Catecholamine-stimulated Ion Transport in Duck Red Cells

Gradient Effects in Electrically Neutral [Na + K + 2Cl] Co-Transport

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ABSTRACT The transient increase in cation permeability observed in duck red cells incubated with norepinephrine has been shown to be a linked, bidirectional, co-transport of sodium plus potassium. This pathway, sensitive to loop diuretics such as furosemide, was found to have a [Na + K] stoichiometry of 1:1 under all conditions tested. Net sodium efflux was inhibited by increasing external potassium, and net potassium efflux was inhibited by increasing external sodium. Thus, the movement of either cation is coupled to, and can be driven by, the gradient of its co-ion. There is no evidence of *trans* stimulation of co-transport by either cation. The system also has a specific anion requirement satisfied only by chloride or bromide. Shifting the membrane potential by varying either external chloride (at constant internal chloride) or external potassium (at constant internal potassium in the presence of valinomycin and DIDS [4,4′-diisothiocyano-2,2′-disulfonic acid stilbene]), has no effect on norepinephrine-stimulated net sodium transport. Thus, this co-transport system is unaffected by membrane potential and is therefore electrically neutral. Finally, under the latter conditions—when E_m was held constant near E_K and chloride was not at equilibrium—net sodium extrusion against a substantial electrochemical gradient could be produced by lowering external chloride at high internal concentrations, thereby demonstrating that the anion gradient can also drive co-transport. We conclude, therefore, that chloride participates directly in the co-transport of [Na + K + 2Cl].

INTRODUCTION

Beta-adrenergic catecholamines, including norepinephrine, epinephrine, and isoproterenol, promote an increase in ion transport across the duck red cell

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membrane (for review, see McManus and Schmidt, 1978*a*). The cells respond with a transient change in volume and ion content. Although the ion transport pathway stimulated by these agents is insensitive to ouabain (Riddick, 1969; Kregenow, 1973; Schmidt and McManus, 1977*b*), it is blocked by the diuretic agent furosemide (Schmidt and McManus, 1974). In previous reports from this laboratory, it was shown that ion movements activated by norepinephrine, as well as by cell shrinkage, represent a co-transport of [Na + K] across the plasma membrane (Schmidt and McManus, 1974, 1977*a*, *b*, *c*). Recent evidence suggests that this sytem also has a specific anion requirement satisfied only by chloride or bromide (Kregenow and Caryk, 1979).

This paper reports a determination of the stoichiometry of [Na + K] co-transport under a variety of conditions, as well as an investigation of the effects of ion gradients, anion substitution, and membrane potential on norepinephrine-stimulated ion movements across the duck red cell membrane.

An important prediction of the co-transport model is that coupling between solute movements allows one ion to drive the net uphill movement of a co-ion, but only if the driver ion gradient has a higher potential. By suitable manipulation of the system we have been able to meet this criterion for both sodium and potassium, and have shown as well that chloride is actually co-transported with the cations in an electrically neutral complex consisting of [Na + K + 2Cl]. When the membrane potential is altered with the potassium ionophore valinomycin, and anion permeability is reduced with DIDS, the chloride gradient can be set away from equilibrium. Under these conditions we have shown that the anion can also function as driver ion for the co-transport system.

Preliminary reports of some of these results have appeared previously (McManus and Schmidt, 1978*b*; Haas and McManus, 1981).

MATERIALS AND METHODS

Preparation of Cells

Fresh, heparinized blood was obtained from White Pekin ducks as previously described (Schmidt and McManus, 1977*a*). After centrifugation and removal of plasma and buffy coat, the cells were washed three times in 5 vol of ice-cold, isotonic (323 mosmol) choline chloride or tetramethylammonium (TMA) chloride. Where indicated, preincubation was then carried out for 90 min at 41°C, 10% hematocrit, in a standard medium containing 9 mM KCl, 1.0 mM KH_2PO_4, 20 mM TMA-TES (*N*-Tris [hydroxymethyl] methyl-2-aminoethane-sulfonic acid [Sigma Chemical Co., St. Louis MO], pH adjusted to 7.4 at 41°C with TMA hydroxide), 10 mM glucose, and sufficient NaCl to adjust osmolality to 323 mosmol. When elevation of cell sodium content, Na_c,[1] was desired, 0.1 mM ouabain was added. TES buffers were prepared as previously described (Schmidt and McManus, 1977*a*).

[1] As in previous papers (Schmidt and McManus, 1977*a*, *b*, *c*), Na_c, K_c, etc. represent the amount of a substance in the cells (millimoles/kilogram cell solid), whereas $[Na]_c$, $[K]_c$, etc. refer to the concentration (millimoles/liter cell water). $[Na]_o$, $[K]_o$, etc. represent the concentration (millimolar) in the external medium.

Alteration of Cell Ionic Composition

NYSTATIN A modification of the technique developed by Cass and Dalmark (1973) was used to alter cell sodium and potassium contents. Fresh, washed cells were suspended (2% hematocrit) in loading solutions containing 150 mM total salt (NaCl, KCl, and chlorine chloride in varying combinations) plus 60 mM sucrose. Nystatin (Squibb, New York) was added from a stock solution (20 mg/ml in dimethyl sulfoxide) to a final concentration of 40 μg/ml, and the cells were incubated for 30 min at 4°C. This procedure was then repeated once more using the same loading solutions without nystatin. To restore the membrane to its normal cation impermeability, nystatin was eluted by incubation for 5 min at 25°C in 50 vol of loading solution fortified with 0.5% bovine serum albumin (fraction V), and 0.1 mM ouabain. Fraction V albumin, as commercially supplied (Sigma Chemical Co.), contained sodium (0.13 mmol/g dry weight). This contamination was reduced to <0.002 mmol/g dry weight by treating a 10% solution with cation exchange resin (AG 50W-X; Bio-Rad Laboratories, Richmond, CA). The acidic solution was then back-titrated to pH 7.4 (25°C) with TMA hydroxide. The elution procedure was repeated a total of five times by alternate centrifugation, also at 25°C, and resuspension. Finally, the cells were resuspended at 4°C in solutions of the same ionic composition as those used for loading and elution. Immediately before the test incubation, they were given a final wash in ice-cold, isotonic choline chloride.

Nystatin does not increase permeability of the plasma membrane to choline to the same degree as to sodium and potassium. Therefore, cells loaded in solutions containing high levels of choline, and low [Na + K], tend to remain somewhat shrunken after elution and resuspension in isotonic media. By itself, however, cell shrinkage stimulates co-transport (Schmidt and McManus, 1977*a*), and therefore an elevated level of activity is to be expected in the absence of catecholamine. Nevertheless, the magnitude of co-transport observed when norepinephrine is added to moderately shrunken cells is comparable to that seen in cells of normal volume (M. Haas and T. J. McManus, unpublished experiments).

ANION SUBSTITUTION Replacement of cell chloride with other mobile, monovalent anions (X = bromide, nitrate, acetate, or methylsulfate) was accomplished by overnight incubation at 4°C, 2% hematocrit, in solutions identical to the standard preincubation medium, except that chloride was replaced by X. A second 2-h preincubation followed using the same solutions at 41°C. The pH of the TES buffers was adjusted to be 7.4 at the different temperatures. In this manner, cell chloride concentration was reduced to <0.1 mM. A similar procedure was followed in experiments where cell chloride was only partially replaced. In this instance, the preincubation solutions contained appropriate mixtures of chloride and X. When elevated cell sodium was also desired, ouabain (0.1 mM) was added to the second (41°C) preincubation.

