ADP + Orthophosphate (P_i) Stimulates an Na/K Pump-mediated Coefflux of P_i and Na in Human Red Blood Cell Ghosts

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ABSTRACT The Na/K pump in human red blood cells that normally exchanges 3 Na_i for 2 K_o is known to continue to transport Na in a ouabain-sensitive and ATP-dependent manner when the medium is made free of both Na_o and K_o. Although this Na efflux is called "uncoupled" because of removal of ions to exchange with, the efflux has been shown to be comprised of a coefflux with cellular anions. The work described in this paper presents a new mode of operation of uncoupled Na efflux. This new mode not only depends upon the combined presence of ADP and intracellular orthophosphate $(P_i)_i$ but the Na efflux that is stimulated to occur is coeffluxed with $(P_i)_i$. These studies were carried out with DIDS-treated resealed red cell ghosts, suspended in buffered $(NMG)_2SO_4$, that were made to contain, in addition to other constituents, varying concentrations of ADP and P_i together with Na_2SO_4, $MgSO_4$ and hexokinase. While neither ADP nor P_i was effective alone, ouabain-sensitive uncoupled Na efflux, (measured with ^{22}Na) could be activated by [ADP + P_i] where the $K_{0.5}$ for ADP in the presence of 10 mmol $(P_i)_i$/liter ghosts was 100–200 μmol/liter ghosts and the $K_{0.5}$ for $(P_i)_i$, in the presence of 500 μmol ADP/liter ghosts was 3–4 mmol/liter ghosts. [ADP + P_i] activation of this Na efflux could be inhibited by as little as 2 μmol ATP/liter ghosts but the inhibition could be relieved by the addition of 50 mM glucose, given entrapped hexokinase. While ouabain-sensitive Na efflux was found to be coeffluxed with P_i (measured with entrapped $[^{32}P]H_3PO_4$), this was not so for SO_4 (measured with $^{35}SO_4$). The stoichiometry of Na to P_i efflux was found to be ~2 to 1. Na efflux as well as $(P_i)_i$ efflux were both inhibited by 10 mM Na_o ($K_{0.5} \cong 4$ mM). But, whereas 20 mM K_o ($K_{0.5} \cong 6$ mM) inhibited the efflux of $(P_i)_i$, as would be expected from previous work, Na efflux was actually increased. When K_o influx was measured in this situation there was a 1 for 1 exchange of Na_i for K_o, that is, of course, downhill with respect to the gradient of each ion. Surprisingly AsO_4 was unable to replace P_i for activation of Na efflux but Na efflux could be inhibited by vanadate and oligomycin. In terms of mechanism, it is likely that ADP acts to promote the

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formation of the phosphoenzyme (EP) by $(P_i)_i$ that would otherwise be inhibited by Na_i. The subsequent translocation of Na and P_i via the E_1P to E_2P transition could also provide for their release to the outside. Evidence in support of this interpretation is based on the finding that P_i efflux is inhibited by Na_o and that Na_o, in inhibiting ouabain binding, inhibits the formation of EP. Two modified Albers-Post reaction schemes are presented that depict possible intermediates that underlie [ADP + P_i]-dependent coefflux of Na and P_i and the effects of K_o.

