# Anion-coupled Na Efflux Mediated by the Human Red Blood Cell Na/K Pump

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ABSTRACT The red cell Na/K pump is known to continue to extrude Na when both Na and K are removed from the external medium. Because this ouabain-sensitive flux occurs in the absence of an exchangeable cation, it is referred to as uncoupled Na efflux. This flux is also known to be inhibited by 5 mM $Na_o$ but to a lesser extent than that inhibitable by ouabain. Uncoupled Na efflux via the Na/K pump therefore can be divided into a $Na_o$-sensitive and $Na_o$-insensitive component. We used DIDS-treated, $SO_4$-equilibrated human red blood cells suspended in HEPES-buffered ($pH_o$ 7.4) $MgSO_4$ or $(Tris)_2SO_4$, in which we measured $^{22}Na$ efflux, $^{35}SO_4$ efflux, and changes in the membrane potential with the fluorescent dye, diS-$C_3$ (5). A principal finding is that uncoupled Na efflux occurs electroneutrally, in contrast to the pump's normal electrogenic operation when exchanging $Na_i$ for $K_o$. This electroneutral uncoupled efflux of Na was found to be balanced by an efflux of cellular anions. (We were unable to detect any ouabain-sensitive uptake of protons, measured in an unbuffered medium at pH 7.4 with a Radiometer pH-STAT.) The $Na_o$-sensitive efflux of $Na_i$ was found to be 1.95 ± 0.10 times the $Na_o$-sensitive efflux of $(SO_4)_i$, indicating that the stoichiometry of this cotransport is two $Na^+$ per $SO_4^{=}$, accounting for 60–80% of the electroneutral Na efflux. The remainder portion, that is, the ouabain-sensitive $Na_o$-insensitive component, has been identified as $PO_4$-coupled Na transport and is the subject of a separate paper. That uncoupled Na efflux occurs as a cotransport with anions is supported by the result, obtained with resealed ghosts, that when internal and external $SO_4$ was substituted by the impermeant anion, tartrate $_{i,o}$, the efflux of Na was inhibited 60–80%. This inhibition could be relieved by the inclusion, before DIDS treatment, of 5 mM $Cl_{i,o}$. Addition of 10 mM $K_o$ to tartrate $_{i,o}$ ghosts, with or without $Cl_{i,o}$, resulted in full activation of Na/K exchange and the pump's electrogenicity. Although it can be concluded that Na efflux in the uncoupled mode occurs by means of a cotransport with cellular anions, the molecular basis for this change in the internal charge structure of the pump and its change in ion selectivity is at present unknown.

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## INTRODUCTION

The red cell Na/K pump has been extensively studied with regard to its kinetic properties and ligand dependencies of ion translocation (Cavieres, 1977; Glynn 1985; Kaplan, 1989). Thus the pumped exchange of Na/K is inhibited by cardiotonic steroids, such as ouabain, utilizes MgATP, and has an obligatory requirement for the simultaneous presence of inside Na ($Na_i$) and external K ($K_o$). On the other hand, by an alteration in the ligand composition on one or the other side of the membrane, the pump can be made to operate in several kinds of partial modes, such as Na/Na exchange when $K_o$ is removed from the external medium. The principal focus of this paper is on the partial reaction referred to as uncoupled Na efflux, an ouabain-sensitive Na efflux that occurs when all exchangeable cations, i.e., $Na_o$ and $K_o$, are removed from the external medium (Garrahan and Glynn, 1967*a*; Sachs, 1970; Eilam and Stein, 1973; Lew et al., 1973; Glynn and Karlish, 1976). This flux requires $Na_i$ and $Mg_i$, utilizes ATP, and is inhibited by low concentrations of $Na_o$ (i.e., 5 mM). The initial thrust of the present studies was concerned with establishing whether the operation of the uncoupled Na efflux mode transferred net charge, as in the pump's 3 $Na_i$/2 $K_o$ exchange (cf. Hoffman et al., 1979) and if so, the relationship between the flux of Na and the size of the electrogenic potential. To our surprise we found that uncoupled Na efflux occurred electroneutrally. This led to a search for the ionic basis of the electroneutrality that resulted in finding that the uncoupled efflux of Na was in reality a cotransport mechanism involving the movement through the membrane of cellular anions in concert with Na. Cotransport of anions and Na was supported by measurements of anion/Na stoichiometries as well as by finding that uncoupled Na efflux was inhibited when impermeant anions, such as tartrate, were substituted for permeable anions, such as $SO_4$ or Cl. However, we do not as yet know the molecular basis for the pump's transition from electrogenic Na/K exchange to electroneutral Na cotransport with anions. Preliminary accounts of the present work have previously appeared (Dissing and Hoffman, 1983*a*, *b*; Hoffman et al., 1985).

## MATERIALS AND METHODS

### *Preparation of Red Cells Loaded with $SO_4$*

Blood from healthy donors was freshly drawn into heparin and centrifuged at 12,000 *g* for 2 min (Rotor SS34, Dupont Co., Wilmington, DE). The packed red cells were then resuspended and washed twice at 4°C, with 10 vol of 160 mM NaCl. The buffy coat was then removed and the cells were resuspended (unless otherwise specified) in 25 vol of 95 mM $Na_2SO_4$, 5 mM $NaH_2PO_4$, 3 mM glucose, and 5 mM adenosine (pH 7.4 with NaOH) and incubated at 37°C in a reciprocating water bath for 20 min to allow for $SO_4$/Cl exchange to occur. (In some experiments the solution was modified to contain up to 10 mM Cl that was substituted for an equal number of equivalents of $SO_4$.) This procedure was repeated twice and the $SO_4$-equilibrated cells were resuspended in the same solution (final hematocrit of 10%) that included either $^{24}NaCl$ (30 $\mu$Ci/ml) or $Na_2{}^{35}SO_4$ (50 $\mu$Ci/ml). The suspension was then incubated for approximately 3 h at 37°C for isotope loading, and, since the solution was $K_o$-free, to raise the intracellular Na ($Na_i$). During the last 5 min of the incubation period, 4,4′-diisothiocyanosilbene-2,2′disulfonic acid (DIDS, obtained from Sigma Chemical Co., St. Louis, MO) was added directly to the suspension to a final concentration of 50 $\mu$M (see later).

Depending on the particular experiment, as specified in the legends, the cells were subsequently washed twice, at pH 7.4, in either 95 mM $(Tris)_2SO_4$, or 180–240 mM $MgSO_4$ + 15 mM HEPES. These media were used where isotope fluxes and fluorescence measurements were made. For pH-STAT experiments (see later) the cells were washed three times with unbuffered 220 mM $MgSO_4$. The final centrifugation in all cases was carried out at 27,000 g for 10 min and the packed cells were kept at 4°C for use as described below. It is important to note that all solutions used were made bicarbonate-free by preequilibration with hydrated $N_2$ for 30 min at approximately pH 5 before titration to the specified pH values.

### *Determination of Cell Electrolytes and Water*

$Na_i$ and $K_i$ content of packed cells or ghosts was determined by flame photometry after suitable dilution, together with correction for medium trapped in the intercellular space. It should be noted that during the 3-h incubation period in 95 mM $Na_2SO_4$ solution (K-free) the packed cells, analyzed after the final centrifugation, gained $Na_i$ to a concentration usually between 18 and 24 mmol/liter of cells.

The $SO_4$ content was determined (Hoffman and Laris, 1974) by hemolyzing the $^{35}SO_4$-equilibrated, packed cells in 0.15 M perchloric acid and relating the $^{35}SO_4$ radioactivity per volume of packed cells to the specific activity of $^{35}SO_4$ in the incubation medium. After equilibration and washing in 95 mM $(Tris)_2SO_4$, when intracellular $SO_4$ had completely replaced Cl, the estimated intracellular $SO_4$ was ~42 mmol/liter of cells. Comparable values were obtained for cells washed in 220 mM $MgSO_4$/HEPES media. The Cl content of these $SO_4$-loaded cells was determined with a chloridometer (Cotlove Buchler Instruments Inc., Saddlebrook, NJ) to be <0.4 mmol/liter of cells. The latter determinations were not corrected for possible contaminating glutathione.

The percent water (g/g) was determined on the packed cells, after the final washing with the various solutions, by net weight/dry weight difference after drying at 90°C for 24 h. Osmolalities were determined with an osmometer (Advanced Instruments Inc., Needham, MA). Fig. 1 presents the osmolalities of the solutions used and the water contents of the packed cells after washing with varying concentrations of either $MgSO_4$ or $(Tris)_2SO_4$. It is clear that the cells were near their normal volume (63–65% water) when suspended in 200–220 mM $MgSO_4$ or 95 mM $(Tris)_2SO_4$. Although most of the experiments reported in this paper (see Results) were carried out on slightly shrunken cells, no substantive differences in results were observed when cell volume was in the normal range by use of solutions at lower osmolalities.

### *Treatment with DIDS*

We had previously found (Hoffman et al., 1979) as had others (Knauf et al., 1977) that treatment of $SO_4$-loaded red cells with DIDS essentially eliminated (>99% inhibition) transport of $SO_4$ by the Band 3 anion exchange mechanism. Thus treatment with DIDS reduced the background and helped to optimize measurements of $SO_4$ permeability (efflux) of cells in which Na efflux was also to be measured. Since $SO_4$ is known to form an ion pair with Na ($NaSO_4^-$) (p. 76 in Martell and Smith, 1974), treatment with DIDS also eliminates this type of transport by Band 3 for Na as well as $SO_4$ (Becker and Duhm, 1978). Table I shows the effects of varying DIDS concentration on the efflux and influx of Na in $SO_4$-loaded cells. Because 50 $\mu$M DIDS was the lowest concentration tested that produced maximum inhibition of the fluxes, this concentration was used in all subsequent experiments unless otherwise specified.

