saturation of furosemide-sensitive sodium influx which occurs at 17.1 mM $[Na]_0$.

Fig. 8 shows the effect of varying $[Rb]_0$ on both sodium and rubidium influx. The furosemide-sensitive rubidium influx, illustrated in the left panel, is half-
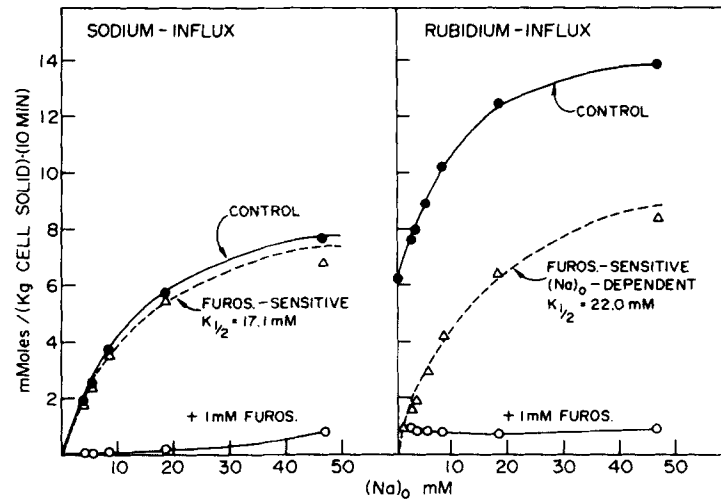
FIGURE 7.   Sodium and rubidium influxes (millimoles/kilogram cell solids · 10 min) as a function of $[Na]_o$. Fluxes were determined for the first 10 min after addition of norepinephrine (10^{-6} M). Incubation solutions contain (mM): ouabain = 0.1; $[Rb]_o$ = 17; $[Cl]_0$ = 163; Mg-TES = 10. pH_0 = 7.4; 25°C; 323 mosmol. Choline was substituted for $[Na]_o$. Solid lines connecting both open and closed circles were drawn by eye. The dashed lines are derived from a linear least-squares fit of the data on a reciprocal plot (Lineweaver-Burk).
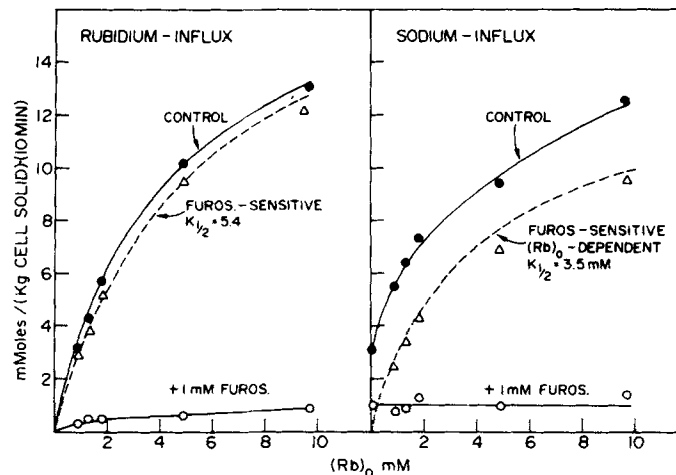

FIGURE 8.   Rubidium and sodium unidirectional influxes (millimoles/kilogram cell solids · 10 min) as a function of $[Rb]_o$. Fluxes were determined for the first 10 min after addition of norepinephrine (10^{-6} M). Incubation solutions were prepared as described in Fig. 7, except that $[Na]_o$ = 97 mM and choline was substituted for $[Rb]_o$. Solid lines connecting both open and closed circles were drawn by eye. The dashed lines are derived from a linear least-squares fit of the data on a reciprocal plot (Lineweaver-Burk).

saturated at 5.4 mM $[Rb]_o$. When $[Rb]_o$ is absent, a furosemide-sensitive sodium influx remains (right panel) which is not significantly different from that observed in the absence of norepinephrine (Table III). If this component represents a furosemide-sensitive exchange of $[Na]_o$ for $[Na]_c$ it does not appear to be affected by norepinephrine. The $[Rb]_o$-dependent furosemide-sensitive sodium influx was calculated in the same manner as described for Fig. 7. Half-maximum saturation occurs at 3.5 mM $[Rb]_o$.

Fig. 9 is a plot of the $[Na]_o$-dependent and $[Rb]_o$-dependent components of rubidium influx against the corresponding components of sodium influx. Data are from the same experiments as shown in Figs. 7 and 8. The dashed line represents what would be expected if a single rubidium ion is transported into
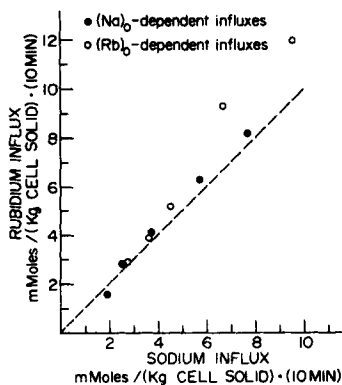