INTRODUCTION

This is the third paper in a series that is concerned with uncoupled Na efflux in human red blood cells (see Dissing and Hoffman, 1990; Marín and Hoffman, 1994). This flux, first described by Garrahan and Glynn (1967*a*), is known to be a partial reaction of the red cell Na/K pump on the basis that the Na efflux is inhibited by cardiotonic steroids, such as ouabain, and is dependent on MgATP as an energy source. The reason this flux is called uncoupled is that it occurs in the absence of external Na (Na_o) and K (K_o). In fact, low concentrations of Na_o (e.g., 5 mM) inhibit a major portion of the Na efflux whereas K_o converts the efflux to an exchange of K_o with internal Na (Na_i). Having found that uncoupled Na efflux was electroneutral, it was subsequently established that intracellular anions, from two separate sources, were cotransported out and coupled to the efflux of Na (Dissing and Hoffman, 1990; Marín and Hoffman, 1994). One source of anions was cytoplasmic (SO_4 or Cl) and these were effluxed in a Na_o-sensitive as well as in a ouabain-sensitive manner (Dissing and Hoffman, 1990). The other source of anions was completely unexpected for it turned out to be orthophosphate (P_i) that originated, not from the intracellular pool of P_i but from the terminal gamma phosphate of ATP (Marín and Hoffman, 1994). This result strongly implied that the Na-coupled efflux of P_i was transferred via the pump's (E) phospho-intermediate (EP). This P_i efflux was ouabain-sensitive but Na_o-insensitive. Thus, it is evident that there are two types of ATP-dependent uncoupled Na efflux. For convenience these are classified as types IA and IB, where type IA refers to cytoplasmically based anions that are cotransported with Na in a Na_o-sensitive manner and type IB refers to the transfer of Na together with the gamma phosphate of ATP in a Na_o-insensitive manner. While other characteristics of these two types of uncoupled Na efflux are considered later, this classification provides a perspective for a third type of uncoupled Na efflux, designated type II, that is the subject of this paper.

The present study arose as a result of questioning the fate of the product, ADP, in uncoupled Na efflux. If ADP stayed bound to the enzyme (although this is not a prerequisite), then perhaps the pump could turn over, in a downhill manner, by the cyclic binding and release of P_i. It turned out that when both ADP and P_i were entrapped in resealed human red cell ghosts, uncoupled Na efflux was, indeed, stimulated to take place. This efflux was, like type IA uncoupled Na efflux, inhibitable by both ouabain and Na_o. But the dramatic and unsuspected result was that P_i, cytoplasmically based, was coeffluxed in concert with Na. The characteristics of the P_i efflux, as in type IB uncoupled Na efflux, are consistent with the transport of P_i occurring via EP. Thus, type II uncoupled Na efflux is defined as being

[ADP + P$_i$]-dependent and shares certain properties with both types IA and B as described below. A preliminary account of some of this work was previously reported (Marín and Hoffman, 1986).

MATERIALS AND METHODS

The studies reported in this paper utilize resealed human red blood cell ghosts that had been prepared to contain different added constituents. The procurement of blood and the initial hemolysis and washing of the ghosts up to the stage where the ghosts were concentrated, before being transferred to the reversal medium, were carried out following the same protocol as described in Marín and Hoffman (1994). For the present purposes the composition of the reversal solution unless otherwise specified, was as follows: 12.5 mM Na_2SO_4, 5 mM $MgSO_4$, 25 mM $Tris_2SO_4$, from 0 to 10 mM Tris H_2PO_4, from 0 to 500 μM $Tris_2$-ADP, 50 mM glucose, 70 U Hexokinase, 50 μM Trypan blue, 40 μM P^1,P^5-di(adenosine-5′)pentaphosphate (Ap5A), together with sufficient $(NMG)_2SO_4$ (*N*-methyl glucamine sulfate) to bring the osmolarity of the solution to 300 mosM and adjustment of the pH to 7.5 at 0°C. When K was present it was added as K_2SO_4 in substitution for an equivalent concentration of $(NMG)_2SO_4$. To measure their unidirectional effluxes, the reversal solution also contained either 2 μCi ^{22}Na/ml reversal solution or 5 μCi/ml reversal solution of either $^{32}PO_4$ or $^{35}SO_4$. These isotopes were introduced during the 15-min period that followed the mixing of the ghosts and reversal solution that provided for equilibration of the added constituents. The resealing of the ghosts was then carried out by incubating the suspension for 45 min at 37°C, in a reciprocating water bath. 5 min before the end of the resealing period, DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid) was added to a final concentration of 50 μM in the resealing medium. (DIDS is assumed to act only on the outside of transporting ghosts since resealing of the ghosts was essentially complete by 40-min incubation.) At the end of the resealing period, the suspension was cooled in an ice bath for 5 min and then centrifuged at 48,000 *g* (4°C) for 10 min. The resealed ghosts (now bluish) were washed twice with a solution containing 25 mM $Tris_2SO_4$ and requisite amounts of $(NMG)_2SO_4$ to bring up the osmolarity to 300 mosM. The ghosts (50% suspension) were kept at 4°C until used as described below.