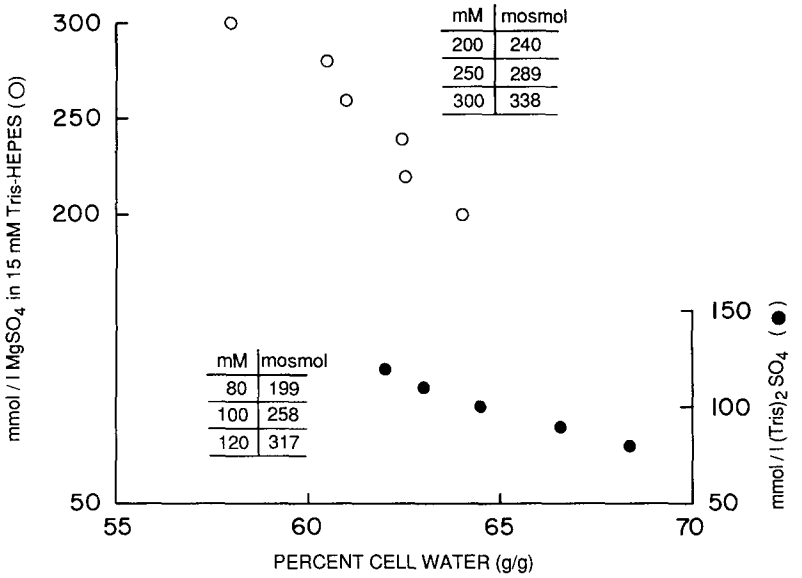


FIGURE 1. The water content of cells equilibrated with varying concentrations of either $MgSO_4$ (in 15 mM Tris-HEPES, pH 7.4) or $(Tris)_2SO_4$. In addition, the osmolalities of three concentrations of each solution are also given. The text should be consulted for the concentrations of the solutions used in the various experiments to interpolate the relative cell volume. l, liter.

### *Preparation of Ghosts*

Red cells washed three times with 160 mM NaCl as described above were resuspended to 50% hematocrit in this solution. Ghosts were then prepared by a modification of the method of Bodemann and Passow (1972). The cells were hemolyzed by rapidly injecting the suspension into 40 vol of a stirred solution that contained 1.6 mM acetic acid + 4 mM $MgSO_4$ at pH 5.8–6.0 where the temperature was maintained at 0°C. After 5 min the pH of the hemolysis

TABLE I

*Effect of Varying Concentrations of DIDS on the Flux of Na in $SO_4$-loaded Red Cells*

| | DIDS | | | | | | |
|---|---|---|---|---|---|---|---|
| | *μM* | | | | | | |
| Na flux | 0 | 5 | 10 | 20 | 50 | 75 | 100 |
| | *mmol/liter cells × h* | | | | | | |
| Efflux | 1.35 | 0.76 | 0.76 | 0.70 | 0.61 | 0.66 | 0.73 |
| Influx | 1.79 | 0.62 | 0.60 | 0.60 | 0.49 | 0.49 | 0.60 |

The cells were suspended in a medium that contained 25 mM $Na_2SO_4$, 75 mM $MgSO_4$, 75 mM sucrose, and 15 mM HEPES/Tris buffer (pH 7.3, 23°C). $Na_i$ was ~ 40 mmol/liter of cell water. Treatment of the cells with DIDS and the details of the flux measurements are described in Materials and Methods. The results of a single experiment are presented.

mixture was titrated to pH 7.4 (with NaOH) and allowed to sit at 0°C for 25 min. The mixture was then centrifuged for 5 min at 48,000 *g* at 0°C, the supernatant was removed, and the packed ghosts were resuspended, at 0°C, to a hematocrit of 10%, in a solution that contained (in millimolar) 25 Na, 40 tartrate, 50 Tris, 2.5 Mg, 4 $Na_2ATP$, and 0.25 EDTA with either 5 mM $MgCl_2$ (to entrap $Cl_i$ before DIDS treatment) or an osmotically equivalent concentration of Mg-tartrate (pH 7.2 at 37°C). After one wash the two types of ghosts (those with and without 10 mM $Cl_i$) were resuspended again in their same respective solutions except for the addition of $^{22}Na$ (0.1 μCi/ml) or $^{24}Na$ (10 μCi/ml) and incubated for 15 min at 0°C to equilibrate the isotope. The flasks were then transferred to a reciprocating water bath where the ghosts were resealed for 45 min at 37°C. (When $SO_4$ or Cl efflux was to be measured, a portion of the ghosts was loaded respectively with $^{35}SO_4$ or $^{36}Cl$ in place of labeled Na.) After the resealing period the ghosts were treated for 15 min at 23°C with 50 μM DIDS (final concentration) added directly to the suspensions. The suspensions were then centrifuged and the ghosts were washed twice with a solution that contained (in millimolar) 50 Tris, 40 tartrate, 15 Mg together with 5 mM $MgCl_2$ (i.e., 10 mM $Cl_o$) or its equivalent of Mg-tartrate. The ghosts were packed and placed on ice until used in efflux (see Table V) or fluorescence (see Figs. 8 and 9) measurements.

### *Flux Measurements*

Efflux was initiated by pipetting the packed labeled cells or ghosts into flasks containing the various media (as specified in the legends) at 37°C. The final hematocrit was ~2%. Four or five aliquots were removed between 3 and 28 min and centrifuged at 10,000 *g* (Eppendorff 3200, Brinkmann Instruments Co., Westbury, NY). Samples of the supernatants (as well as of suspensions) were removed for radioactivity determinations by gamma or beta (in Ultrafluor, National Diagnostics, Sommerville, NJ) counting respectively of $^{24}Na$ or $^{22}Na$, $^{35}SO_4$, and $^{36}Cl$. Efflux rate constants $^{o}k_I$ (per hour) were calculated (Hoffman, 1962) from the equation:

$$^{o}k_I = \ln [R_\infty - R_0)/(R_\infty - R_t)]t^{-1},$$

where $R$ is the radioactivity of the suspension and $R_0$ is the radioactivity of the supernatant at time zero and $R_t$, at time $t$. The slope of the line was computed by linear regression (least squares fit). The efflux, $^{o}M_I$, (in millimoles per liter of cells or ghosts × hour) was calculated from the relation:

$$^{o}M_I = {}^{o}k_I \times I_i,$$

where $I_i$ is the cellular concentration of Na, $SO_4$, or Cl in millimoles per liter of cells or ghosts. All efflux measurements were carried out in triplicate.

Influx was initiated by pipetting packed red cells into media, as specified in the text, containing either $^{24}Na$ 2 μCi/ml or $^{35}SO_4$ (5 μCi/ml) at 37°C at a final hematocrit of 10%. Samples were removed at four time points between 3 and 28 min. The samples were pipetted into 10 vol of the appropriate ice-cold, isotope-free medium to stop the influx and immediately centrifuged at 11,000 *g* for 1 min. The packed cells were resuspended and washed twice with 10 vol of the same iced medium. The packed cells were then hemolyzed by the addition of water and counted for $^{24}Na$ or $^{35}SO_4$ after precipitation of the protein with perchloric acid.

The influx, $^{i}M_I$ (in millimoles per liter of cells × hour), was calculated from:

$$^{i}M_I = I_0 \times R,$$

where $I_o$ is the medium concentration of either Na or $SO_4$ (in millimoles per counts per minute) and $R$ (in counts per minute per liter of cells × hour) is the rate of isotope uptake into 1 liter of cells calculated from linear regression analysis of the time course (four time points), taking into account the hematocrit of the suspension. All influx measurements were carried out in triplicate.

### *Proton Uptake*

Estimates of proton uptake were carried out with a pH-STAT (Radiometer, Copenhagen, Denmark). Packed red cells washed with 220 mM $MgSO_4$ (unbuffered) were suspended, at 30% final hematocrit (5 ml total volume), in the same solution, contained in a chamber that was stirred and thermostatically controlled at 37°C. Proton influx was titrated with 0.2 or 0.5 mM $H_2SO_4$ with the pH-STAT. It should be noted that because the cells were DIDS-treated, pH equilibration via Band 3 was sufficiently inhibited (Dissing and Hoffman, 1982; Milanick and Hoffman, 1982) to optimize, for the time period studied (15–30 min), possible changes in medium pH that might be due to $Na_i/H_o^+$ exchange (see later). With the initial $pH_o$ set to ~7.30 with $pH_i$ at 7.40 the system was sensitive enough to detect a ouabain-sensitive proton uptake equivalent to ouabain-sensitive uncoupled Na efflux: ouabain-sensitive uncoupled Na efflux from the 1.5 ml of cells in the chamber would be expected to average 0.3 μmol Na/15 min. Therefore experimentally when 0.3 μmol $OH^-$ was added to the chamber (either as tetramethylammonium hydroxide or NaOH) $pH_o$ was raised by 0.55 pH units. This would represent a minimum change in $pH_o$ if the exchange of $Na_i$ for $H_o^+$ were one for one.

### *Membrane Potential Measurements*

Membrane potential ($E_m$) was determined by use of the fluorescent dye, diS-$C_3$(5), as described by Hoffman and Laris (1974). Cells were suspended at a hematocrit of 0.2% in a cuvette in which the suspension was stirred and temperature-controlled at 37°C. The concentration of diS-$C_3$(5) in the cuvette was 1.6 μM. It was shown in separate experiments that this dye concentration had no effect on any of the flux measurements reported in this paper.

### *ATP Measurements*

ATP was measured by the luciferase method as described by Strehler (1974) with a luminometer (Turner Designs Inc., Mountain View, CA). The ATP determination was carried out on a perchloric acid extract of cells hemolyzed at 0°C.

### *Notation*

Subscripts "i" and "o" refer respectively to cellular (inside) and medium (outside) concentrations of ions unless otherwise specified.

## RESULTS

### *Characterization of the Cell Preparation*

Since most of the work described in this paper has been carried out on $SO_4$-loaded, DIDS-treated red cells, it was important to know whether the Na/K pump in these cells behaved the same as in normal cells. Because of this as well as to establish properties of uncoupled Na efflux that are basic to the work that follows, we first studied the separate effects of varying $Na_i$ (Figs. 2 and 3) and $Na_o$ (Figs. 4 and 5) on Na efflux in the presence and absence of $K_o$. Figs. 2 and 3 show respectively the

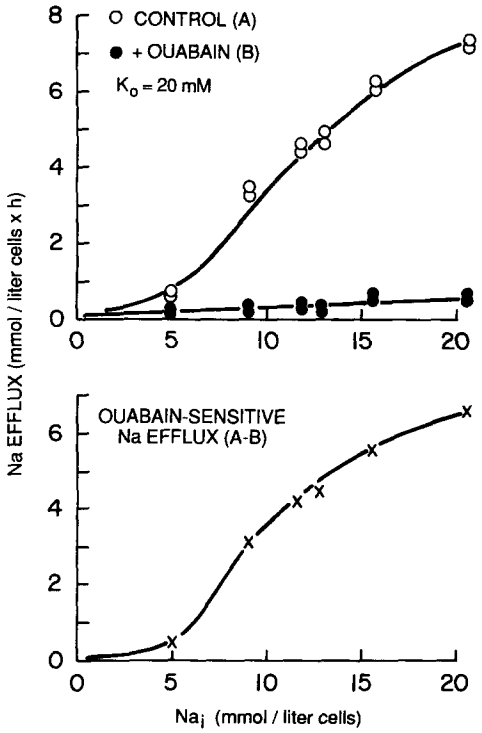


FIGURE 2. $K_o$-dependent Na efflux from $SO_4$-loaded, DIDS-treated red cells as a function of varying concentrations of $Na_i$. The efflux of $^{24}Na$ was measured, at 37°C, from cells suspended in a medium containing 10 mM $K_2SO_4$ + 180 mM $MgSO_4$ + 15 mM Tris-HEPES (pH 7.40). (*Upper panel*) Na efflux in the absence (O, *A*) and presence (●, *B*) of 50 μM ouabain. (*Lower panel*) Ouabain-sensitive Na efflux taken as the difference in the points comprising curves *A* and *B*. The red cells used in these experiments were from the same preparation as those used in connection with the results shown in Fig. 3, where $Na_i$ varied reciprocally with $K_i$.