Use of Valinomycin to Shift Membrane Potential

The potassium ionophore, valinomycin, (Sigma Chemical Co. or Calbiochem-Behring Corp., San Diego, CA) was used to increase potassium conductance, and thus alter the membrane potential (E_m) according to the constant-field equation (Goldman, 1943; Hodgkin and Katz, 1949):

$$E_m = \frac{-RT}{F} \ln \frac{[Cl]_o + B[K]_c}{[Cl]_c + B[K]_o} \quad (1)$$

where $B = P_K/P_{Cl}$. When the valinomycin concentration is high enough, P_K is

increased manyfold over P_{Cl} (Hunter, 1977; Knauf et al., 1977), and E_m approaches E_K, the potassium equilibrium potential. Under these conditions, $[Cl]_o$ can be varied at constant $[Cl]_c$ without a significant effect on E_m. We estimated the ratio P_K/P_{Cl} in the presence of valinomycin by measuring net efflux of potassium from resting cells, in the absence of catecholamine, into isotonic sodium or rubidium chloride solutions. If sufficient valinomycin is present, the rate of potassium efflux into NaCl or (NaX) is limited only by the permeability of the following anion (Hunter, 1977). Thus, net efflux gives an estimate of P_{Cl} (or P_X). If the external solution contains only RbCl, however, the ionophore promotes a potassium-rubidium exchange. In this case, potassium efflux is limited by P_K, because valinomycin selects rubidium over potassium (Andreoli et al., 1967). This approach, therefore, offers an estimation of P_K. Table I shows that in the presence of 2×10^{-6} M valinomycin, P_K exceeds P_{Cl} by a factor of approximately four. Exposure to 10^{-5} M DIDS (Sigma Chemical Co.), which

TABLE I

ESTIMATION OF P_K/P_{Cl} IN THE PRESENCE OF 2×10^{-6} M VALINOMYCIN

DIDS 10^{-5} M	Potassium efflux		$P_K(val)/P_{Cl}$
	RbCl medium	NaCl medium	
	mmol/kg cell solid · 2 min		
0	195.8	51.9	3.8
+	198.4±2.7	26.4±0.5	7.5±0.2

Data represent net effluxes of potassium into potassium-free NaCl and RbCl media. Results are averages of separate determinations: two for control cells, and four for DIDS-treated cells. Data for DIDS-treated cells are shown ± SEM. The ratio of valinomycin-induced potassium permeability, $P_K(val)$, to chloride permeability, P_{Cl}, was calculated from (K efflux into RbCl)/(K efflux into NaCl). Cells were preincubated with or without 10^{-5} M DIDS for 90 min in the standard preincubation medium. After two washes with ice-cold, isotonic TMA chloride, a 2-min test incubation in the presence of valinomycin was performed at 3% hematocrit, 41°C. Only cells pretreated with DIDS were exposed to DIDS in the test incubation. Valinomycin was added from a 0.67-mM stock solution (in absolute ethanol) <0.2 min after withdrawal of the initial sample. The time of ionophore addition marked the beginning of the test incubation.

lowers P_{Cl} in red cells (Knauf et al., 1977), results in a further increase in P_K/P_{Cl}. This effect is caused entirely by lowering P_{Cl}, because DIDS had no effect on P_K. In the presence of 2×10^{-6} M valinomycin plus DIDS, P_K/P_{Cl} is raised to 7.5 (range 7.2–8.1 in four separate determinations). Using this value to calculate E_m according to Eq. 1, it is apparent that $[Cl]_o$ can be varied from 1 to 150 mM with only a 2.8-mV change in E_m, if all other parameters are held constant.

To vary $[Cl]_o$ while holding $[Cl]_c$ constant, it is necessary to inhibit anion exchange. Without such inhibition, any alteration of $[Cl]_o$ will lead to a rapid exchange of internal chloride with the external replacement anion, or with hydroxyl ions (Jennings, 1978), leading to changes in intracellular pH. With DIDS present, however, we have observed a maximum change in cell chloride of only 12 mM/liter cell water, from 96 to 84, after 10 min of incubation in the presence of 1 mM $[Cl]_o$, using methylsulfate as the other external anion. Although no direct measurements of cell pH were made, cell water contents were not significantly altered (mean value = 1.476

± 0.004 kg H_2O/kg cell solid, $n = 16$) when $[Cl]_o$ was varied from 1 to 150 mM, which suggests cell pH was also unaffected.

Preparation of Reagents and Test Incubation Media

Before test incubations, the cells were washed three times in ice-cold, isotonic choline or TMA salt solutions. Unless otherwise noted, test incubation media contained TMA-TES (20 mM), glucose (10 mM), and ouabain (0.1 mM). Concentrations of sodium, potassium, and rubidium salts used are noted in the legends to the tables and figures. All media were pH 7.4 at 41°C. Osmolality was adjusted to 323 mosmol by addition of choline or TMA salts. Unless specified otherwise, the incubation hematocrit was 3%. Procedures for making stock solutions of ouabain and furosemide have been described in detail elsewhere (Schmidt and McManus, 1977*a*). A fresh aqueous solution of 10^{-3} M norepinephrine (levarterenol bitartrate; Winthrop Laboratories, New York) was prepared never more than 3 min before each test incubation to minimize oxidative breakdown of the catecholamine. Zero time of the test incubation was marked by addition to the cell suspension of sufficient hormone to yield a final concentration of 10^{-6} M. Whenever used, DIDS was added from a 1-mM aqueous stock solution to give a final concentration of 10^{-5} M.

The chloride salts of TMA and choline were both recrystallized routinely from absolute ethanol and ligroine, respectively, and stored in a sealed container at −20°C to reduce water uptake. TMA salts of chloride, bromide, nitrate, acetate, and methylsulfate were obtained from Eastman Organic Chemicals, Rochester, NY. Choline chloride and TMA hydroxide were obtained from Sigma Chemical Co. Sodium and potassium methylsulfate were obtained from ICN Pharmaceuticals, Plainview, NY. Solutions of TMA nitrate were filtrated routinely through activated charcoal to remove yellow discoloration, a procedure that caused no change in osmolality.

After test incubations, samples were centrifuged in specially fabricated, precooled nylon tubes, and the separated cells were analyzed for ion and water contents as previously described (Schmidt and McManus, 1977*a*).

Presentation of Data

Because cell water content changed rapidly during many of the incubations, ion contents are expressed in millimoles per kilogram cell solid and water in kilogram H_2O per kilogram cell solid. Because the rates of these ion transport processes can vary significantly between red cells from different ducks, we have chosen to present results from representative experiments. Each experiment, however, has been replicated one or more times with cells from different animals. Qualitatively, the results have been totally consistent. For the purposes of elucidating stoichiometric relationships between co-transported ions, data are usually presented in terms of net changes of ion content over a given time interval. In each case, however, the initial concentrations of ions and the amount of water in the cells is given in the legends.

RESULTS

Ion Gradient Effects

In a previous paper (Schmidt and McManus, 1977*b*), it was shown that norepinephrine, when added to duck red cells, stimulated an external sodium-dependent potassium influx, as well as an external potassium-dependent sodium influx. Furosemide inhibited both of these fluxes. With rubidium

substituting for external potassium in the presence of ouabain, a stoichiometry of 1:1 for furosemide-sensitive [Na + Rb] uptake was demonstrated. This co-transport system is reversible, as shown by the marked stimulation of net sodium efflux produced by removal of external potassium (Schmidt and McManus, 1977*b*).

The stoichiometry and internal ion dependence of norepinephrine-stimulated co-transport efflux was investigated by incubating cells in media containing neither sodium nor potassium (Fig. 1). Intracellular potassium concentration was varied between 0.3 and 101 mM by preincubation with nystatin according to the technique of Cass and Dalmark (1973). $[Na]_c$ was held constant at ~60 mM. Choline made up the balance of intracellular cations. In Fig. 1, net losses of sodium and potassium from cells in a choline medium are plotted against initial value of $[K]_c$. Despite a substantial, outwardly directed, sodium gradient, there was very little loss of that ion when $[K]_c$ was close to zero. As internal potassium increased, so did the net efflux of [Na + K]. At each level of $[K]_c$, the net movements of sodium and potassium were approximately equal in both control and norepinephrine-stimulated cells. Furosemide blocked net cation efflux almost completely, although a small drug-insensitive component can be detected.

Fig. 2 represents an experiment in which a similar procedure was used to vary $[Na]_c$ at a constant $[K]_c$ of ~50 mM. Again, very little cation loss was observed when $[Na]_c$ was close to zero, despite a large, outwardly directed gradient for potassium. Increasing $[Na]_c$ promoted a net efflux of both cations, which once more demonstrated a stoichiometry of 1:1, both in the presence and absence of norepinephrine, over the entire range of $[Na]_c$. Norepinephrine also stimulated, and furosemide inhibited, this $[Na]_c$-dependent cation flux.

In each of the experiments depicted in Figs. 1 and 2, net loss of chloride was equivalent to that of [Na + K] during the same interval (data not shown). Analysis of the regression of net chloride loss on net [Na + K] loss yielded the following equation: $\Delta Cl_c = (0.90 \pm 0.06)\ (\Delta K_c + \Delta Na_c) - (4.8 \pm 2.0)$. The regression coefficient was 0.96. The fact that equivalent net losses of cation and anion occurred in the absence of external sodium and potassium demonstrates that co-transport has no *trans* requirement, i.e., that efflux does not have to be coupled to influx.

Another important property of the system is illustrated in Fig. 3. As shown above, cells stimulated by catecholamine release approximately equal amounts of sodium and potassium into a medium free of these ions. When, however, 100 mM potassium is added to the external solution, replacing an equivalent amount of choline, net sodium loss is reduced despite an unchanged sodium gradient. Similarly, when sodium is added to the medium, potassium efflux is reduced. Clearly, net sodium efflux depends on the potassium gradient, and conversely, net potassium efflux depends on the sodium gradient.