The Ap5A (Lienhard and Secemski, 1973) and the Trypan blue (Kaplan and Hollis, 1980) were incorporated within the ghosts in order to inhibit any remaining residual adenylate kinase activity and nucleoside phosphorylase activity, respectively. The use of DIDS not only provided for the entrapment of SO_4 (Dissing and Hoffman, 1990), PO_4 (Bodemann and Hoffman, 1976) and AsO_4 (Kenney and Kaplan, 1988*b*) within the ghosts by preventing efflux via the Band 3 anion exchanger, but also lowered the background membrane conductance resulting in more accurate estimates of ouabain-sensitive anion permeability (Hoffman, 1992). Note also that in all cases studied the ghosts were prepared to contain a final concentration of Na_i of 25 mmol/liter ghosts. The concentration of K_i, unless specifically incorporated, was always nominally zero (<50 μmol/liter ghosts). It should also be stated that all solutions used were preequilibrated by gassing with N_2 to reduce contamination of CO_2/bicarbonate (see Marín and Hoffman, 1994).

Nucleotide Determinations

The concentrations of ADP and ATP were determined by methods previously described (Kennedy, Lunn, and Hoffman, 1986; Dissing and Hoffman, 1990). It should be noted that the final concentration of ADP measured at the end of the resealing process was less than that present in the reversing solution. Averaged for all experiments where the initial ADP concentration was 1 mM, the concentration of ADP (±SEM) at the end of resealing was 589 ± 46 μM, which fell to 473 ± 28 μM at the end of the standard 35-min flux period that was also

carried out at 37°C. The change in ADP concentration reflects two effects. The first involves the dilution of the ADP added in the reversal solution with the ghost's intracellular volume. The second concerns the breakdown that occurs during the resealing incubation as well as during the incubation associated with the flux measurement. Thus, where a concentration of 500 μM is given as the ADP content of the ghosts, it should be recognized that this is an approximation based on the above averaged results. A similar approximation would apply to the values when the ADP concentrations were experimentally varied (cf, Table I).

Flux Measurements

Na efflux for each experimental condition was carried out in quadruplicate (or as specified) in the presence and absence of 10^{-4} M ouabain. For each replicate, Na efflux was started by adding 200 μl of a 50% ghost suspension to 6 ml of flux medium (final hematocrit 1–4%) contained in an Erlenmeyer flask preincubated for 5 min at 37°C. 600 μl aliquots were taken at 5, 15, 25, and 35 min except for the results presented in Table II. The ghosts were pelleted in a microcentrifuge for 90 s (model 235C, Allied Fisher Scientific Philadelphia, PA), and 500-μl samples of the supernatant were assayed for radioactivity with a gamma counter. 500 μl samples of the total suspension were also counted.