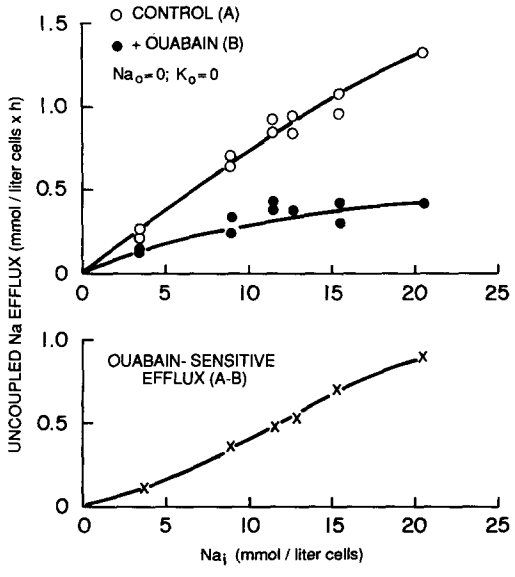


FIGURE 3. Uncoupled Na efflux from preequilibrated $SO_4$-loaded, DIDS-treated red cells as a function of the intracellular Na concentration ($Na_i$). The efflux of $^{24}Na$ was measured at 37°C, from cells suspended in a medium containing 200 mM $MgSO_4$ + 15 mM Tris-HEPES (pH 7.40). (*Upper panel*) Na efflux in the absence (O, *A*) and presence (●, *B*) of 50 μM ouabain. (*Lower panel*) Ouabain-sensitive Na efflux taken as the difference in the points comprising curves *A* and *B*. $Na_i$ was varied by varying $Na_o$ (together with $K_o$) during the preincubation time when the cells were loaded with $^{24}Na$ and $SO_4$ before DIDS treatment. $K_i$ varied reciprocally (88–105 mmol/liter of cells) with $Na_i$ such that cell water was the same for all values of $Na_i$. Intracellular ATP concentrations were between 0.69 and 0.75 mmol/liter of cell water. Results of another experiment were similar to those shown here.

activation by $Na_i$ of pump-mediated Na/K exchange and uncoupled Na efflux. In these figures (as well as in Figs. 4 and 5) the lower panel shows the ouabain-sensitive component ($A - B$) taken as the difference between the curves in the upper panel when Na efflux was measured in the absence ($A$) and presence ($B$) of ouabain. It is evident that the activation of Na efflux in the two pump modes are both sigmoid and show half-maximal activation in the same range as the values found by Sachs (1970). It should be understood that red cells from the same preparation were used in both experiments so that the only difference in the conditions is the presence and absence of 20 mM $K_o$. Therefore, it is also apparent that in these cells the size of uncoupled Na efflux, at 15–20 mM $Na_i$, is about 12–15% that of Na/K exchange, although there is some variation in this proportion when the same or different individual's blood is used on different days (see below). Note that in these experiments (Figs. 2 and 3) $Na_i$ and $K_i$ were varied reciprocally but when $K_i$ is substituted by choline in red cells (Garay and Garrahan, 1973) the apparent affinity for $Na_i$ falls to below 1 mM $Na_i$ (but compare Eilam and Stein, 1973).

Another parallel between ouabain-sensitive uncoupled Na efflux and Na/K exchange regards their dependency on $Mg_i$. $Mg_i$ was varied by the use of the divalent ionophore, A23187 (Reed, 1976, Yingst and Hoffman, 1978, 1984; Cavieres, 1980; Flatman and Lew, 1981). Thus cells containing 13.7 mM $Na_i$ were prepared and suspended in $(Tris)_2SO_4$ (details as in the legend of Fig. 5) together with 4 $\mu$M A23187 plus, to control or buffer $Mg_{i,o}$, 0.1 mM EDTA. Ouabain-sensitive Na efflux (in millimoles per liter of cells × hour) when $Mg_o$ was either nominally zero (not added) or 0.8 mM was, respectively, for the uncoupled mode 0.02 and 0.88 and for Na/K exchange 0.25 and 3.7. Free $Mg_i$ was estimated to be ~0.7 mM for the conditions when $Mg_o$ was 0.8 mM and EDTA was 0.1 mM (Perrin and Sayce, 1967). In the absence of added A23187 ± 0.1 mM EDTA the corresponding (control) values were 0.83 and 4.5, respectively, for uncoupled and Na/K exchange, all measurements being the average of duplicates. These results indicate that these two pump modes not only respond to changes in $Mg_i$ but also display at 0.8 mM $Mg_o$ (in the presence of A23187 and EDTA) fluxes similar to their respective control values, in addition to showing that the A23187 method is useful for altering the transport rate (see later).

The effect of $Na_o$ on uncoupled Na efflux from $SO_4$-loaded, DIDS-treated cells is shown in Fig. 4. As found by Garrahan and Glynn (1967*a*), Sachs (1970), and Lew et al. (1973), uncoupled Na efflux is inhibited by $Na_o$, the maximum inhibition being at ~5 mM $Na_o$. Increasing $Na_o$ above this concentration activates Na/Na exchange (Garrahan and Glynn, 1967*b*, Sachs, 1970), a process thought to be unrelated to uncoupled Na efflux. A special characteristic that should be noted is that the inhibition of uncoupled Na efflux by ouabain exceeds significantly the inhibition by $Na_o$, as also seen by Sachs (1970) and Lew et al. (1973). The reduction of uncoupled Na efflux by 5 mM $Na_o$ ranged in our experiments ~65–80% of the inhibition observed with ouabain (see below). This is important because the differences in the effects of $Na_o$ and ouabain help to distinguish between two different components of uncoupled Na efflux. The first component, that is the $Na_o$-sensitive portion of uncoupled Na efflux, is the primary subject of this paper, and is correlated with the transport of anions (e.g., $SO_4$) that originate in the cytoplasm. The second compo-

nent that is $Na_o$-insensitive, has been correlated with an efflux of $PO_4$ that is transported by the pump directly from substrate ATP (see Marín and Hoffman, 1988) and will be taken up in detail in a subsequent paper now in preparation.

It should also be mentioned that there is no significant ouabain-sensitive influx of $Na_o$ at 5 mM $Na_o$. As found in separate measurements ouabain-sensitive Na influx, at 5 mM $Na_o$, was 0.02 ± 0.01 mmol Na/liter of cells × h (mean ± SEM, $n = 5$) where, for the same cells when $Na_o$ was zero, ouabain-sensitive uncoupled Na efflux was 1.05 ± 0.14.

The results presented in Fig. 5 show that $Na_o$ when raised to the level that inhibits uncoupled Na efflux is without effect on Na/K exchange, at least when $K_o$ is 20 mM. The small inhibition of uncoupled Na efflux by $Na_o$ that is sometimes seen (Garrahan

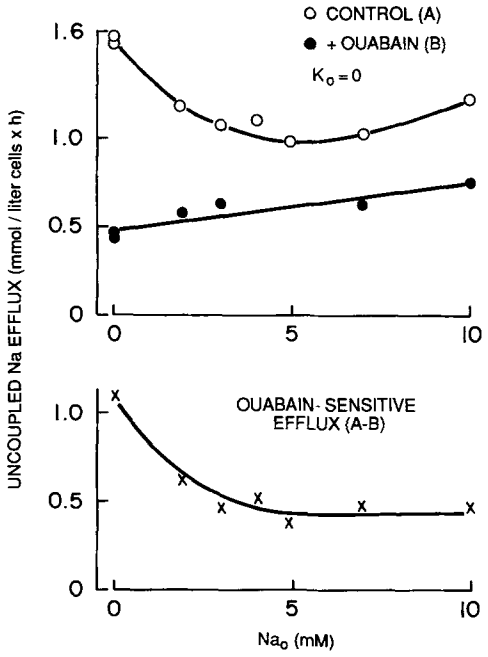


FIGURE 4. Uncoupled Na efflux from $SO_4$-loaded, DIDS-treated red cells as a function of varying concentrations of external Na ($Na_o$). The efflux of $^{24}Na$ was measured, at 37°C, from cells suspended in a medium containing 200 mM $MgSO_4$ + 15 mM Tris-HEPES (pH 7.40). (*Upper panel*) Na efflux in the absence (O, *A*) and presence (●, *B*) of 50 μM ouabain. (*Lower panel*) Ouabain-sensitive Na efflux taken as the difference in the points comprising curves *A* and *B*. $Na_i$ was 21 mmol/liter of cells.

and Glynn, 1967*a*; Lew et al., 1973) when $K_o$ is 10 mM may be due to the differences in the values of $K_o$ used or to differences in cell/ghost preparations. However, it would appear that when $K_o$ is saturating, uncoupled Na efflux is inoperative during the pump's exchange of $Na_i$ for $K_o$.