FIGURE 9. $[Na]_o$-dependent and $[Rb]_o$-dependent components of rubidium influx vs. the corresponding components of sodium influx. Results are derived from the experiments represented in Figs. 7 and 8. $[Na]_o$-dependent sodium influxes are from the control curve represented in the left panel of Fig. 7. $[Na]_o$-dependent rubidium influxes represent only that component of rubidium influx which varies with $[Na]_o$. $[Rb]_o$-dependent rubidium influxes are from the control curve in the left panel of Fig. 8. $[Rb]_o$-dependent sodium influxes represent only that component of sodium influx which varies with $[Rb]_o$.

the cell with each sodium. At fixed $[Rb]_o$, $[Na]_o$-dependent rubidium influx and $[Na]_o$-dependent sodium influx exhibit a 1:1 stoichiometry. At fixed $[Na]_o$, $[Rb]_o$-dependent rubidium influx is somewhat greater than the $[Rb]_o$-dependent sodium influx, particularly at the higher values of $[Rb]_o$.

DISCUSSION

Similarities between the response of duck red cells to norepinephrine and to hypertonicity (Schmidt and McManus, 1977a) are so numerous that they merit compilation. First, volume changes brought about by both agents are unaffected by ouabain, yet are highly dependent on both $[Na]_o$ and $[K]_o$. In both cases, swelling occurs in the presence of elevated $[K]_o$ only when $[Na]_o$ exceeds 25 mM. Besides its obvious effect on sodium uptake, $[Na]_o$ contributes to a ouabain-insensitive potassium transport as well. Lowering of $[Na]_o$ leads to net potassium loss, whereas a transient net uptake of K_c against its electrochemical gradient can

be demonstrated at high [Na]$_o$. In both cases, elevating [K]$_o$ not only reduces potassium loss, but also stimulates sodium gain. Furthermore, removing potassium entirely from the incubation medium results in a net extrusion of cell sodium against its electrochemical gradient. Isosmotic replacement of 20 mM [K]$_o$ by [Rb]$_o$ results in identical uptakes of sodium, chloride, and water in both systems. Finally, furosemide blocks all net salt and water shifts, whether uptake or loss, induced by either hypertonicity or norepinephrine. This is in contrast to β-adrenergic blockers which inhibit only the norepinephrine response (Riddick et al., 1971; Kregenow, 1971). Kregenow (1976) has recently confirmed our original report (Schmidt and McManus, 1974) on the inhibition of this system by furosemide. In addition, he has shown that this agent does not affect the increase in cyclic-AMP promoted by catecholamines. These results suggest that furosemide acts at a transport site which is activated by either hypertonicity or norepinephrine, and does not interfere with agonist binding or with activation of the adenyl cyclase system. Furosemide's effect on duck red cells resembles its inhibition of ouabain-insensitive cation transport in human red cells (Dunn, 1970, 1973; Sachs, 1971; Wiley and Cooper, 1974) which do not respond to catecholamines by significant ion movements or volume response (McManus and Schmidt, unpublished experiments).

The only significant difference between the effects of hypertonicity and norepinephrine is in the time course. While the response to norepinephrine is nearly complete in 30 min, swelling in 400 mosmol hypertonic solutions does not stop for at least 90 min (Schmidt and McManus, 1977a). Similar kinetic differences have been observed in the absence of ouabain (Kregenow, 1971, 1973). Although the same transport mechanism appears to be involved in both cases, net salt movements are approximately three times faster in isotonic solutions containing norepinephrine.

A simple scheme describing the stimulation of sodium plus potassium transport by hypertonicity or norepinephrine can be written as follows:

$$X_0 + n\{Na\}_o + m\{K\}_o \rightleftharpoons n\{Na\}-X-m\{K\} \rightleftharpoons X_c + n\{Na\}_c + m\{K\}_c,$$
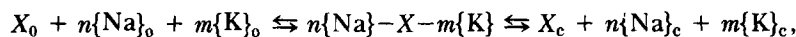
where X represents a hypothetical membrane molecule possessing a minimum of two binding sites, one for sodium and one for potassium. The letters n and m represent stoichiometric numbers. In this case, the brackets denote the individual molecules or ions rather than concentrations or amounts. Neither the sequence, relative affinity, nor stoichiometry of cation binding is specified. It is assumed that the mobility of the carrier in the membrane phase is the same whether it is loaded or empty. In this way, net transport becomes feasible since the unloaded carrier can return at the same rate as it crosses, when loaded, in the direction of net uptake or loss. Furthermore, the system is uncoupled in the sense that net movement is not dependent on the loading of carrier for the return trip. Thus, it is possible to obtain net sodium plus potassium efflux in the absence of [K]$_o$ (Fig. 4). In the final analysis, the direction of net transport becomes a function of the respective gradients of both sodium and potassium across the membrane with due regard for the effect of the membrane potential on ion movements. A thermodynamic approach to this will be developed in

detail after we have demonstrated the role of membrane potential in the next paper in this series (Schmidt and McManus, 1977b).