The effect shown in the left panel of Fig. 3 was examined more closely by repeating the experiment with rubidium instead of potassium in the external medium (Table II). It is apparent that rubidium uptake was stimulated by the catecholamine in ouabain-treated cells even in a sodium-free medium, which confirms previous observations (Schmidt and McManus, 1977*b*). Note

that rubidium uptake [ΔRb_c] was approximately equal in each case to [$\Delta K_c - \Delta Na_c$]. The latter expression represents net loss of potassium in excess of that which can be accounted for by 1:1 co-transport with sodium. The fact that it was equivalent to the rubidium taken up from a sodium-free medium also confirms our previous report that catecholamines stimulate a sodium-

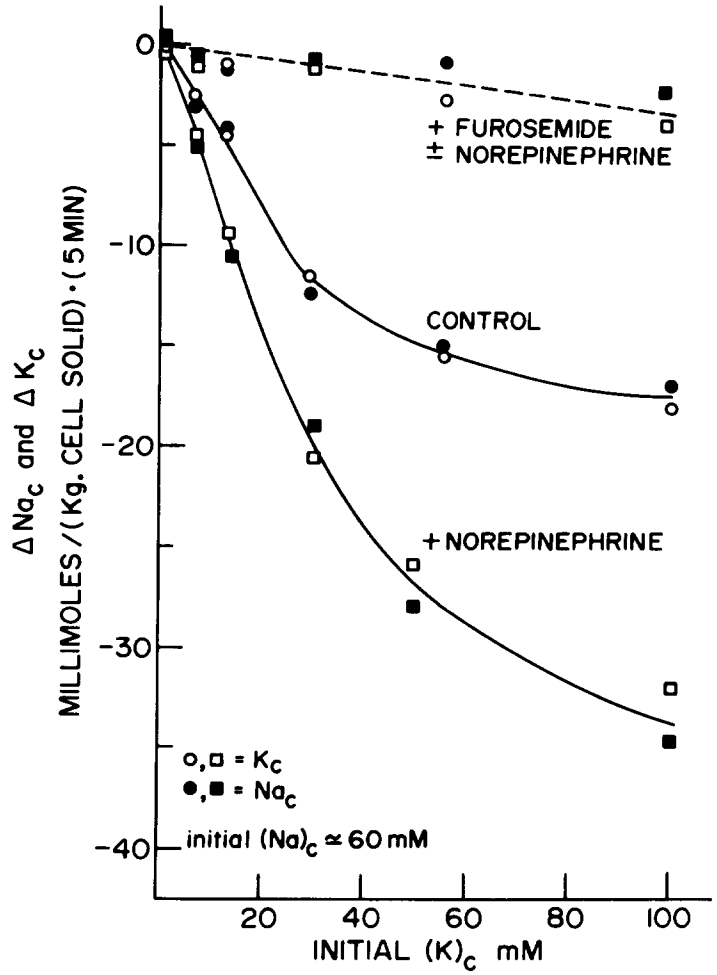

FIGURE 1. Effect of internal potassium on net [Na + K] efflux. Co-ion gradients are maximized by incubating cells in a medium free of sodium and potassium. Cells, preloaded by the nystatin method to contain $[Na]_c = 60.5 \pm 1.7$ (SEM, $n = 24$) mM/liter cell water, were incubated at 41°C, 3% hematocrit, in a medium containing 10 mM glucose, 10 mM Mg-TES, 0.1 mM ouabain, and enough choline chloride to maintain osmolality at 323 mosmol. Co-transport was stimulated by adding 10^{-6} M norepinephrine. Furosemide concentration was 1 mM. Potassium was varied in the nystatin loading solutions, using choline chloride as osmotic replacement, to yield different levels of $[K]_c$ as noted on the abscissa. Initial cell water was 1.268 ± 0.031 kg H_2O/kg cell solid. Identical results were obtained in the absence of ouabain.

independent, 1:1 K/Rb exchange in these cells (McManus and Haas, 1981). In addition, as $[Rb]_o$ was increased, the amount of sodium lost from the cells decreased—an effect similar to that observed when raising $[K]_o$ (Fig. 3). There was also a progressive decrease in $[\Delta K_c + \Delta Rb_c]$ as $[Rb]_o$ increased. This latter

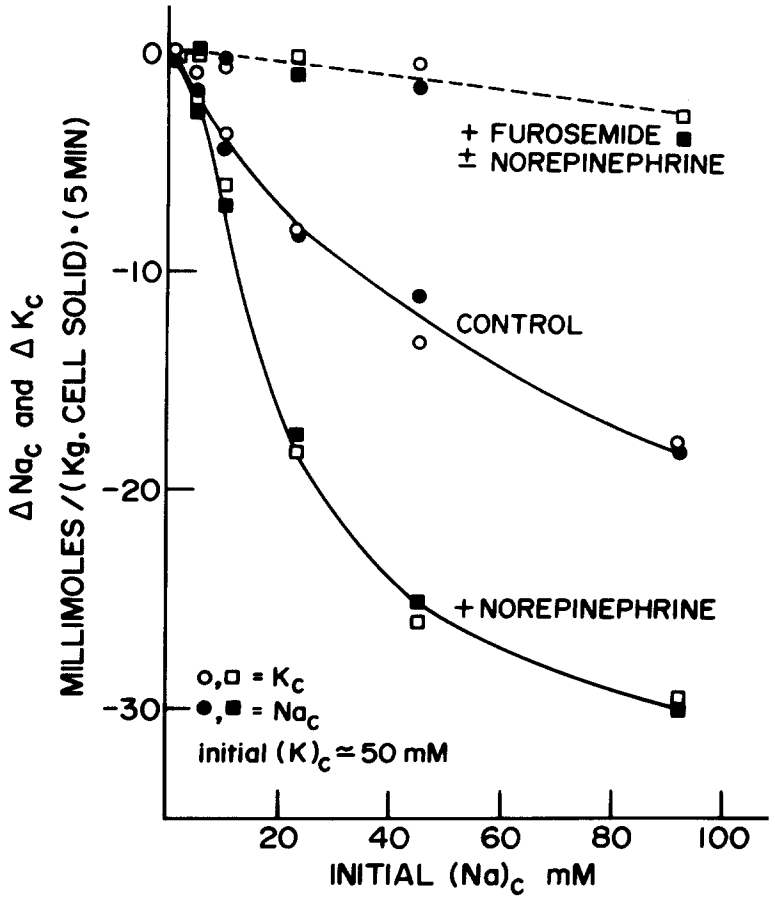

FIGURE 2. Effect of internal sodium on net [Na + K] efflux. As in Fig. 1, the incubation medium contained neither sodium nor potassium. Cells were preloaded by the nystatin technique to contain $[K]_c = 50.2 \pm 1.1$ (SEM, $n = 24$) mM/liter cell water. Sodium was varied in the nystatin loading solutions, using choline chloride as osmotic replacement, to yield different levels of $[Na]_c$ as noted on the abscissa. All other incubation conditions were identical to those in Fig. 1. Initial cell water was 1.393 ± 0.031 kg H_2O/kg cell solid.

sum is equivalent to the decrease in total cell potassium that would be expected if potassium, rather than rubidium, was present in the external medium (see, for example, Fig. 6 of Schmidt and McManus, 1977*b*). In each case, $[\Delta K_c + \Delta Rb_c]$ was approximately equal to $[\Delta Na_c]$. Thus, increasing $[Rb]_o$ reduces net [Na + K] efflux via the co-transport pathway, presumably

by lowering the net force driving the co-ions in the efflux direction (Schmidt and McManus, 1977*c*).