*Uncoupled Na Efflux and $E_m$.*

The basis for the present work began with measurements of $E_m$ in association with uncoupled Na efflux. If the phrase "uncoupled Na efflux" is taken literally, then it could be that this Na efflux is electrogenic, representing a net outward flow of current across the red cell membrane. It was of course of interest to test this possibility given the availability of a method (Hoffman et al. 1979) sensitive enough

to resolve ouabain-sensitive changes in $E_m$ that could be associated with uncoupled Na efflux. The method is based on the use of a membrane permeable fluorescent dye, in our case, diS-$C_3$(5), that because it bears a net (positive) charge is distributed across the membrane in accordance with the $E_m$ (Sims et al. 1974). Changes in $E_m$ induce a redistribution of the dye between the cell and the bulk phase resulting in a change in the relative fluorescence that can be calibrated in mV (see Hoffman and Laris, 1974). The results of this type of experiment are shown in Fig. 6. In the upper tracing, the fluorescence intensity of cells, operating in the uncoupled Na efflux mode by being suspended in a $Na_o$- and $K_o$-free solution, has come to equilibrium before the addition of 10 mM $K_o$. The addition of $K_o$ activates the Na/K pump and results in a downward deflection (hyperpolarization of $E_m$) that is reversed by the addition of ouabain. These changes in fluorescence intensity have been correlated with changes in $E_m$, indicating the electrogenicity of the Na/K pump that is

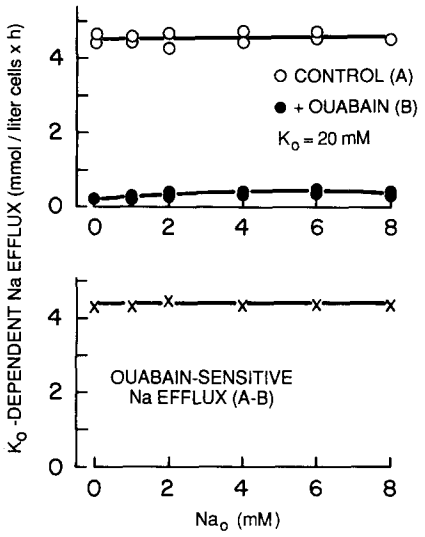


FIGURE 5. The effect of $Na_o$ on $K_o$-dependent Na efflux from $SO_4$-loaded, DIDS-treated red cells. The efflux of $^{24}Na$ was measured, at 37°C, from cells suspended in a medium composed of 10 mM $K_2SO_4$, 85 mM $(Tris)_2SO_4$ and 15 mM Tris-HEPES (pH 7.40). The addition of $Na_2SO_4$ replaced an osmotically equivalent concentration of $(Tris)_2SO_4$. (*Upper panel*) shows the Na efflux in the absence (O, *A*) and presence (●, *B*) of 50 μM ouabain. (*Lower panel*) Ouabain-sensitive Na efflux taken as the difference in the points comprising curves *A* and *B*. $Na_i$ was ~13 mmol/liter cells.

associated with the pump's three-$Na_i$ for two-$K_o$ exchange stoichiometry (Hoffman et al., 1979). In this particular experiment ouabain-sensitive Na efflux was determined to be 5.08 ± 0.09 mmol Na/liter of cells × h (mean ± SEM, $n$ = 5) in the presence of 10 mM $K_o$ and 0.91 ± 0.05 in the absence of $K_o$. The former value, of course, represents Na/K exchange while the latter, uncoupled Na efflux. If uncoupled Na efflux was electrogenic the change in fluorescence intensity expected on the addition of ouabain (lower tracing, Fig. 6) should be ~50% of that seen in the upper tracing after ouabain. This is based on the net current flow for a three-to-two stoichiometry taking the ratio of the uncoupled Na efflux to be one-third of the ouabain-sensitive Na/K exchange (assuming that the membrane resistance is not altered by $K_o$). It is clear from the result (lower tracing) that no such change occurred indicating that uncoupled Na efflux is an electroneutral process. The addition of 5 mM $Na_o$ to cells operating in the uncoupled Na efflux mode had no effect on the fluorescence

intensity of the suspension and did not discernibly alter the lack of response to ouabain added subsequently (data not shown).

### *Independence of Uncoupled Na Efflux and Proton Influx*

Having established that uncoupled Na efflux is not electrogenic raised the question of what was the basis for its electroneutral operation. One possibility is that external protons could be taken up in a one-for-one exchange with $Na_i$. This was tested by

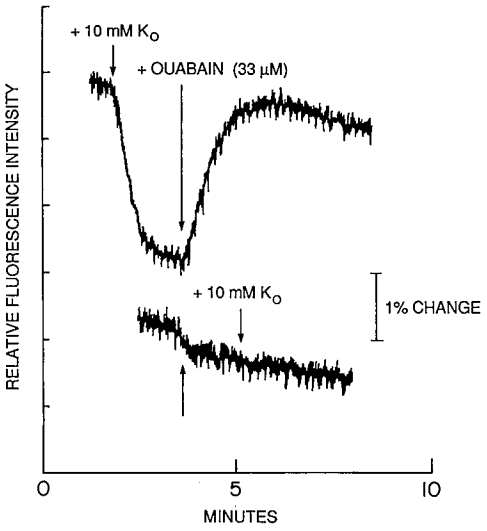


FIGURE 6. The membrane potential ($E_m$) changes in red cells associated with ouabain-sensitive Na/K transport and uncoupled Na efflux. $E_m$ changes are estimated from changes in the relative fluorescence of the dye, diS-$C_3$(5), as described in Materials and Methods. The Na-loaded, $SO_4$-equilibrated, and DIDS-treated cells were suspended, at 37°C, in 95 mM $(Tris)_2SO_4$ (pH 7.40) at an hematocrit of 1 part cells/300 parts medium. The fluorescence intensity tracings, representative of many similar observations, were recorded after dye equilibration (~10–15 min). A change in the fluorescence intensity in the downward deflection is associated with a hyperpolarization (cell interior negative with respect to the outside). A separately performed calibration procedure (see Hoffman and Laris, 1974) with these red cells indicated that a 1% change in the fluorescence intensity was approximately equivalent to a 2-mV change in $E_m$. $Na_i$ was 24.6 mmol/liter of cells. (*Upper curve*) Na/K pump is activated upon the addition of 5 mM $K_2SO_4$ to the cell suspension in $(Tris)_2SO_4$ medium, producing a decrease in the fluorescence intensity associated with the pump's electrogenicity. Ouabain reverses these effects and returns the fluorescence intensity to its original value. (*Lower curve*) When the cells are engaged in uncoupled Na efflux (i.e., in the absence of $K_o$ and $Na_o$) ouabain has no effect on the fluorescence intensity (beyond its dilution effect on the dye concentration in the suspension) and prevents any subsequent change with K addition.

suspending $SO_4$-loaded, DIDS-treated cells in an unbuffered medium where $pH_o$ was set and then maintained at various values with a pH-STAT (see Materials and Methods and the legend to Fig. 7) that pari passu provides a measure of changes in $pH_o$. It is clear from the results presented in Fig. 7 that there is no proton uptake between $pH_o$ 7.4 and 6.6. The ouabain-sensitive difference between the curves in *A* are shown in *B*, again emphasizing the fact that at $pH_o$ 7.4, the $pH_o$ used in the present studies, countertransport of protons (or cotransport of hydroxyl ions) does not occur. These results make untenable the interpretation proferred by Goldshleg-

ger et al. (1989) that the uncoupled Na efflux as seen in red cells is the result of combined Na/proton and anion/hydroxyl (or bicarbonate) exchange.

While it is not our main concern it should be noted that as $pH_o$ is decreased below $pH_o$ 6.6 there is a proton uptake that has both a ouabain-sensitive as well as a

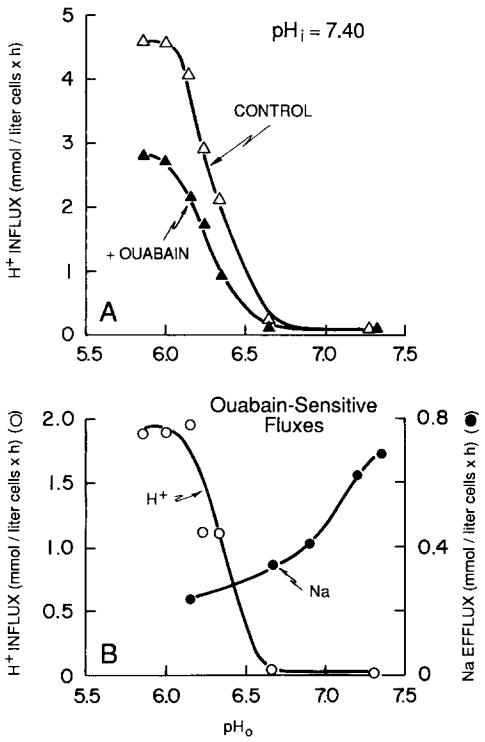


FIGURE 7. The effect of pH on uncoupled Na efflux and proton influx on $SO_4$-loaded, DIDS-treated red cells. (*A*) Effect of altering pH on the influx of protons in the presence and absence of 50 $\mu$M ouabain. The ouabain-sensitive component (i.e., the difference curve) is shown in *B* together with the ouabain-sensitive uncoupled efflux of Na that was obtained in a separate experiment. The red cells used in the proton influx measurements were Na and $SO_4$-loaded by incubation for ~4 h at 37°C with 95 mM $Na_2SO_4$ 5 mM adenosine, 2 mM glucose, and 5 mM $Na/PO_4$ buffer (pH 7.40). During the last 15 min, 50 $\mu$M DIDS was added after which the cells were washed three times with unbuffered 220 mM $MgSO_4$. The cells, at 30% hematocrit, were then added to the chamber of a pH-STAT. The $pH_o$ adjusted to the indicated values with $H_2SO_4$, and after 5 min of equilibration, the influx of protons was measured over the next 20 min by following the $H_2SO_4$ added by the titrator to keep $pH_o$ constant at the preset value. Thus, $pH_i$ was fixed at a constant pH value (the measured $pH_i$ was pH 7.40) by treatment with DIDS (see Materials and Methods) after which $pH_o$ was varied as indicated. The $pH_i$ changed <0.1 pH unit over the flux period due to the cell's high buffer capacity. $Na_i$ was 18 mmol/liter of cells. The cells used for the Na efflux determinations were preincubated in the same way (except for the addition of tracer $^{24}Na$) as the cells used in the proton flux measurements through the DIDS treatment but were then washed at 4°C in a medium that contained 180 mM $MgSO_4$ + 20 mM HEPES (pH 7.40 at 23°C) and finally suspended in the same medium but where the $pH_o$ was adjusted [with $Mg(OH)_2$] to the desired values. The Na efflux values are the average of triplicates. $Na_i$ was 16 mmol/liter of cells. Na efflux was measured in the presence and absence of 50 $\mu$M ouabain but only the ouabain-sensitive difference curve is presented in *B*.

ouabain-insensitive component. The ouabain-insensitive component represents Na/proton exchange and is antagonized by $Na_o$ (Dissing and Hoffman, 1982; Milanick and Hoffman, 1982). The ouabain-sensitive component (Fig. 7*B*) presumably reflects the transport by the Na/K pump of protons substituting for either $K_o$ or $Na_o$ with a

possible change in the pump's stoichiometry (Hara and Nakao, 1986; Polvani and Blostein, 1988; Goldshlegger et al., 1989). Note also that ouabain-sensitive Na efflux decreases as $pH_o$ decreases (results are derived from a separate experiment) perhaps indicative of a change in transport stoichiometry. Evaluation of this possibility would require further study (see Discussion).