This model as presented here has several characteristics which can be tested with the data reported thus far. For example, at constant $[K]_o$, both sodium and potassium influx will depend on $[Na]_o$. Similarly, at fixed $[Na]_o$, both fluxes will depend on $[K]_o$. The model further predicts that half-maximum influxes of both ions will occur at similar $[Na]_o$ with constant $[K]_o$, and at similar $[K]_o$ at constant $[Na]_o$. A kinetic analysis of the norepinephrine-stimulated unidirectional influxes generally supports this cotransport scheme. Whereas 5.4 mM $[Rb]_o$ half-saturates furosemide-sensitive rubidium influx, 3.5 mM $[Rb]_o$ half-saturates the co-ion component of sodium influx (Fig. 8). Also, a similar $[Na]_o$ half-saturates furosemide-sensitive, sodium influx (17 mM $[Na]_o$) and sodium-dependent, rubidium influx (22 mM $[Na]_o$) (Fig. 7). Similar results were reported by Wiley and Cooper (1974) for an apparent cotransport of sodium plus potassium into human red cells. They estimated that 22 mM $[Na]_o$ is required for half-maximal stimulation of $[Na]_o$-dependent, furosemide-sensitive potassium influx. On the other hand, 7 mM $[K]_o$ half-saturates a $[K]_o$-dependent, furosemide-sensitive sodium influx.

For duck cells, two components of rubidium influx appear to be stimulated by norepinephrine and inhibited by furosemide. One is sensitive to $[Na]_o$ and the other is not. When $[Na]_o$-dependent rubidium influx is plotted against $[Na]_o$-dependent sodium influx, the slope is close to one (Fig. 9), suggesting that one molecule of rubidium enters with each molecule of sodium.

The second component is seen when sodium is omitted from the incubation (Fig. 7). Two interpretations of this furosemide-sensitive but sodium-independent rubidium influx can be offered. First, it may represent the unidirectional component of a rubidium-potassium exchange pathway which normally carries only potassium. Although this is also catecholamine activated, it could be separate from the cotransport system. The observation that net potassium efflux in the presence of $[Rb]_o$ is greater than in the absence of either $[Rb]_o$ or $[K]_o$ (Fig. 6) is consistent with an exchange mechanism of this type. However, this exchange could also occur by way of the same system responsible for cotransport. For example, in the absence of $[Na]_o$, rubidium could enter the cell on a half-loaded carrier or by occupying the sodium site as well. The data suggest that the former possibility is more likely. If $[Rb]_o$ could compete for the empty sodium site in the absence of $[Na]_o$, one would expect rubidium influx to decrease as $[Na]_o$ is added, making fewer sites available for rubidium. Just the opposite is observed (Fig. 7). Exchange could then take place by return of the carrier complexed only with potassium, or with potassium plus sodium.

Although no direct demonstration of cotransport of sodium plus potassium out of the cell was sought in this investigation, the furosemide-sensitive net loss of both ions into $[K]_o$-free solutions (Fig. 4) readily fits this concept. In light of the report by Wiley and Cooper (1974) that furosemide also inhibits ouabain-insensitive sodium plus potassium influxes in human red cells, it is possible that the ouabain-insensitive, ethacrynic acid-inhibited "pump II" of Hoffman and Kregenow (1966) could be a manifestation of outward cotransport. The system

in duck cells is obviously much more active, particularly when stimulated by norepinephrine. For example, it is possible to demonstrate ouabain-insensitive net sodium extrusion into high $[Na]_o$, $[K]_o$-free solutions without lowering the sodium electrochemical gradient, as was found necessary in human red cells (Sachs, 1971). More recent work in our laboratory (manuscript in preparation) has demonstrated that in the absence of both $[Na]_o$ and $[K]_o$, net sodium loss increases as $[K]_c$ is elevated. Furthermore, with fixed $[Na]_c$, net potassium loss approximates net sodium loss over a wide range of $[K]_c$. This coupled net efflux is markedly stimulated by norepinephrine and inhibited by furosemide. A similar pathway might account for ouabain-insensitive sodium extrusion, observed in mammalian kidney slices, which is inhibited by the same diuretics (for a review, see Whittembury and Proverbio, 1976).

While the unidirectional influxes measured at 25°C in duck red cells support the hypothesis that sodium and potassium enter the cell together (Figs. 7-9), it must be emphasized that kinetic parameters derived from these data cannot be used to predict net movements at 41°C. At 25°C, co-ion unidirectional influxes saturate at approximately 10 mM $[Rb]_o$ and 45 mM $[Na]_o$, respectively. From this we can state only that, under these conditions, a point is reached where influx becomes independent of external cation concentration. This could be secondary to the saturation of available binding sites or to a limitation of movement of free and loaded carrier complexes across the membrane. Raising the incubation temperature to 41°C may increase the fluidity of the membrane, causing more available binding sites or a higher rate of translocation or both. Our data cannot distinguish between these possibilities. Such changes might account for the finding at 41°C that *net* influxes of both sodium and potassium continue to be stimulated by $[K]_o$ above 10 mM (Fig. 3) and by $[Na]_o$ above 45 mM (Fig. 2). These concentrations are sufficient to saturate unidirectional influxes at 25°C. The direction of net movements can, however, be predicted on the basis of the thermodynamic driving forces acting on the cotransport system, as we will show in our next paper (Schmidt and McManus, 1977 *b*).

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