Increasing $[Rb]_o$, however, also promotes K/Rb exchange. This effect is best explained by a counter-transport process with a relatively low affinity for $[Rb]_o$. At first, co-transport and exchange appeared to be independent, because stimulating inward co-transport by raising $[Na]_o$ had no effect on

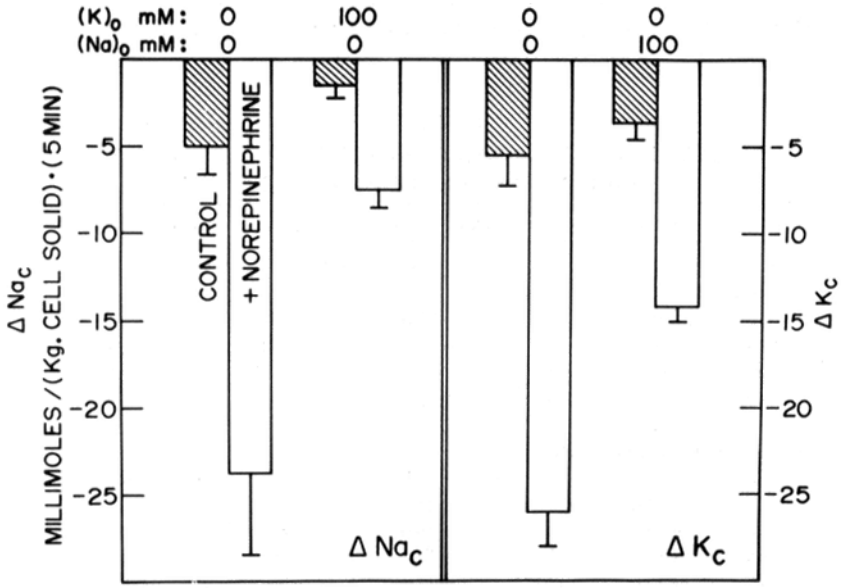

FIGURE 3. Inhibition of net sodium efflux by raising external potassium (left panel), and net potassium efflux by raising external sodium (right panel). Hatched bars represent control values, whereas clear bars are results obtained in the presence of 10^{-6} M norepinephrine. Initial cell sodium was increased by preincubating for 3 h at 41°C, 10% hematocrit, in the presence of 0.1 mM ouabain. The preincubation medium contained 155 mM Na, 2 mM K, 10 mM glucose, and 10 mM Na-TES buffer (pH 7.4 at 41°C). After washing three times in isotonic choline chloride, cells were test incubated at 41°C, 5% hematocrit. Choline replaced sodium and potassium, maintaining medium osmolality at 323 mosmol. Initially, the cells contained (millimoles per liter cell water): $[Na]_c = 29.3 \pm 0.4$ (SEM, $n = 24$), $[K]_c = 135 \pm 2$. Initial cell water was 1.324 ± 0.009 kg H_2O/kg cell solid. Results shown represent means of three separate experiments, ± SEM (error bars).

K/Rb exchange (McManus and Haas, 1981). Further studies have led us to conclude that these two processes may, in fact, represent different modes of a single, highly complex transport system. For example, they manifest the same degree of sensitivity to the high-affinity diuretic bumetanide. Also, the inhibitory potency of this agent is enhanced by increasing $[Rb]_o$ to the same extent for both co-transport and exchange (Haas and McManus, 1982).

In any tightly coupled co-transport system, transfer of energy between solute species may result in net movement of the "driven" solute against its gradient at the expense of the exergonic, downhill flow of the "driver" solute. Thus, the conservative movement of one species is coupled to the dissipative movement of one of its co-transported companions. In principle, any one of the solutes involved in the co-transport complex may serve as the "driver" solute. A necessary requirement for such a "secondary active" transport process is that the gradient of the "driver" solute be larger and of the opposite sign to that of the "driven" solute. The experiment shown in Fig. 4 illustrates

TABLE II

EFFECT OF EXTERNAL RUBIDIUM ON NET CATION MOVEMENTS IN SODIUM-FREE MEDIA

$[Rb]_o$	ΔRb_c	ΔK_c	$[\Delta K_c + \Delta Rb_c]$	ΔNa_c	$[\Delta K_c - \Delta Na_c]$
mM			*mmol/kg cell solid · 5 min*		
CONTROL					
0	0	−11	−11.0	−10.0	−1.0
10	+0.5	−12	−11.5	−6.0	−6.0
20	+1.6	−1	+0.6	−0.6	−0.4
40	+1.9	−3	−1.1	−0.2	−2.8
+ NOREPINEPHRINE (10^{-6} M)					
0	0	−33	−33.0	−34	+1
5	+8.8	−37	−28.2	−29	−8
10	+15.0	−41	−26.0	−25	−16
15	+18.5	−47	−28.5	−26	−21
20	+23.0	−45	−22.0	−23	−22
30	+28.5	−48	−19.5	−22	−26
40	+31.2	−45	−13.8	−18	−27

To raise $[Na]_c$, cells were preincubated for 3 h in the presence of ouabain and high external sodium, as described in the legend to Fig. 3. They were then washed three times in isotonic choline chloride, and test incubated at 5% hematocrit, 41°C, in sodium-free media with varying $[Rb]_o$. Initially, the cells contained (millimoles per liter cell water): $[Na]_c$ = 68.4 ± 1.5 (SEM, n = 11), $[K]_c$ = 130 ± 1. Initial cell water was 1.477 ± 0.004 kg H_2O/kg cell solid. Positive signs indicate net uptake and negative signs indicate net loss.

this characteristic of catecholamine-stimulated co-transport. Cells were preloaded by the nystatin method to contain constant $[K]_c$, but varying $[Na]_c$, as in the experiment shown in Fig. 2. In this case, however, external potassium was raised to 94 mM, thus creating an inwardly directed gradient for that ion, favoring its uptake by the cells. Indeed, when furosemide was added to inhibit co-transport, they did show a slight uptake of potassium. In the absence of furosemide, however, net potassium extrusion occurred at all levels of $[Na]_c$ above 10 mM. The amount of potassium transported in this paradoxical fashion—out of the cell, but against its gradient—increased as expected with

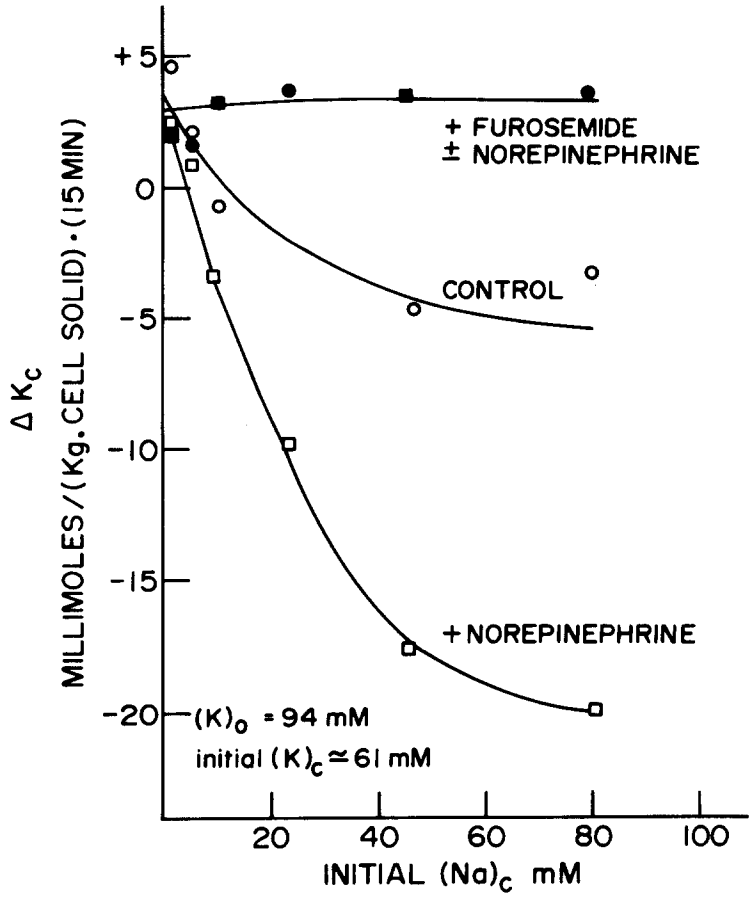

FIGURE 4. Net efflux of potassium against its electrochemical gradient driven by an outwardly directed sodium gradient. Cells were preloaded by the nystatin technique to contain $[K]_c$ = 61.3 ± 0.1 (SEM, n = 18) mM/liter cell water. $[Na]_c$ was varied as indicated on the abscissa. This experiment is identical in design to that shown in Fig. 2, except that the incubation medium contained $[K]_o$ = 94 mM, and incubation time was extended to 15 min. As before, incubations were carried out 41°C, 3% hematocrit, in the presence of 0.1 mM ouabain. Co-transport was stimulated by adding 10^{-6} M norepinephrine. Furosemide concentration was 1 mM. Initial cell water was 1.454 ± 0.032 kg H_2O/kg cell solid.

increasing $[Na]_c$ and was greatly stimulated in the presence of norepinephrine. In previously reported experiments (Schmidt and McManus, 1977*b*), it was further shown that fresh cells incubated with norepinephrine in the presence of ouabain could extrude sodium against its gradient, provided the downhill efflux of potassium driving this transport was first maximized by reducing $[K]_o$ to zero.