### *Relation of Uncoupled Na Efflux and $(SO_4)_i$ Efflux*

Since Na efflux in the uncoupled mode was not engaged in an exchange with external protons, we tested the possibility that cellular anions were cotransported with $Na_i$. It was found, as shown in Table II, that not only was there a ouabain-sensitive efflux of $(SO_4)_i$ but that this efflux (${}^oM_{SO_4}^{\Delta ouab}$) was always less than the ouabain-sensitive efflux of Na (${}^oM_{Na}^{\Delta ouab}$) under the same conditions. When the comparison is made in terms of monovalent equivalents, Na efflux exceeds $SO_4$

TABLE II

*Ouabain-sensitive Efflux of Na and $SO_4$ from $SO_4$-loaded Red Cells*

| Expt. | Medium | $Na_i$ | ${}^oM_{Na}$ | | ${}^oM_{SO_4}$ | | ${}^oM_{Na}^{\Delta ouab}$ | ${}^oM_{SO_4}^{\Delta ouab}$ |
|---|---|---|---|---|---|---|---|---|
| | | | Control | +ouab | Control | +ouab | | |
| | | *mmol/liter cells* | *mmol/liter cells × h* | | | | | |
| 1 | $MgSO_4$ | 25 | 1.48 ± 0.10 | 0.77 ± 0.05 | 1.45 ± 0.04 | 1.19 ± 0.03 | 0.71 ± 0.13 | 0.26 ± 0.05 |
| | | 3.7 | 0.25 ± 0.02 | 0.07 ± 0.01 | 1.23 ± 0.02 | 1.23 ± 0.02 | 0.18 ± 0.02 | 0.00 ± 0.05 |
| 2 | $MgSO_4$ | 20.1 | 1.66 ± 0.09 | 0.94 ± 0.14 | 1.58 ± 0.07 | 1.36 ± 0.08 | 0.72 ± 0.16 | 0.22 ± 0.10 |

The $Na_i$ concentration was varied in the two different batches of cells used in expt. 1 by preincubation for 3.5 h (at ~10% hematocrit) in either 90 mM $Na_2SO_4$ (to raise $Na_i$) or $K_2SO_4$ (to lower $Na_i$) with 5 mM $Na/PO_4$ or $K/PO_4$ buffers (pH 7.4), respectively, together with 5 mM adenosine and 3 mM glucose. During preincubation, the cells were also loaded with either $^{24}Na$ or $^{35}SO_4$ by splitting each batch into two subgroups. For efflux the cells were washed and suspended in 180 mM $MgSO_4$ + 10 mM HEPES (pH 7.4) ± 50 μM ouabain. The cells used in expt. 2 were pretreated in the same way as the $Na_2SO_4$ exposed cells in expt. 1 as well as being suspended in the same efflux medium as used in expt. 1. All cells after preincubation, but before washing with the final suspension medium, were treated with 50 μM DIDS for 15 min at 37°C. The values given are the means ± SEM where $n$ = 3 and 5 for Na and $SO_4$ efflux, respectively.

efflux by ~27% and 39%, respectively, in experiments 1 and 2. It should also be noted that the ouabain-sensitive efflux of $SO_4$ depends on the level of $Na_i$ (experiment 1) similar to the dependence of uncoupled Na efflux on $Na_i$ (Fig. 3). This is what would be expected if the transport of Na and $SO_4$ were linked.

Since we had also observed in other experiments that $SO_4$ efflux was inhibited by 5 mM $Na_o$ to about the same extent as the inhibition with ouabain, we studied the relationship between the $Na_o$-sensitive efflux of $SO_4$ and the $Na_o$-sensitive efflux of Na, under the same uncoupled mode conditions. The results of such measurements are presented in Table III. The mean value (±SEM) of the ratio of the two fluxes for the four experiments shown is 1.95 ± 0.10, indicating that the stoichiometry of this cotransport is two $Na^+$ per $SO_4^=$. This $Na_o$-sensitive cotransport of Na and $SO_4$ appears to account in an electroneutral manner for most of the uncoupled Na efflux but it leaves unexplained the remainder part of the efflux, i.e., the $Na_o$-insensitive

component that is also ouabain-sensitive. This latter component, as stated before, is due to the cotransport of $PO_4$ and is the subject of a separate paper (see Marín and Hoffman, 1988).

The previously discussed results were carried out with $SO_4$-loaded cells. While it would be desirable to perform similar experiments where Cl was the principal anion, the high background permeability (conductance) of the membrane, even in the presence of DIDS, precludes measurements of ouabain-sensitive Cl efflux (Dissing and Hoffman, 1983*b*). Nevertheless it is possible to test the influence of $Cl_{i,o}$ on the efflux of $SO_4$, in the uncoupled mode, from $SO_4$-loaded, DIDS-treated cells by substituting either 5 or 10 mM Cl for an osmotically equivalent concentration of $Na_2SO_4$ in the loading solutions prior to treatment with DIDS. These concentrations of Cl on both sides of the membrane were also present in the $(Tris)_2SO_4$ medium used in the remainder of the experiment. Thus ouabain-sensitive $SO_4$ efflux values in

TABLE III

*$Na_o$-sensitive Efflux of Na and $SO_4$ from $SO_4$ loaded, DIDS-treated Cells*

| Expt. | ${}^{o}M_{Na}^{\Delta Na}$ | ${}^{o}M_{SO_4}^{\Delta Na}$ | Stoichiometry $Na^+/SO_4^=$ |
|---|---|---|---|
| | *mmol/liter cells × h* | | |
| 1 | 0.46 ± 0.09 | 0.25 ± 0.13 | 1.85 |
| 2 | 0.47 ± 0.04 | 0.23 ± 0.07 | 2.03 |
| 3 | 0.46 ± 0.09 | 0.27 ± 0.11 | 1.67 |
| 4 | 0.43 ≥) ± 0.04 | 0.19 ± 0.07 | 2.23 |

The pretreatment of the cells for all experiments was the same as that described in the legend to Table II where the cells were equilibrated with $SO_4$, $^{24}Na$, and $^{35}SO_4$ and treated with DIDS. The final suspension medium for the cells in expts. 1, 2, and 3 was 240 mM $MgSO_4$ + 10 mM HEPES (pH 7.20); for expt. 4, the medium was 100 mM $(Tris)_2SO_4$. When present $Na_2SO_4$ was substituted for an osmotically equivalent concentration of either $MgSO_4$ or $(Tris)_2SO_4$. The final suspension medium in expts. 1 and 2 also contained 300 μM acetazolamide. The symbols, ${}^{o}M_{Na}^{\Delta Na}$ and ${}^{o}M_{SO}^{\Delta Na}$ indicate the difference in the respective fluxes of Na and $SO_4$ in the presence and absence of 5 mM $Na_o$. The values given are the means ± SEM where $n = 6$ for expts. 1, 2, and 4; $n = 5$ for expt. 3.

cells containing 0, 5, and 10 mM $Cl_i$, respectively, were 0.11 ± 0.02, 0.01 ± 0.02, and 0.00 ± 0.02 mmol/liter of cells × h (means ± SEM, $n = 4$). The value of ${}^{o}M_{SO}^{\Delta ouab}$ at zero $Cl_i$ was lower than usual because of the low value of $Na_i$ (12 mmol/liter of cells). In a separate but similar experiment ${}^{o}M_{Na}^{\Delta ouab}$ was found to be the same independent of the presence of $Cl_i$. These results indicate that in uncoupled Na efflux Cl is preferred to $SO_4$ for cotransport.

### *Effect of Impermeant Anions on Uncoupled Na Efflux*

Since uncoupled Na efflux appears to be cotransported with an anion, such as $SO_4$, it was important to know how this flux might be affected by substitution of a relatively impermeant anion, such as tartrate, for $SO_4$. Because of tartrate's relative impermeability, it was necessary to use ghosts in order to entrap tartrate inside before their reconstitution as described in Materials and Methods. The results of three experiments of this type are shown in Table IV. Here ouabain-sensitive Na

efflux was measured from ghosts containing tartrate$_i$ without and with 10 mM $Cl_i$ and suspended in tartrate$_o$ in the absence and presence of $Cl_o$ and/or 10 mM $K_o$. One important result is that in all three experiments uncoupled Na efflux in the absence of a readily permeant anion is inhibited relative to the same flux in the presence of 10 mM $Cl_{i,o}$. The inhibition ranges ~50–70% similar to the level of inhibition seen in $SO_4$-loaded cells as the difference between the ouabain-sensitive component and the $Na_o$-sensitive component referred to before (e.g., Fig. 4). This would be the approximate level of inhibition expected if the $Na_o$-sensitive component required a permeable anion (e.g., $SO_4$) in order to carry out cotransport.

The second important result is that the activation of the ouabain-sensitive Na/K pump by $K_o$ is essentially the same independent of the presence or absence of $Cl_{i,o}$

TABLE IV

*Effect of Impermeant Anions (Tartrate) on the Uncoupled Efflux of Na in Red Cell Ghosts*

| Ghost/medium Composition | ${}^oM_{Na}^{\Delta ouab}$ | | |
|---|---|---|---|
| | Expt. A | Expt. B | Expt. C |
| | | *mmol/liter cells × h* | |
| $Tartrate_{i,o}$ | 0.45 ± 0.17 | 0.46 ± 0.07 | 0.25 ± 0.13 |
| $Tartrate_{i,o}$ + $Cl_{i,o}$ | 1.18 ± 0.16 | 0.92 ± 0.13 | 0.88 ± 0.23 |
| $Tartrate_{i,o}$ + $K_o$ | 5.85 ± 0.27 | — | 4.78 ± 0.49 |
| $Tartrate_{i,o}$ + $Cl_{i,o}$ + $K_o$ | 5.54 ± 0.13 | — | 5.62 ± 0.31 |

The ghosts were made, as described in Materials and Methods, to contain 15–20 mM Na, 50 mM Tris, 40 mM tartrate, 2.5 mM Mg, 0.25 mM EDTA, 4 mM $Na_2$ ATP, and a tracer amount of $^{24}Na$ (pH 7.4 at 23°C.). After resealing for 45 min the ghosts were divided into two portions and suspended in a solution containing 50 mM Tris, 15 mM Mg, and 40 mM tartrate where one portion also had added 5 mM $MgCl_2$ while the other portion was osmotically compensated with additional tartrate (Cl-free). Thus, one portion of ghosts contained Cl while the other did not. Both portions were then treated with 100 μM DIDS for 15 min during resealing at 37°C before washing and suspension in the same solutions (i.e., with or without $Cl_o$) containing either 10 mM K (as tartrate) or an equivalent concentration of $(Tris)_2$ tartrate. The ouabain-sensitive efflux of Na (${}^oM_{Na}^{\Delta ouab}$) is presented as the difference in efflux measured in the absence and presence of 50 μM ouabain. The ghosts contained ~96% water and <0.1–0.4 mmol K/liter of ghosts. In experiments A, B, and C the concentration of $Na_i$ was 17.9, 14, and 16.7 mmol/liter of ghosts, respectively. The values in the table represent the means ± SEM, where $n = 3$ in all experiments. Measurements of $E_m$ changes of the ghosts used in experiments A and B are presented in Figs. 8 and 9, respectively.