The rate constant for Na efflux was estimated (cf, Hoffman, 1962*a*) from the relation ${}^{o}k_{Na} = \ln(1 - R_s/R_{eq})t^{-1}$, where ${}^{o}k_{Na}$ is the rate constant in h^{-1}, R_s is the radioactivity of each supernatant sample taken at time, t, and R_{eq} is the radioactivity of the suspension mixture. Thus, ${}^{o}k_{Na}$ was determined from the slope (calculated by least squares) of the $\ln(1 - R_s/R_{eq})$ plotted against t. An initial rapid loss of radioactivity was routinely observed during the first 5 min of Na efflux that represented only ~15% of the total counts. To circumvent this loss, rate constants were calculated for the incubation period between 5 and 35 min, during which the slope of the ln transform was linear. The same method was used to assay $^{35}SO_4$ or $^{32}PO_4$ ($^{32}P_i$) efflux. The rate constants for $^{35}SO_4$ or $^{32}P_i$ efflux were calculated as mentioned above. The ouabain-sensitive fluxes (in mmol/liter packed ghosts × h) for each experimental condition were calculated by subtracting the mean rate constant obtained in the presence of ouabain from that in the absence of ouabain, and multiplying the difference by the intracellular concentration of Na, P_i or SO_4, respectively. The Na concentration of the resealed packed ghosts was determined by flame photometry in ghosts previously washed in an isotonic $MgCl_2$ solution. The SO_4 concentration of the resealed ghosts was determined by hemolyzing the $^{35}SO_4$ packed ghosts in 0.15 M perchloric acid and relating the $^{35}SO_4$ radioactivity in the hemolysate (in counts/liter packed ghosts) to the specific activity of the $^{35}SO_4$ in the resealing medium (counts $^{35}SO_4$/mmol SO_4). The P_i concentration of the packed ghosts was determined following the method of Forbush (1983*a*) in ghosts previously washed in an isotonic $MgCl_2$ solution and hemolyzed with distilled water.

K influx determinations were initiated by adding about 250 μl of a 50% ghost suspension to 6 ml of incubation medium that contained 94 or 85 mM $(NMG)_2SO_4$ together, respectively, with either 0.5 or 5 mM K_2SO_4, 20 mM $Tris_2SO_4$ (pH 7.4 at 37°C), 36 $\mu Ci^{42}K$. In addition, the incubations were carried out in the presence and absence of 10^{-4} M ouabain. 1-ml samples of the suspension were taken at 5, 15, 25, and 35 min and washed three times (to remove medium radioactivity) at 4°C by centrifugation (10 min at 6,000 *g*) and resuspension in unbuffered hypertonic (220 mM) $MgCl_2$. The hypertonicity was found to be important because preliminary experiments showed that shrunken ghosts retained their radioactivity during the washing treatment, while ghosts washed more than once with unbuffered isotonic $MgCl_2$ (~110 mM) lost radioactivity after two washes, presumably from rehemolysis. After washing, the packed ghosts were hemolyzed with 2 ml distilled water. The radioactivity of the hemolysate was then assayed in a gamma counter. The specific activity of the medium, which did not change during

the incubation, was determined separately for each K_o concentration used. Influxes for each experimental condition were performed in quadruplicate. The influxes were calculated as described in Marín and Hoffman (1994).

Reagent Sources

All reagents other than the following were purchased from Sigma Chemical Co. (St. Louis, MO) (see Marín and Hoffman, 1994). Oligomycin (61% A, 34% B, 5% C) was obtained from Calbiochem Corp. (La Jolla, CA). The radioactive isotopes, ^{22}Na, ^{42}K, $^{35}SO_4$, and [^{3}H] ouabain were from New England Nuclear Corp. (Boston, MA); [^{32}P]H_3PO_4 was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA).

RESULTS

As mentioned in the introduction, this paper is concerned with defining, in human red blood cells, the characteristics of uncoupled Na efflux of the second type, the type that is driven by P$_i$ and ADP. This stands in contrast to type I uncoupled Na efflux, that was originally described by Garrahan and Glynn (1967*a*), and refers to ouabain-sensitive, ATP-dependent uncoupled Na efflux that, in analogy with type II, is coupled to anion transport (Dissing and Hoffman, 1990; Marín and Hoffman, 1994).

Dependence of Uncoupled Na Efflux on [P$_i$ + ADP]