Effect of Anion Substitution

The role of chloride in [Na + K] co-transport was studied by first preincubating cells in media containing either chloride or a substitute anion to which the membrane is also permeable. Once the internal and external chloride was thus replaced, the cells were incubated as above in a medium free of sodium and potassium, using TMA as the replacement cation. The anion present was either chloride or the substitute used in the preincubation. Table III shows results from a typical experiment. A significant, furosemide-sensitive net loss of sodium, representing [Na + K] co-transport, occurred only in the presence of chloride or bromide. Using the valinomycin technique of Hunter (1977),

TABLE III

EFFECT OF ANION SUBSTITUTION ON NET SODIUM EFFLUX

Anion	P_{anion}/P_{Cl}	1 mM Furosemide	$-\Delta Na_c$
			mmol/kg cell solid·5 min
Cl	1.0	0	9.6
		+	1.2
Br	1.2	0	8.3
		+	1.2
NO_3	2.5	0	0.9
		+	0.6
$MeSO_4$	0.6	0	0.8
		+	1.0

Cells were preincubated and washed in appropriate buffers, so that the only mobile anion present was one of the following: Cl, Br, NO_3, or methylsulfate ($MeSO_4$). The test incubation was carried out at 3% hematocrit, 41°C, in a TMA medium free of sodium and potassium, with the same anion used in the preincubation. Norepinephrine (10^{-6} M) and ouabain (0.1 mM) were added to every flask. Initially, the cells contained (millimoles per liter cell water): $[Na]_c = 9.6 \pm 0.4$ (SEM, $n = 8$), $[K]_c = 165 \pm 2$. Initial cell water was 1.423 ± 0.019 kg H_2O/kg cell solid. The anion permeability ratio (P_{anion}/P_{Cl}) was estimated from the net efflux of potassium in the presence of valinomycin (2×10^{-6} M) into media containing only NaX, where X = Cl, Br, NO_3, or $MeSO_4$ (see Table I).

we were able to demonstrate that duck red cells are readily permeable to both nitrate and methylsulfate. Nevertheless, these anions did not support furosemide-sensitive net sodium efflux.

In a previous paper (Schmidt and McManus, 1977*c*), it was reported that lowering chloride in both the cell and medium to the level of ~20 mM by replacement with acetate resulted in norepinephrine-stimulated uptake of [Na + K] apparently independent of chloride. This observation suggested that co-transport has no specific anion requirement, and either acetate or chloride could follow net [Na + K] uptake to satisfy electroneutrality. This interpretation is in conflict, however, with the results presented in Table III. To resolve this apparent discrepancy, the original experiment was repeated with

the following modifications: cells were preincubated in chloride, acetate, or nitrate solutions, as in the experiment shown in Table III, except that 20 mM chloride was added to the acetate and nitrate media, thus maintaining $[Cl]_c$ at ~20 mM. They were then reincubated for 2 h at 41°C in media of the same anion composition with one-half of them exposed to 10^{-5} M DIDS, a potent inhibitor of the red cell anion exchange pathway (Cabantchik and Rothstein, 1974). The final incubation media again had the same anion composition, but with the addition of enough sodium and potassium to ensure a net uptake upon activation of co-transport.

Table IV lists net changes in ion content after 10 min of exposure to

TABLE IV

EFFECT OF PARTIAL REPLACEMENT OF CHLORIDE ON THE STOICHIOMETRY OF NET CATION-CHLORIDE CO-TRANSPORT

$[Cl]_o$	Chloride replacement	1 mM furosemide	10^{-5} M DIDS	$\Delta[Na_c + K_c]$	ΔCl_c
mM					
144	None	0	0	+20.51	+20.30
		0	+	+21.08	+20.71
		+	0	+0.58	−0.93
		+	+	−0.33	+0.31
20	Acetate	0	0	+7.06	+1.20
		0	+	+7.42	+5.41
		+	0	−0.33	−0.22
		+	+	+0.39	+1.00
20	Nitrate	0	0	+6.44	+0.99
		0	+	+6.78	+3.76
		+	0	+1.71	−0.15
		+	+	+1.07	+0.87

Net changes in cell ion contents are given in mmol/kg cell solid·10 min. Positive signs indicate net uptake, and negative signs indicate net loss. All incubation media were isotonic (323 mosmol) containing 125 mM Na, 22 mM K, and 0.1 mM ouabain. Co-transport was stimulated by adding 10^{-6} M norepinephrine. Initially, the cells contained the following concentrations of chloride (millimoles per liter cell water): 111.7 ± 0.9 (all-chloride flasks), 20.3 ± 0.6 (acetate flasks), and 18.3 ± 0.5 (nitrate flasks). Initial cell cation concentrations were (millimoles per liter cell water): $[Na]_c$ = 15.5 ± 0.4, and $[K]_c$ = 161.6 ± 2.1.

norepinephrine. When chloride levels were normal, net uptakes of [Na + K] and chloride were equal and unaffected by exposure to DIDS. In acetate or nitrate media containing 20 mM chloride, uptake of [Na + K] was reduced, but not accompanied by an equivalent amount of chloride, which confirmed the previous observation (Schmidt and McManus, 1977*c*). Although the DIDS-treated cells in the low chloride media showed no change in cation uptake, chloride uptake was markedly increased.

These results demonstrate that the chloride dependence of [Na + K] co-

transport (Table III) is caused by the actual participation of the anion in the co-transport process. In making this interpretation, it must first be recalled that norepinephrine, under the above conditions, promotes an uptake of *isotonic* fluid from the medium (Schmidt and McManus, 1977*b*). If chloride is an obligatory participant in this process, then its concentration in the increment of fluid taken up will be equal to that of the cations, but substantially higher than the chloride concentration of the cytosol in the low-chloride cells. Therefore, when the isotonic increment taken up as a result of co-transport mixes with the cytosol, there will be a tendency for the ratio of intracellular/extracellular chloride to increase, and for the equivalent ratio of the substitute anion to decrease. This perturbation would lead to a heteroexchange of internal chloride for external acetate or nitrate via the DIDS-sensitive anion exchanger. Thus, there appeared to be a net uptake of [Na + K] without an equivalent amount of chloride in the low-chloride cells (Table IV). DIDS inhibited this heteroexchange enough so that a substantial increase in cell chloride was observed.

Crucial to this interpretation is the assumption that heteroexchange can occur with the substitute anions. Nitrate is known to participate in anion heteroexchange via a DIDS-sensitive pathway (Wieth, 1970). In separate experiments (data not shown), we have found that DIDS inhibits ~25% of ^{14}C-acetate efflux from duck red cells incubated at 2°C in an acetate medium containing 20 mM chloride. These results are consistent with observations made on red cells from several other species (Deuticke, 1977).

Effect of Membrane Potential

When external chloride is lowered, at constant internal chloride, by replacement with sucrose or with an *impermeable* anion such as gluconate, norepinephrine-stimulated uptake of salt and water is inhibited (Schmidt and McManus, 1977*c*). Because chloride is at thermodynamic equilibrium in red cells, its distribution across the membrane gives an estimate of E_m, as calculated from the Nernst equation. Thus, at low external chloride, the cell interior becomes positive. The decrease in uptake of [Na + K] could be a result of this depolarization, or, in consideration of the data presented above (Tables III and IV), a consequence of the change in the direction and magnitude of the chloride chemical potential gradient.

This question of the role of membrane potential vs. chloride gradient cannot be answered so long as any change in the level of this anion affects both. Fortunately, there is a way to resolve this problem. When red cells are treated with DIDS to inhibit anion exchange and partially inhibit anion conductance, the addition of valinomycin increases P_K/P_{Cl} to the point where membrane conductance is dominated by potassium. Consequently, E_m can be held constant near E_K as $[Cl]_o$ is varied over a wide range at constant $[Cl]_c$. When this was done (Fig. 5), it was found that the change in norepinephrine-stimulated sodium transport brought about by lowering $[Cl]_o$ is unaffected by addition of valinomycin. After a preincubation to increase Na_c, cells were placed in a medium containing the same concentration of sodium as the cell

interior, but with potassium elevated to the point where membrane potential in the presence of valinomycin was close to the physiological level. At normal levels of $[Cl]_o$, where the chloride gradient was inwardly directed, the cells took up [Na + K] in response to the hormone. At low $[Cl]_o$, where the chloride gradient was reversed, they lost [Na + K]. In separate experiments (data not shown), the permeability of DIDS-treated cells to the replacement anion,