(Table IV, experiments A and C, bottom two rows). These results indicate that an anion is not involved in the normal exchange of $Na_i$ for $K_o$ by the pump. Thus, although the pump is operating electrogenically in these circumstances (see Fig. 8), there is no obvious explanation for how the net charge movement of Na is electrically compensated (see Discussion).

### *Membrane Potentials of Ghosts Containing Impermeant Anions*

The results presented in Fig. 8 show changes in dye fluorescence intensity, and therefore relative changes in $E_m$, that are associated with the four different conditions listed in Table IV for the same ghosts used in experiment A (Table IV). Considering traces *C* and *D* first, it is clear that the addition of $K_o$ in activating

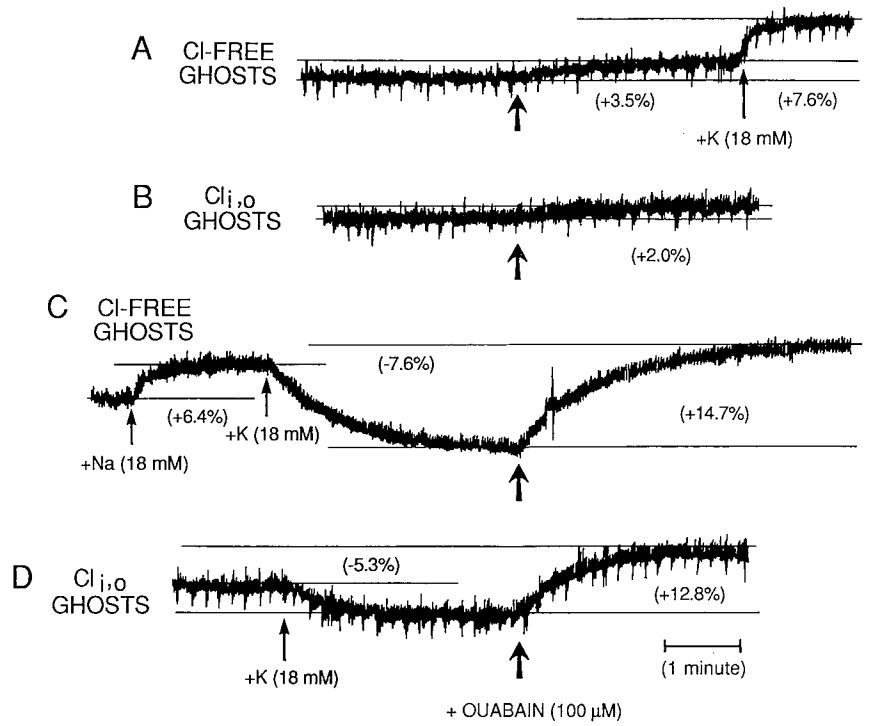


FIGURE 8. $E_m$ changes associated with uncoupled Na efflux and Na/K exchange in red cell ghosts in which the principal (impermeant) anion on both sides of the membrane is tartrate. The figure shows tracings of experimental recordings. The $E_m$ changes are estimated from changes in the relative fluorescence ($\Delta Fl^{ce}$) of the dye, diS-$C_3(5)$, as described in Materials and Methods. The two types of ghosts used were the same as those used in experiment A in Table IV (see legend for details). The $Fl^{ce}$ recorded in curves *A*, *B*, *C*, and *D* correspond directly to the conditions given in Table IV for the top to bottom rows, respectively. As in Fig. 6, the same convention is used where a downward deflection represents a hyperpolarization. The K or Na salts of tartrate were used for the indicated additions (and final concentrations) and were made directly to the stirred suspension of ghosts in the spectrofluorometer. The percent change in $\Delta Fl^{ce}$ was calculated from the relation, [(initial $Fl^{ce}$ − final $Fl^{ce}$)/(initial $Fl^{ce}$)] × 100, where the scale for the relative fluorescence intensity values (ordinate) had been calibrated from zero to maximum. No attempt was made to calibrate, in terms of millivolts, the percent $Fl^{ce}$ changes (given in brackets after each addition) that result from the various additions because of the uncertainties caused by the change in the ratio of permeabilities, $P_{anion}$ to $P_{cation}$, that evidently obtains in these type ghosts. Note, for instance, the depolarization that occurs in curve *A* by the addition of K after ouabain. This is discussed further in the text.

pump-mediated Na/K exchange induces a hyperpolarization that is reversed by ouabain, similar to the result presented in Fig. 6 (upper trace). An important point to be emphasized is that the change in $E_m$ seen upon the addition of ouabain is only slightly larger in Cl-free ghosts (trace *C*) than in $Cl_{i,o}$-ghosts (trace *D*), i.e., 14.7% compared with 12.8%. The difference in the ouabain-sensitive $\Delta Fl^{ce}$ is presumably real since the conductance of the membrane would be expected to be somewhat higher in the presence of a permeable anion, such as Cl.

Traces *A* and *B* of Fig. 8 show that ouabain added to ghosts engaged in uncoupled Na efflux (see Table IV) results in a slight depolarization (3.5% and 2.0%, respectively) of $E_m$. This is different than the electroneutral response obtained with $SO_4$-loaded cells (Fig. 6, lower trace). Nevertheless, that the electrogenic responses seen in traces *A* and *B* are significant is discussed below in connection with Fig. 9. If these electrogenic responses are real then they would presumably reflect the $Na_o$-insensitive component (see Fig. 4) of uncoupled Na efflux.

The tartrate-containing ghosts, as illustrated in Fig. 8, are interesting in another connection because they appear to have a lower membrane conductance to cations (as well as anions) than either ghosts or intact cells containing $SO_4$ or Cl as the principal anion. Thus it is possible to see in tartrate ghosts changes in $E_m$ that reflect changes in diffusion potentials of Na and K associated with their respective concentration gradients. For instance, in trace *A* (Fig. 8) the addition of 18 mM $K_o$, after the addition of ouabain, results in a depolarizing $\Delta$ $Fl^{ce}$ of +7.6%; in trace *C*, the addition of 18 mM $Na_o$ produces a similar result (+6.4%). Since $E_m$ in the absence of $Na_o$ or $K_o$ would be expected to be hyperpolarized due to the outward diffusion gradient for $Na_i$ (the ghosts contain ~25 mM $Na_i$ and <1 mM $K_i$), the addition of $Na_o$ would decrease $E_m$ by decreasing the outwardly directed Na gradient, while the addition of $K_o$ would decrease $E_m$ by offsetting the Na diffusion potential. The internal consistency of these relative effects in the traces shown in Fig. 8 support this interpretation. Note that if the addition of 18 mM $K_o$ (trace *A*) produces a percent $\Delta Fl^{ce}$ of +7.6%, this same change should also take place when 18 mM $K_o$ is added to ghosts as in trace *D*. Therefore, the hyperpolarization that results (−5.3%) would be expected to be −7.6% larger if there was no associated K diffusion potential. If this is so, then the sum, (−5.3) + (−7.6) = −12.9%, would represent the actual extent of the activation of Na/K exchange and should be the same as the extent of inhibition seen with ouabain (12.8%). This same argument holds for the case represented by trace *C* where the activation by $K_o$ addition [(−7.6) + (−7.6) = −15.2%] is approximately the same as the inhibition by ouabain (14.7%). This discussion in emphasizing the tightness of tartrate ghosts regarding passive diffusion of ions provides a rationale for the small but evident electrogenic component associated with ouabain-sensitive uncoupled Na efflux as seen in traces *A* and *B*. Here the presence of Cl (trace *B*), by increasing the membrane conductance, decreases the percent $\Delta$ $Fl^{ce}$ relative to the value seen in the absence of Cl (+2.0% compared with +3.5%). The same consequence of Cl inclusion can also be seen in the differences between traces *C* and *D* for the inhibition by ouabain of percent $\Delta$ $Fl^{ce}$ of the electrogenicity of Na/K exchange by the pump (+12.8% compared with +14.7%).

In order to test the reality of the small electrogenic component associated with uncoupled Na efflux, as just discussed, this flux was inhibited in another way. Fig. 9 presents the results of an experiment where the $Mg_i$ content of tartrate ghosts (the same ghosts as used in experiment B of Table IV) was altered by the addition of EDTA and A23187, as mentioned earlier. It is apparent that the electrogenic components displayed by the pump in the Na/K and uncoupled modes (traces *A* and *B*, respectively) are both prevented by the removal of $Mg_i$ (traces *C* and D, respectively). Although these results, in tartrate ghosts, indicate that there is an

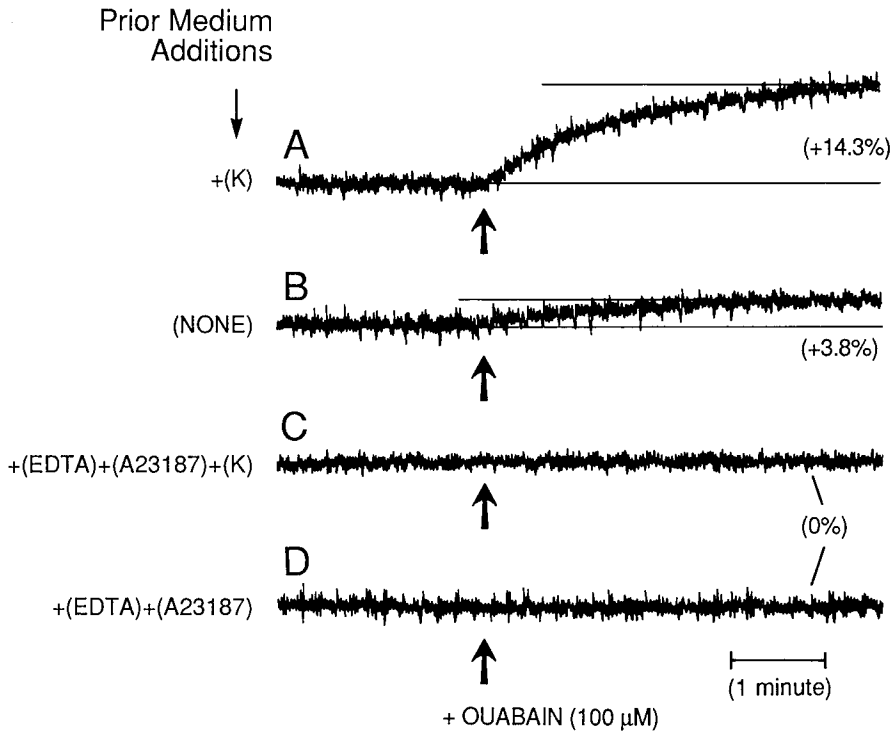


FIGURE 9. An evaluation of the $E_m$ changes that are associated with uncoupled Na efflux in tartrate ghosts in the absence of permeant anions. The figure shows tracings of experimental recordings. The preparation of ghosts, the fluorescence measurements and analyses were carried out as described in the legend to Fig. 8 and in Materials and Methods. The measurements were made on the same ghosts as used in experiment B presented in Table IV. Curves *A* and *B*, depicting the $Fl^{\alpha}$ changes that occur in the presence and absence of added K, are similar to their relevant counterparts in Fig. 8. The final concentration of K, when added, was 10 mM and in curves *C* and *D*, the divalent ionophore, A23187, was 10 μM and EDTA was 5 mM. The purpose of the pretreatment of the ghosts with A23187 and EDTA was to reduce internal Mg (see Yingst and Hoffman, 1984) in order to inhibit uncoupled Na efflux. Curve *C* shows that in the absence of $Mg_i$, the 14.3% change in $Fl^{\alpha}$ seen upon the addition of ouabain in curve *A* is inhibited; similarly, the 3.8% change in $Fl^{\alpha}$ seen after ouabain addition in curve *B* is also inhibited (curve *D*).

electrogenic component of uncoupled Na efflux, it is not clear why this was not detected in measurements such as that described in Fig. 6, unless it was shunted in the latter case by the intact cells having a higher conductance.