Evidence that type II, defined as ouabain-sensitive, [ADP + P$_i$]-dependent, uncoupled Na efflux, occurs in human red cell ghosts is presented in Table I. Here it is apparent that ouabain-sensitive uncoupled Na efflux ($^oM_{Na}^{ouab}$) requires for activation the combined presence of P$_i$ and ADP since there is no activation of Na efflux by ADP in the absence of P$_i$ (Experiment A) or by P$_i$ in the absence of ADP (Experiment B). Because the principal anion present is SO_4, it is also clear (Experiment A) that, in the absence of P$_i$, SO_4 will not substitute for P$_i$ in [ADP + P$_i$]-promoted uncoupled Na efflux. The concentration of P$_i$ that gives one-half maximal activation ($K_{0.5}^{Pi}$) of $^oM_{Na}^{ouab}$ in the presence of 500 μM ADP approximates 3–4 mM P$_i$ (Experiment A) whereas $K_{0.5}^{ADP}$ in the presence of 10 mM P$_i$ is ~ 100–200 μM ADP (Experiment B) indicating that ADP presumably binds to the pump with low affinity (see Glynn, 1985). It should be mentioned that the average maximum value of $^oM_{Na}^{ouab}$ obtained in these two experiments (0.7 mmol Na/liter ghosts × h) is also representative of the results as presented in subsequent tables, and is ~70% of the maximum value that is normally seen for type I uncoupled Na efflux that is driven by ATP (Dissing and Hoffman, 1990). On the other hand, the ouabain-insensitive component is considerably larger (20- to 30-fold) in ghosts used to study type II compared to type I uncoupled Na efflux (see Dissing and Hoffman, 1990) but the basis for this increase and the extent to which it represents a leakage component is not clear. The fact that the ouabain-insensitive component is large in these DIDS-treated ghosts, independent of whether they contain P$_i$ and/or ADP, may reflect a protective effect of ATP if not other differences in their preparative protocols.

To test the extent to which low concentrations of ATP could be involved with [ADP + P$_i$]-dependent Na efflux, ghosts were loaded to contain ~2 μmol ATP/liter ghosts (maintained constant, over the measured flux interval, with an entrapped

regenerating system as described in the legend to Table II) together with hexokinase, Ap5A and Trypan blue. The results presented in Table II show that this concentration of ATP almost completely inhibited [ADP + P_i]-dependent uncoupled Na efflux in the absence but not in the presence of 50 mM glucose. Presumably, hexokinase in this situation acts as a scavenger to reduce the concentration of ATP to ineffectual levels (see legend) by catalyzing the formation of glucose-6-PO_4 from glucose, thereby preventing the inhibition. (This type of competition between the Na/K pump and the hexokinase reaction for ATP has been exploited before for a different

TABLE I

The Concentration Dependence of [Orthophosphate (P_i) + ADP] and Their Role in Supporting Uncoupled Na Efflux (Type II) from Reconstituted Human Red Blood Cell Ghosts

Experiment	P_i	ADP	${}^ok_{Na}$		${}^oM_{Na}^{ouab}$
			Alone	+Ouabain	
	mM	*µM*	h^{-1}		*mmol/liter ghosts × h*
A	0	500	0.605 ± .002	0.604 ± .003	0.03 ± .09
	2.5	500	0.617 ± .002	0.606 ± .002	0.28 ± .07
	5	500	0.630 ± .002	0.606 ± .003	0.60 ± .09
	10	500	0.632 ± .002	0.605 ± .002	0.68 ± .07
B	10	0	0.596 ± .003	0.595 ± .002	0.03 ± .09
	10	25	0.603 ± .003	0.601 ± .001	0.05 ± .08
	10	100	0.614 ± .002	0.602 ± .002	0.30 ± .07
	10	250	0.630 ± .002	0.600 ± .002	0.75 ± .07