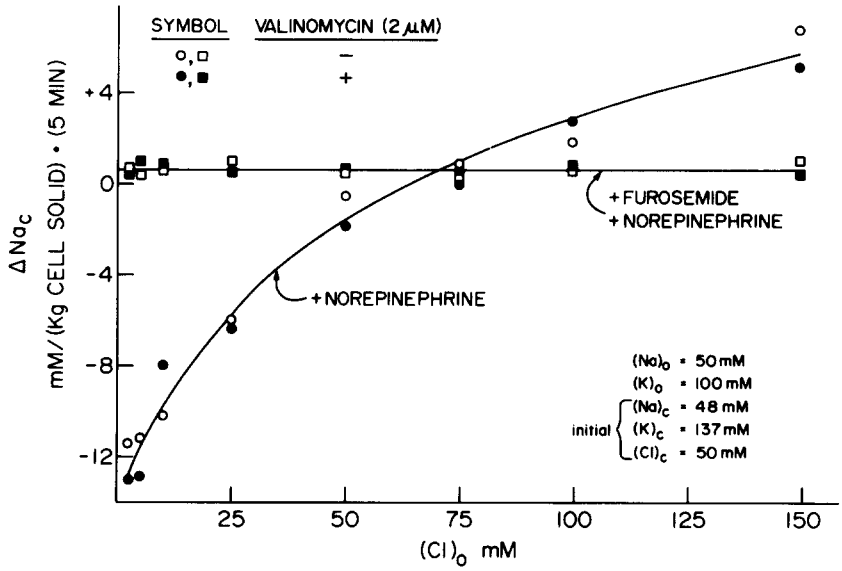

FIGURE 5. Effect of varying $[Cl]_o$ at constant $[Cl]_c$ on net sodium movements in the presence and absence of valinomycin (2×10^{-6} M). Cells were preincubated overnight at 4°C to increase Na_c and also to replace ~50% of $[Cl]_c$ with methylsulfate. The following morning, an additional preincubation of 90 min at 41°C was carried out in the presence of 0.1 mM ouabain and 10^{-5} M DIDS. All test incubations were performed at 41°C, 3% hematocrit, in media containing 50 mM Na, 100 mM K, 10^{-5} M DIDS, 10^{-6} M norepinephrine, and 0.1 mM ouabain, with or without 2×10^{-6} M valinomycin and 1 mM furosemide. Methylsulfate substituted for external Cl, maintaining medium osmolality at 323 mosmol. Initially, the cells in this experiment, as well as in that shown in Fig. 6, contained (millimoles/liter cell water): $[Na]_c = 47.9 \pm 0.7$ (SEM, $n = 64$), $[K]_c = 137.2 \pm 1.3$, and $[Cl]_c = 50.2 \pm 0.2$. Initial cell water was 1.472 ± 0.006 kg H_2O/kg cell solid. In the presence of valinomycin, P_K/P_{Cl} was estimated to be 7.5 (Table I), which yielded an E_m of -7.9 ± 0.5 mV (SEM, $n = 8$), as calculated from the constant-field equation (Eq. 1) over the entire range of $[Cl]_o$ from 2.5 to 150 mM. E_{Cl} varied from +78.9 mV at 2.5 mM $[Cl]_o$ to −28.9 mV at 150 mM $[Cl]_o$. In the absence of valinomycin, it was assumed that $P_{Cl} \gg P_K, P_{Na}$ and that $P_{MeSO_4} = 0.5\ P_{Cl}$. Again, using the constant-field equation, E_m was calculated to vary from approximately −0.4 to −18.3 mV. The determination of P_K/P_{Cl} in the presence of valinomycin (Table I) was carried out before preincubation on the cells used in this experiment, as well as the one shown in Fig. 6.

methylsulfate, was found to be about one-half their chloride permeability. Thus, variation of $[Cl]_o$ in the absence of valinomycin caused membrane potential to vary over a fairly wide range. Nevertheless, the effect of lowering chloride was the same in either case. These results strongly support the notion that the important parameter affected by lowering $[Cl]_o$ is the chemical potential gradient of chloride rather than the membrane potential.

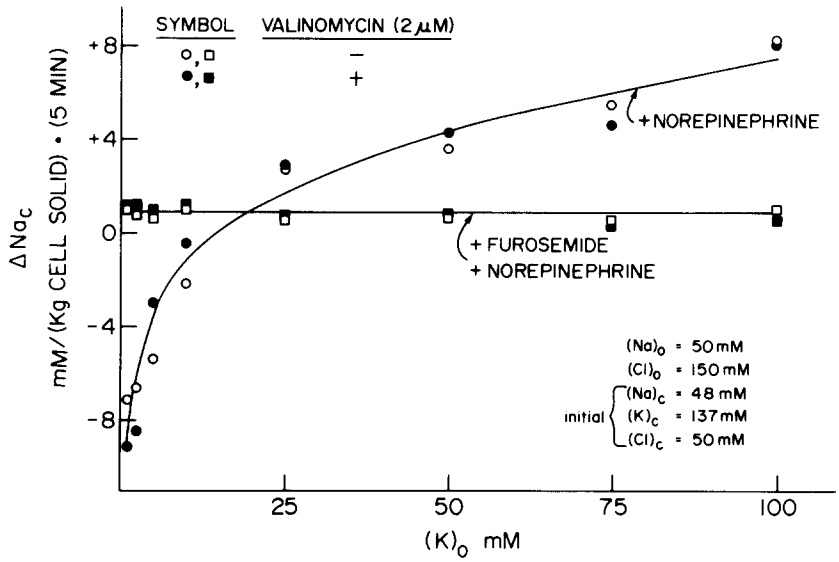

FIGURE 6. Effect of varying $[K]_o$ at constant $[K]_c$ on net sodium movements in the presence and absence of valinomycin (2×10^{-6} M). Cells were preincubated overnight at 4°C to increase Na_c and pretreated with DIDS the following morning as described in the legend to Fig. 5. Initial cell ion and water contents before the final test incubation are also listed in the legend to Fig. 5. Test incubations were performed at 41°C, 3% hematocrit, in media containing 50 mM Na, 150 mM Cl, 10^{-5} M DIDS, 10^{-6} M norepinephrine, and 0.1 mM ouabain, with or without 2×10^{-6} M valinomycin and 1 mM furosemide. TMA was substituted for external potassium, maintaining medium osmolality at 323 mosmol. In the presence of valinomycin, increasing $[K]_o$ from 1 to 100 mM caused E_K to vary from −129.6 to −8.3 mV. This changed E_m from −79.5 to −10.2 mV as calculated from Eq. 1 with $P_K/P_{Cl} = 7.5$ (Table I). Valinomycin from two suppliers was compared with nearly identical results. An earlier report (Haas and McManus, 1981) of 50% inhibition of co-transport by valinomycin was not confirmed with subsequent lots of ionophore. The original material may have been contaminated with a nonspecific inhibitor.

An alternative way of looking at this system is to vary $[K]_o$ in DIDS-treated cells in the absence or presence of valinomycin (Fig. 6). The potassium gradient was varied over a wide range at constant E_m in the absence of the ionophore or at variable E_m in its presence. Again, the system appears to be insensitive to the membrane potential. This is particularly apparent at very

low $[K]_o$ where valinomycin hyperpolarized the membrane to almost −80 mV, as calculated from Eq. 1 (see figure legends for further details). If net sodium loss at this level of $[K]_o$ was sensitive to E_m, addition of valinomycin would be strongly inhibitory. This, however, was not the case.

It should be noted that catecholamine-stimulated net sodium transport is driven by the chloride, as well as the potassium, gradient (Fig. 5; also see below). In the experiment shown in Fig. 6, therefore, where the chloride gradient was directed inward, the cells gained sodium at all levels of $[K]_o$ above ~20 mM. This uptake occurred despite the fact that the potassium gradient remained outwardly directed at all levels of $[K]_o$.

Chloride as Driver Ion

Coupling between solute gradients is clearly the most convincing way to demonstrate the presence of co-transport or counter-transport processes. Because chloride is normally at equilibrium in the red cell, it has been difficult by this approach to prove its direct participation in [Na + K] co-transport. Definitive confirmation would require the demonstration of net cation transport against an electrochemical gradient driven by chloride at constant E_m. This experiment became feasible with the development of the DIDS-valinomycin system described above (Figs. 5 and 6). Cells were incubated in the presence of enough sodium to provide a substantial inwardly directed gradient and enough potassium to maintain E_m constant at −12.4 mV in the presence of DIDS plus valinomycin (Fig. 7). At low $[Cl]_o$, net efflux of sodium against its gradient was observed, driven by the outwardly directed gradient of chloride. At high $[Cl]_o$, where the direction of the chloride gradient was reversed, a furosemide-sensitive net influx of sodium was observed.

The sigmoid appearance of the curve is not a kinetic property of the system, but is caused by the limiting effect of cell sodium at low $[Cl]_o$. These cells were not preincubated to raise Na_c, as in Figs. 5 and 6, because an inwardly directed gradient was desired. Thus, at $[Cl]_o$ <25 mM, co-transport depleted the cells of sodium almost completely during the period of observation. In experiments designed to study the kinetics of co-transport with respect to its dependence on $[Cl]_o$ at saturating concentrations of sodium and potassium, a curve similar to Fig. 5 was obtained with no evidence of sigmoid shape. Under these conditions, the half-maximal value with respect to $[Cl]_o$ was 75 mM (Haas and McManus, 1982).

These results offer strong evidence that chloride can play the role of driver ion as well as sodium and potassium.