## DISCUSSION

The studies reported in this paper have been concerned with an analysis in human red blood cells of uncoupled Na efflux, a ouabain-sensitive process known to be mediated by the Na/K pump when both $Na_o$ and $K_o$ are absent (Garrahan and Glynn, 1967*a;* Lew et al., 1973; Karlish and Glynn, 1974). The primary finding is

that not only does the uncoupled efflux of Na occur electroneutrally (Fig. 6) but that this efflux of Na is accompanied by a cotransport of intracellular anions (Tables II and III). Thus the efflux of $SO_4$, like the efflux of Na, from DIDS-treated, $SO_4$-loaded red cells, can be inhibited by ouabain (Table II). Because we found that the ouabain-sensitive efflux of Na (Fig. 4 and Table II) exceeded the ouabain-sensitive efflux of $SO_4$, we also studied the 5 mM $Na_o$-sensitive components of Na and $SO_4$ efflux. The stoichiometry of these latter two fluxes was found (Table III) to be two Na per $SO_4$ accounting in part (60–80%) for the electroneutrality of the process. The residual portion, that is, the $Na_o$-insensitive, ouabain-sensitive component (see Fig. 4) is accounted for by the cotransport of the gamma-$PO_4$ from ATP (without mixing with cytoplasmic orthophosphate) as discussed by Marín and Hoffman (1988). Thus, the total ouabain-sensitive Na efflux appears to be a cotransport comprised of anions coming from two different cellular sources: one cytoplasmic (e.g., $SO_4$ or Cl), the other from substrate ATP (i.e., $PO_4$). The cytoplasmic component appears to be completely inhibited by substitution of $SO_4$ with the relatively impermeant anion, tartrate (Table IV, Fig. 8), the inhibition being relieved by the addition of a low concentration of a permeant anion (Cl). The slight electrogenicity displayed by the residual $PO_4$-component of uncoupled Na efflux (Fig. 8) could be a result of the general decreased membrane conductance due to the use of tartrate or could represent, in a magnified way, an electrogenic component that was otherwise masked in the absence of tartrate (Fig. 6). It should also be mentioned that, in the absence of $Na_o$, the addition of 10 mM $K_o$ to tartrate ghosts activates ouabain-sensitive Na/K exchange (Table IV) in concert with the pump's becoming electrogenic (Fig. 8) whether or not Cl is present. Furthermore, the activation of the Na/K pump by $K_o$ simultaneously acts to completely inhibit the residual $PO_4$ efflux from tartrate ghosts as well as the $SO_4$ and $PO_4$ effluxes from DIDS-treated $SO_4$-loaded intact cells as described above (see Marín and Hoffman, 1988).

An electrogenic process, such as the ouabain-sensitive exchange of three $Na_i$ for two $K_o$, is usually thought to comprise a movement of charge, initiated in this case by a net efflux of Na, that is necessarily electrically compensated by either an outward movement of an anion or an inward movement of a cation. We therefore anticipated that when the pump was performing Na/K exchange a ouabain-sensitive movement of $SO_4$ would have been evident, similar in kind to that measured when the pump was operating in its uncoupled mode. But this was not so. In addition it was also surprising that the electrogenicity of pump activation by $K_o$ in tartrate ghosts was essentially the same in the presence of Cl as in its absence (Fig. 8, Table IV). This emphasizes in a different way the elusive nature of the mechanism of charge compensation by the Na/K pump. Since net $H_o^+$ movement is excluded (see Fig. 7) other candidates that should be surveyed for their possible involvement in charge compensation include the various extracellular cations as well as the anions present inside red cells and ghosts (in addition to $HCO_3^-$) under the conditions studied. Whether Baker's (1964) finding that uncoupled Na efflux from crab nerves bathed in glucose was accompanied by the loss of aspartate and glutamate but not during Na/K exchange is relevant to the results discussed above is not known.

The foregoing considerations also emphasize the conceptual difference between an electrogenic and an electroneutral process, since in the latter case, there would presumably be no separation of charge after a completed cycle of the pump. This raises the question of the nature of the ionic mechanism that underlies the pumped cotransport of Na together with anions, such as $SO_4$. The scheme, as presented in Fig. 10, depicts the transfer of $Na_i$ to $Na_o$ that has $SO_4$ moving in an unspecified

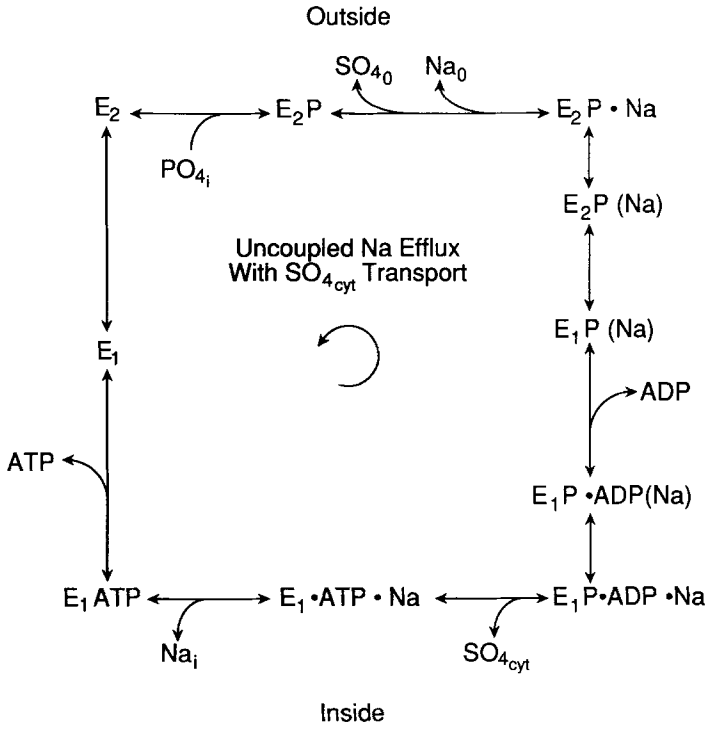


FIGURE 10. Transphosphorylation scheme (modified from Karlish et al. 1978) depicting uncoupled Na efflux with the simultaneous transport of $SO_4$. $E_1$ and $E_2$ stand for two different conformations of the enzyme, Na,K-ATPase, that are thought to occur during translocation of the ions. E can reversibly be phosphorylated, bind nucleotide and Na during the transport cycle. (Na) symbolizes occluded Na, a complexed form of Na that makes it inaccessible from either side of the membrane during translocation. $SO_4$ is depicted as moving through the membrane in an unspecified manner as Na is translocated via the indicated intermediates in the transphorylation cycle. Presumably the stoichiometry of net transport involves two Na per $SO_4$ accounting for the electroneutrality of the cotransport process (see Discussion).

fashion from the inside to the outside in concert with Na (also see later). It is, of course, not necessary that $SO_4$ traverses the membrane together with or linked to Na, only that both types of ions would be released (and taken up) in an unspecified order. Since in uncoupled Na efflux an external cation is not available for $Na_i$ to exchange with, the translocation cycle could be poised to release Na externally once a movable anion becomes available for transfer. In the absence of an available anion

the estimated electrogenic membrane potential that would be produced if each pump unit transported two or three Na alone would be <0.05 mV, i.e., below the detection limits of the methods presently available, indicating that this type mechanism would not be distinguishable from an electroneutral one. Thus this approach focuses on the release mechanism and would seem to be independent of any interaction between anions and Na during the internal membrane process of translocation. Such a mechanism would be compatible with the $E_1$ to $E_2$ transition in being sensitive to changes in the membrane potential (see DeWeer et al., 1988) that has been seen in a variety of cell types but not in red cells (Milanick and Hoffman, 1986). The basis for this difference between red cells and other cell types is not yet clear, nor is whether the underlying characteristics of transport are the same or not.

On the other hand, it is also possible that in the process of cotransport anions could, along with Na, travel together through the pump. This could mean that the "pocket" in the Na,K-ATPase complex that is involved in the occlusion of Na might also occlude small anions but not large ones. This notion was tested on purified, chymotrypsin-treated pig kidney Na,K-ATPase carried out in collaboration with I. M. Glynn, Y. Hara, and D. E. Richards. The method used was identical to that described in Glynn et al. (1984) where occlusion of the ion was estimated from the difference in the retention of the ion ($^{22}Na$) by the Na,K-ATPase treated with ATP compared to ADP. It was first found that $^{22}Na$ occlusion (tested at 0.2 mM Na in the presence of 0.5 mM $MgBr_2$) was not affected by the size of the anion, whether the anion, tested as the Tris salt (100 mM), was Cl, Br, HEPES, or glutamate. On the other hand, there was no detectable occlusion of $^{77}Br$ examined under the same conditions. In separate experiments of the same series it was also observed that the Na-ATPase activity, a component part of the Na,K-ATPase, was likewise unaffected by the various anions. While these results indicate that anion occlusion is unlikely to occur in pig kidney Na,K-ATPase, the findings may not represent the case for red cells. This is because the types of uncoupled Na efflux displayed by human red cells and pig kidney Na,K-ATPase incorporated into vesicles appear to be different from each other (see below). Independent then of anion occlusion, a possible mechanism for electroneutral cotransport is that anions could ride tandem by electrostatic interaction with the occluded Na as the latter undergoes its conformational transition through the $E_1$ to $E_2$ forms in the membrane (see Fig. 10). It is an open question whether or not these considerations also apply to the mechanism for the cotransport of $PO_4$ with Na (manuscript in preparation; see Marín and Hoffman, 1988).