DISCUSSION

These results demonstrate that norepinephrine stimulates co-transport efflux as well as influx. Net sodium efflux depends on cell potassium, and net potassium efflux depends on cell sodium. Under all conditions tested (Figs. 1 and 2), [Na + K] was lost from the cells in a ratio of 1:1. Net efflux via this pathway was also affected by raising the level of either ion in the external medium. For example, increasing $[K]_o$ inhibits net sodium loss, whereas

increasing $[Na]_o$ inhibits net potassium loss (Fig. 3). These solute gradient effects are an implicit feature of the co-transport model originally proposed for this system (Schmidt and McManus, 1974, 1977*b*; McManus and Schmidt, 1978*a*).

The observation that increasing external sodium inhibits net potassium efflux appears to conflict with previous reports (Gardner et al., 1975; Kregenow, 1977, 1978) that increasing $[Na]_o$ stimulates efflux of tracer potassium from catecholamine-treated avian red cells. An explanation for this discrep-

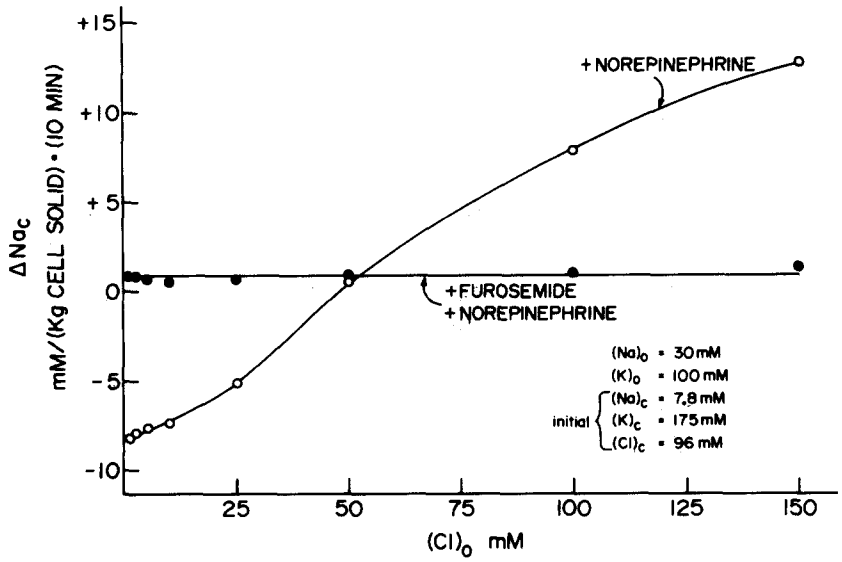

FIGURE 7. Net flux of sodium driven by the chloride gradient at constant E_m. Cells pretreated with DIDS (10^{-5} M) were reincubated at 41°C, 3% hematocrit, in media containing 30 mM Na, 100 mM K, 2×10^{-6} M valinomycin, 10^{-5} M DIDS, 10^{-6} M norepinephrine, and 0.1 mM ouabain, with or without 1 mM furosemide. Methylsulfate substituted for external Cl, maintaining medium osmolality at 323 mosmol. Initially, these cells contained (millimoles/liter cell water): $[Na]_c = 7.8 \pm 0.1$ (SEM, $n = 16$), $[K]_c = 174.9 \pm 0.4$, and $[Cl]_c = 95.6 \pm 0.7$. Initial cell water was 1.476 ± 0.004 kg H_2O/kg cell solids. E_m was calculated from Eq. 1 with $P_K/P_{Cl} = 7.5$ (Table I) to be -12.4 ± 0.4 mV (SEM, $n = 8$) over the entire range of $[Cl]_o$ from 1 to 150 mM.

ancy is provided by the statement that this effect occurs only in the presence of external potassium. Increasing $[Na]_o$ under such circumstances might be expected to activate sodium uptake by co-transport with potassium. At the low level of sodium normally present in cells, any increase in uptake would also promote co-transport efflux (see Fig. 2). It has already been shown (Kregenow, 1973; Schmidt and McManus, 1977*b*) that a significant, transient increase in cell sodium can follow catecholamine stimulation in media containing elevated $[K]_o$, even in the absence of ouabain. This interpretation of

the apparent *trans* effect of sodium receives further support from the data of Kregenow (1978), which show that raising $[K]_o$ from 2.5 to 18 mM markedly enhances stimulation of tracer potassium efflux by external sodium. In the experiment shown in Fig. 3 (right panel), we have avoided this backflux effect by omitting potassium from the external medium.

It is apparent that net co-transport of [Na + K] depends on the direction and magnitude of the respective cation gradients across the cell membrane. When they are oppositely directed, the direction of net salt and water movement will be determined by the predominant gradient. In this way, contra-gradient net transport of either ion can take place. Thus, in the experiment shown in Fig. 4, the energy driving potassium uphill out of the cells is provided by the large, outwardly directed sodium gradient. This phenomenon of "secondary active" transport, i.e., contra-gradient movement driven by an established solute gradient, rather than a metabolic source, can be demonstrated in this system in two ways: by raising the *cis* concentration of the co-transported solute (Fig. 4), or by lowering its *trans* concentration (Schmidt and McManus, 1977*b*).

Net co-transport of cations can also be driven by the chloride gradient when it is manipulated under conditions where E_m is controlled by valinomycin (Fig. 7). Participation of the halide as a co-ion explains why [Na + K] co-transport has a specific requirement for chloride or bromide (Table III). In response to catecholamine stimulation, therefore, chloride participates in the transport of the neutral complex, [Na + K + 2Cl], across the membrane.

Further confirmation of this concept would require a more direct method for estimating E_m during catecholamine stimulation of net co-transport. Kregenow (1977) has described experiments using a potential-sensitive carbocyanine dye, diS-C_3, to estimate E_m by fluorescence in duck red cells. He reports that exposure of cells to norepinephrine at 2.5 and 18 mM $[K]_o$ had no effect on the signal, implying no change in E_m. These experiments, however, were carried out on unmodified cells. Using the constant-field equation, it can be calculated that E_m would not be changed under these conditions by more than a few millivolts even if P_K and P_{Na} were increased to equal P_{Cl}. These experiments need to be repeated with the sodium and potassium gradients oriented in the same direction, as in Figs. 1 and 2, in order to promote a maximal change in E_m in response to any increase in P_K and/or P_{Na}. Another helpful maneuver would be to lower P_{Cl}, and thus increase membrane resistance, by pretreating the cells with DIDS. This has been helpful in other attempts to test for the electrogenicity of cation transport using the dye technique (Hoffman et al., 1979).

Co-transport in this system appears to be unaffected by inhibitors of anion exchange, such as DIDS (Table IV) or SITS (data not shown). Thus, it is quite different from the electrically neutral, volume-sensitive, ion transport processes described in *Amphiuma* red cells by Cala (1980). That system is thought to involve counter-transport of an alkali metal cation and a proton followed by a chloride-bicarbonate exchange to maintain hydrogen ion equilibrium. Cation plus anion co-transport does not appear to be involved.

There have been numerous recent reports of systems that appear to demonstrate cation plus anion co-transport. For example, [Na + Cl] co-transport has been postulated for the luminal membrane in numerous epithelia, such as rabbit gallbladder and *Necturus* proximal tubule (for review, see Frizzell et al., 1979). An electrically neutral [Na + K + 2Cl] co-transport has been described in Ehrlich ascites tumor cells (Geck et al., 1980). Dunham et al. (1980) report that ~70% of ouabain-insensitive potassium influx in human red cells depends on the presence of chloride or bromide and is inhibited by furosemide. A portion of this flux is probably caused by [Na + K + 2Cl] co-transport, which was originally described in the human red cell by Wiley and Cooper (1974). In that cell, however, it represents only a small fraction of the total cation flux, and is not stimulated by catecholamines (M. Haas and T. J. McManus, unpublished experiments).

In a previous paper (Schmidt and McManus, 1977*c*), the net driving force for co-transport was calculated from the sum of the *electrochemical* gradients for sodium and potassium expressed as a logarithmic function of the concentrations of sodium, potassium, and chloride in cell water and external medium, respectively. This approach implied a role for E_m, as well as the chemical potential gradients, in the movement of cations through this pathway. Chloride was assumed to follow by electrodiffusion. When data for net water movements caused by co-transport were pooled from several experiments with varying internal and external ion concentrations, and plotted against the calculated driving force, a straight line was obtained that passed through zero. In retrospect, it is instructive to consider why a model based, as that was, on incorrect assumptions yielded themodynamic predictions that fit the data so well. The reason becomes apparent when an analogous thermodynamic expression is derived for an electrically neutral co-transport of [Na + K + 2Cl]. In this instance, E_m is without effect (Figs. 5 and 6), and the net driving force can be represented by the sum of the *chemical* potential gradients of the ions involved:

$$\Delta\mu_{net} = \Delta\mu_{Na} + \Delta\mu_{K} + 2\Delta\mu_{Cl}; \tag{2}$$

$$\Delta\mu_{net} = RT \ln \frac{[Na]_o}{[Na]_c} + RT \ln \frac{[K]_o}{[K]_c} + 2RT \ln \frac{[Cl]_o}{[Cl]_c}; \tag{3}$$

and finally,

$$\Delta\mu_{net} = RT \ln \frac{[Na]_o \cdot [K]_o \cdot [Cl]_o^2}{[Na]_c \cdot [K]_c \cdot [Cl]_c^2}. \tag{4}$$

Eq. 4 above and Eq. 3 of Schmidt and McManus (1977*c*) are identical. The reason these two thermodynamic derivations arrived at the same final expression is that chloride appears twice in each case. In Eq. 2 above, the stoichiometry of [Na + K + 2Cl] co-transport dictates a coefficient of two for the chloride term. On the other hand, because the electrochemical potential gradients of both sodium and potassium are dependent on E_m, which in turn was represented by E_{Cl}, Eq. 3 of Schmidt and McManus (1977*c*) also ended up with a squared chloride term.

There is a wider significance to this episode. In any coupled transport system, the actual mechanism may be obscured if one of the transported species is distributed at or near thermodynamic equilibrium. Thus, in the normal red cell, where E_m can be approximated by E_{Cl}, it is not possible to determine whether an effect produced by varying the chloride ratio is caused by a change in E_m or a change in the chemical potential gradient of that ion. Only when E_m is shifted away from E_{Cl}, as by the use of valinomycin, can this distinction readily be made.

Of course, if an equilibrium-distributed species can be completely removed from the experimental setting, then a clue to its possible role in the coupled transport process may be revealed (e.g., Table III). That experiment by itself, however, cannot prove direct participation. Furthermore, total replacement of an ion like potassium, for example, may not be possible. This would certainly be the case in excitable cells, such as nerve or cardiac muscle, or certain epithelia where P_K is very much greater than either P_{Na} or P_{Cl}. Suppose, for example, such a system demonstrates an apparent co-transport of [Na + Cl], and the possible involvement of potassium needs to be determined. Obviously, any change in its gradient would result in a change in E_m. This could affect the transport process directly or indirectly, such as by a change in cell pH. Therefore, the participation of potassium as a co-ion could only be confirmed if it could somehow be shifted far away from equilibrium, as was done for chloride in the experiment shown in Fig. 7.

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REFERENCES

ANDREOLI, T. E., M. TIEFFENBERG, and D. C. TOSTESON. 1967. The effect of valinomycin on the ionic permeability of thin lipid membranes. *J. Gen. Physiol.* **50:**2527–2545.

CABANTCHIK, Z. I., and A. ROTHSTEIN. 1974. Membrane proteins related to anion permeability of human red blood cells. I. Location of disulfonic stilbene binding sites in proteins involved in permeation. *J. Membr. Biol.* **15:**207–226.

CALA, P. M. 1980. Volume regulation by *Amphiuma* red blood cells. The membrane potential and its implications regarding the nature of the ion-flux pathways. *J. Gen. Physiol.* **76:**683–708.

CASS, A., and M. DALMARK. 1973. Equilibrium dialysis of ions in nystatin-treated red cells. *Nat. New Biol.* **244:**47–49.

DEUTICKE, B. 1977. Properties and structural basis of simple diffusion pathways in the erythrocyte membrane. *Rev. Physiol. Biochem. Pharmacol.* **78:**1–97.

DUNHAM, P. B ., G. W. STEWART, and J. C. ELLORY. 1980. Chloride-activated passive potassium transport in human erythrocytes. *Proc. Natl. Acad. Sci. U. S. A.* **77:**1711–1715.

FRIZZELL, R. A., M. FIELD, and S. G. SCHULTZ. 1979. Sodium-coupled chloride transport by epithelial tissues. *Am. J. Physiol.* **236:**F1–F8.

GARDNER, J. D., D. R. KIINO, N. JOW, and G. D. AURBACH. 1975. Effects of extracellular cations and ouabain on catecholamine-stimulated sodium and potassium fluxes in turkey erythrocytes. *J. Biol. Chem.* **250:**1164–1175.

GECK, P., C. PIETRZYK, B.-C. BURCKHARDT, B. PFEIFFER, and E. HEINZ. 1980. Electrically silent cotransport of Na, K, and Cl in Ehrlich cells. *Biochem. Biophys. Acta.* **600:**432–447.

GOLDMAN, D. E. 1943. Potential, impedance, and rectification in membranes. *J. Gen. Physiol.* **27:**37–60.

HAAS, M., and T. J. MCMANUS. 1981. Effect of anion substitution and membrane potential on Na/K co-transport in duck red cells. *Fed. Proc.* **40:**484.

HAAS, M., and T. J. MCMANUS. 1982. Bumetanide inhibition of (Na + K + 2Cl) co-transport and K/Rb exchange at a chloride site in duck red cells: modulation by external cations. *Biophys. J.* **37:**214*a*.

HODGKIN, A. L., and B. KATZ. 1949. The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol. (Lond.)* **108:**37–77.

HOFFMAN, J. F., J. H. KAPLAN, and T. J. CALLAHAN. 1979. The Na:K pump in red cells is electrogenic. *Fed. Proc.* **38:**2440–2441.

HUNTER, M. J. 1977. Human erythrocyte anion permeabilities measured under conditions of net charge transfer. *J. Physiol. (Lond.).* **268:**35–49.

JENNINGS, M. L. 1978. Characteristics of CO_2-independent pH equilibration in human red blood cells. *J. Membr. Biol.* **40:**365–391.

KNAUF, P. A., G. F. FUHRMANN, S. ROTHSTEIN, and A. ROTHSTEIN. 1977. The relationship between anion exchange and net anion flow across the human red blood cell membrane. *J. Gen. Physiol.* **69:**363–386.

KREGENOW, F. M. 1973. The response of duck erythrocytes to norepinephrine and an elevated extracellular potassium. Volume regulation in isotonic media. *J. Gen. Physiol.* **61:**509–527.

KREGENOW, F. M. 1977. Transport in avian red cells. *In* Membrane Transport in Red Cells. J. C. Ellory and V. L. Lew, editors. Academic Press, Inc., New York. 383–426.

KREGENOW, F. M. 1978. An assessment of the co-transport hypothesis as it applies to the norepinephrine and hypertonic responses. *In* Osmotic and Volume Regulation, Benzon Symposium 11. C. B. Jørgensen and E. Skadhauge, editors. Munksgaard, Copenhagen. 379–396.

KREGENOW, F. M., and T. CARYK. 1979. Cotransport of cations and Cl during the volume regulatory responses of duck erythrocytes. *Physiologist.* **22:**73. (*Abstr.*).

MCMANUS, T. J., and M. HAAS. 1981. Catecholamine stimulation of K/K (K/Rb) exchange in duck red cells. *Fed. Proc.* **40:**484. (*Abstr.*).

MCMANUS, T. J., and W. F. SCHMIDT. 1978*a*. Ion and co-ion transport in avian red cells. *In* Membrane Transport Processes. J. F. Hoffman, editor. Raven Press, New York. **1:**79–106.

MCMANUS, T. J., and W. F. SCHMIDT. 1978*b*. Catecholamine-activated sodium-potassium co-transport in duck red cells. *J. Supramol. Struct.* **7:**212. (*Abstr.*).

RIDDICK, D. H. 1969. The influence of norepinephrine on cation transport in duck erythrocytes. Ph.D. Thesis, Duke University, Durham, NC.

SCHMIDT, W. F., and T. J. MCMANUS. 1974. A furosemide-sensitive cotransport of Na plus K into duck red cells activated by hypertonicity or catecholamines. *Fed. Proc.* **33:**1457. (*Abstr.*).

SCHMIDT, W. F., and T. J. MCMANUS. 1977*a*. Ouabain-insensitive salt and water movements in duck red cells. I. Kinetics of cation transport under hypertonic conditions. *J. Gen. Physiol.* **70:**59–79.

SCHMIDT, W. F., and T. J. MCMANUS. 1977*b*. Ouabain-insensitive salt and water movements in

duck red cells. II. Norepinephrine stimulation of sodium plus potassium co-transport. *J. Gen. Physiol.* **70**:81–97.

SCHMIDT, W. F., and T. J. MCMANUS. 1977*c*. Ouabain-insensitive salt and water movements in duck red cells. III. The role of chloride in the volume response. *J. Gen. Physiol.* **70**:99–121.

WIETH, J. O. 1970. Effect of some monovalent anions on chloride and sulphate permeability of human red cells. *J. Physiol. (Lond.)*. **207**:581–609.

WILEY, J. S., and R. A. COOPER. 1974. A furosemide-sensitive co-transport of sodium plus potassium in the human red cell. *J. Clin. Invest.* **53**:745–755.