To help place the results presented in this paper in the proper perspective it is of interest to discuss the semantic limitations that attend the use of the term "uncoupled" because it is now evident that this classification harbors at least three distinct types of Na effluxes. Each of these fluxes take place operationally in a medium free of both $Na_o$ and $K_o$. The first type of uncoupled Na efflux, the kind described by Garrahan and Glynn (1967*a*) and the subject of the present work, comprises a Na efflux that, as developed above, is accompanied by a cotransport of cellular anions in an electroneutral fashion. This flux occurs at $pH_o$ 7.4 and, as also mentioned before, has additional properties that distinguish it from the other types including inhibition by 5 mM $Na_o$ and tartrate, where tartrate is used to replace a transportable substrate. The second type, a kind described in inside/outside vesicles

prepared from red cells by Polvani and Blostein (1988) and with pig kidney Na,K-ATPase incorporated into lipid vesicles by Goldshlegger et al. (1989), concerns apparent changes in pump stoichiometry by the surrogate use of external protons, in place of $K_o$, in a fashion that appears to promote $Na_i/H_o^+$ exchange, as opposed to a $Na_i + OH^-$ outward cotransport. (Even if the latter were the case, by the way, the properties of the first two types of "uncoupled" Na effluxes would still be different from each other.) This $Na_i/H_o^+$ exchange flux depends on the $pH_i$'s being below 6.5 and does not occur when $pH_i$ is 6.8 or above. $Na_i/H_o^+$ exchange seen with pig kidney Na,K-ATPase is evidently electroneutral and is unaffected by whether the anion is Cl, gluconate, or aspartate (Goldshlegger et al., 1989). It is not known whether the uncoupled Na efflux studied in red cell vesicles, while presumably remaining electroneutral, becomes transposed into an anion coupled cotransport system above $pH_o$ 6.8 (Polvani and Blostein, 1988), taking on the properties described here. Because of the likelihood that this transition is made, indicating that there might be a continuum of pump characteristics in red cells, all aspects of red cell uncoupled transport should probably be put into the type one category. The kidney Na,K-ATPase on the other hand becomes electrogenic when $pH_o$ is above 7.5 and may, like the shark rectal gland (type 3), extrude Na in the absence of any countertransported ion (Goldshlegger et al., 1989). The third type is the kind described by Cornelius (1989) with shark rectal gland Na,K-ATPase incorporated into liposomes. In this case the Na efflux appears to be truly uncoupled in the sense that the measured electrogenicity appears to be completely accounted for by the Na efflux. This type of uncoupled Na efflux occurs at pH 7.0 in rectal gland Na,K-ATPase where pig kidney Na,K-ATPase still appears to be electroneutral. As noted before, the pH dependence of electrogenic uncoupled Na transport in pig kidney appears to turn on above pH 7.5. But as with the second type, other properties of this efflux (at pH 7.0 and above) are also different as well from those of the first type. For instance, in the uncoupled mode, for both the rectal gland and pig kidney Na,K-ATPase, the effect of adding $Na_o$ at low concentrations (e.g., 5 mM) is to activate $Na_i$ efflux (Cornelius and Skou, 1988; Goldshlegger et al., 1989). These comparisons emphasize not only differences in Na/K pumps obtained from different sources but also, as illustrated with red cells, that the type of uncoupled Na transport displayed is dependent upon and sensitive to the particular experimental conditions.

The results of a recent study (W. R. Martin, M. Jack-Hays, D. E Richards, R. Marín, and J. F. Hoffman, manuscript in preparation) has provided some insight into tissue and species specificity regarding the consistency in the observed properties of uncoupled Na efflux. Thus, the characteristics of ouabain-sensitive uncoupled Na efflux in DIDS-treated, sulfate-loaded pig and rat red cells were found to be the same as described above for human red cells, in that the Na efflux was inhibited by 5 mM $Na_o$ and that the Na efflux was also accompanied by the cotransport of both cytoplasmic based anions (e.g., $SO_4$) and $PO_4$ from substrate ATP. In contrast, uncoupled Na efflux measured (at pH 7.4) in lipid vesicles containing Na,K-ATPase purified from either pig, rat or human kidney was found to display the same characteristics in all three cases that were different from their red cell counterparts since Na efflux was unaffected when the major anion was switched from Cl to tartrate and 5 mM $Na_o$ activated rather than inhibited Na efflux. The contribution of

uncoupled Na efflux to Na/K exchange in the kidney vesicle preparations is <2% compared with ~15% with the various red blood cells studied. Of course, the extent to which uncoupled Na efflux occurs, if at all, in intact kidney cells is unknown. The molecular basis for the differences in uncoupled Na efflux between the red cell Na/K pump and kidney Na,K-ATPase remains to be defined. It would be interesting to know whether Na,K-ATPase isolated from red blood cells and incorporated into liposomes would show red cell or kidney type properties of uncoupled Na efflux.

The scheme presented in Fig. 10 can be used to help interpret the present findings in terms of the pump's transphosphorylation reaction mechanism. Uncoupled Na efflux is seen here to occur by ATP and $Na_i$ binding to an $E_i$ form that becomes phosphorylated before the bound Na is transmuted to an occluded form before the pump's transition to an $E_2$ form with deocclusion and subsequent release of Na externally, before $E_2$ is dephosphorylated and returns to $E_1$. Note that $(SO_4)_i$ could be assumed to associate, in an unspecified manner (see before), to an $E_1$ form that tracks the transport of Na across the membrane as the pump changes its configuration to an $E_2$ form. The rate limiting step in this sequence of reactions is thought to be dephosphorylation, the step that is evidently also inhibited by low (5 mM) concentrations of $Na_o$ (Beaugé and Glynn, 1979; Lee and Blostein, 1980). The released $PO_4$ is depicted as remaining inside since this scheme is restricted to the $Na_o$-sensitive component of uncoupled Na efflux where the origin of the transported anion (in this instance, $SO_4$) is cytoplasmic; the cotransport of $PO_4$ and Na is considered elsewhere (see Marín and Hoffman, 1988). An important aspect of the scheme that is also left unspecified concerns the stoichiometry of the coupling of anions to Na during a single cycle of uncoupled Na efflux. This is because we do not as yet have a sufficiently detailed balance sheet of fluxes to reconcile the two types (i.e., $SO_4$ and $PO_4$) of electroneutral cotransport of anions and Na with the evidence (Karlish and Glynn, 1974; Glynn and Karlish, 1976) that in uncoupled Na efflux two to three $Na_i$ are transported per ATP hydrolyzed (see Marín and Hoffman, 1988). Also left unspecified is the order of release of the several bound $Na_i$ ions (cf. Hara and Nakao, 1981; Pedemonte, 1988) together with the release of the associated anions. We can again suggest, as we have before in a different context (Kennedy et al. 1986; Hoffman, 1987), that $K_o$ (or $Na_o$) binds to the pump before all of the Na is released in order to account for the blockage of anion extrusion that occurs upon the conversion to Na/K or Na/Na exchange.

Since the human red cell pump's three-$Na_i$/two-$K_o$ exchange stoichiometry (Post and Jolly, 1957) that is known (Hoffman et al., 1979) to be electrogenic (Fig. 8) is converted, in uncoupled Na efflux, to an electroneutral process (Fig. 6), it must mean that the internal charge structure of the pump complex has been altered by the removal of $Na_o$ and $K_o$. Thus it can be thought that $Na_o$ and $K_o$ occupy regulatory signal sites that define the type of transport reaction the pump will carry out. The result of occupancy of these signal sites must be different for $Na_o$ compared with $K_o$ (whether or not occupancy also results, at least for $K_o$, in inward transport) since the $Na_o$-sensitive component of uncoupled Na efflux is distinct from the $Na_o$-insensitive component and in the source of the anions transported. The signal that converts the charge structure from electrogenic to electroneutral Na transport must also provide for the transition that underlies its linkage to anions that results in

cotransport (perhaps reflecting a change in $\alpha$- and $\beta$-subunit interaction). One way to think about this is to consider that the three sites that $Na_i$ interacts with in three-$Na_i$/two-$K_o$ exchange is composed of one neutral and two negatively charged sites. When three $Na_i$ are bound the net transport would result in the extrusion of one net positive charge. If the kinetics are ping-pong (see Sachs, 1986) then the two $K_o$ would go in on the two negative sites that were vacated by $Na_i$, together with say the spontaneous return of the vacated neutral (third) site. In the conversion to the uncoupled mode all three sites would remain neutral while occupied by Na and compensating anions as discussed before. It may be that other sites would also have to be involved to account for the stoichiometry of the two kinds of uncoupled Na efflux. It is of course possible that when the external signal sites are empty, as in the uncoupled mode, the internal charges of the pump remain unchanged, providing a basis for the electrogenic transport of three $Na_i$ by shark rectal gland referred to above in category type 3.

Whereas uncoupled Na efflux represents one type of change in ion selectivity that the red cell Na/K pump can mediate, there are other kinds that are of interest to consider in the present context. One kind, of course, occurs as $pH_o$ is lowered in the absence of both $Na_o$ and $K_o$, as mentioned previously, resulting in $Na_i/H_o^+$ exchange (Fig. 7). This exchange was found by Polvani and Blostein (1988) to take place at pH 6.2, but not at 6.8. In terms of pump reaction signal sites, this could mean that $H_o^+$ not only has to be in sufficient concentration to act as a surrogate $K_o$ (or $Na_o$) but that $H_o^+$ could also be titrating a modifier (signal) site that confers the change in selectivity. Another kind of change in the pump's selectivity is effected by $Na_o$. In this instance, increasing $Na_o$ above 5 mM activates a ouabain-sensitive Na/Na exchange (Garrahan and Glynn, 1967*c*) that is thought to occur in two different ways, one with (Glynn and Karlish, 1974; and Blostein, 1983) and the other without the hydrolysis of ATP. The former kind occurs in the absence of ADP while the latter requires ADP (Glynn and Hoffman, 1971). The Na exchange that takes place in the absence of ADP was shown by Blostein (1983) to result in an exchange of three $Na_i$/two $Na_o$ per ATP hydrolyzed. This was interpreted to indicate that in this circumstance $Na_o$ acted as surrogate $K_o$ ions as they cycled through the pump. This result could mean, in terms of uncoupled Na efflux, that if $Na_o$ at high concentration acts as a congener of $K_o$ then high $Na_o$ should result in inhibition of the $PO_4$ efflux that occurs in the absence of $K_o$. This has been tested (Marín and Hoffman, unpublished results) and it was found that the $PO_4$ flux was independent of the concentration of $Na_o$.

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