The ghosts were DIDS-treated during resealing (see Methods) in the presence of 25 mM Na (as Na_2SO_4), a tracer quantity of ^{22}Na (~1 µCi/ml medium), 5 mM $MgSO_4$, 40 µM Ap5A, 70 U hexokinase, 50 µM Trypan blue, 50 mM glucose, 25 mM $Tris_2SO_4$ (pH 7.5 at 23°C) + the indicated concentrations of PO_4 (as Tris H_2PO_4) and $Tris_2$-ADP together with sufficient $(NMG)_2SO_4$ to bring the final osmolality to 300 mosmol/liter. The outward rate constant of Na (${}^ok_{Na}$, in reciprocal hours) was measured over a 35-min period after washing (at 4°C) and suspension of the ghosts (final hematocrit 2–5%, 37°C) in a medium that was free of Na_o and K_o but contained 95 mM $(NMG)_2SO_4$, 50 mM glucose and 20 mM $Tris_2SO_4$ (pH 7.5 at 23°C). Ap5A, Trypan blue and hexokinase + glucose are used to maintain the entrapped ADP as free of other nucleotides, such as ATP, as possible. The symbol, ${}^oM_{Na}^{ouab}$ (in mmol/liter ghosts × h) in representing the ouabain-sensitive component of Na efflux, is calculated from the difference in the values of ${}^ok_{Na}$, obtained in the presence and absence of 100 µM ouabain, multiplied by Na_i (25 mmol Na/liter ghosts). The results where P_i is varied at constant ADP (Experiment A) is from a different experiment than that where ADP is varied at constant P_i (Experiment B). The values in the table represent the means ± SEM, where $n = 4$. See text for discussion.

purpose [see Hoffman, 1962*b;* 1980]). Thus, in order to avoid any possible inhibitory effects of ATP, hexokinase and glucose were standard inclusions in all of the other experiments reported in this paper. On the other hand, the mechanism that underlies the inhibition of type II Na efflux by ATP is not known but it is reasonable to assume that if ATP binds to its high affinity site on E_1 to form E_1·ATP (Hegyvary and Post, 1971; and Nørby and Jensen, 1971), then P_i and ADP could act as product inhibitors (Kennedy et al., 1986).

Effect of Na$_o$ on Type II Uncoupled Na Efflux

The results presented in Table III and Fig. 1 show that [ADP + P$_i$]-promoted Na efflux is inhibited by Na$_o$ and this inhibition by Na$_o$ occurs whether or not K$_o$ and/or K$_i$ is present. It is also evident from the results depicted in Fig. 1 that the concentration of Na$_o$ needed to inhibit the Na efflux by 50% ($K_{0.5}^{Nao}$) is ~4 mM. This effect of Na$_o$ in inhibiting [ADP + P$_i$]-promoted Na efflux, in the absence of K$_o$, is similar to that seen in type I or ATP-dependent Na efflux. But the two types differ quantitatively in that in type II, maximal inhibition occurs at ~ 10 mM Na$_o$ and is complete (Fig. 1) whereas in type I, the maximum effect occurs at 5 mM Na$_o$ and the inhibition elicited is no more than 80% (Garrahan and Glynn, 1967*a;* Glynn and Karlish, 1976; Dissing and Hoffman, 1990). It has been suggested (Beaugé and

TABLE II

The Effect of ATP on [P$_i$ + ADP]-promoted Ouabain-sensitive Uncoupled Na Efflux (Type II) from Reconstituted Human Red Blood Cell Ghosts

Glucose	$^ok_{Na}$		$^oM_{Na}^{ouab}$
	Alone	+Ouabain	
mM	*h^{-1}*		*mmol/liter ghosts × h*
0	0.490 ± .002	0.489 ± .002	0.03 ± .09
50	0.521 ± .002	0.489 ± .002	0.80 ± .07

By use of the protocol described in the legend to Table I (see also Methods section), ghosts were resealed in the presence of 10 mM P$_i$ + 500 μM ADP together with the other constituents listed. In this instance the ghosts also contained, in addition to hexokinase, a creatine kinase/arginine kinase regenerating system (see Marín and Hoffman, 1994) to maintain ATP at ~2 μmol/liter ghosts in the absence of glucose and nominally zero (< 1 nmol/liter ghosts) in its presence. The regenerating system, present in the reversing/resealing medium, consisted of (millimolar): 1000 arginine, 1 arginine phosphate, 10 creatine, 1 creatine phosphate, 2 ATP, 500 ADP (as mentioned before) together with, respectively, 20 and 300 U/ml reversal medium of creatine kinase and arginine kinase. In this experiment the measured values (means ± SEM, where n = 3) of ATP and ADP, determined after resealing, were, respectively, 2.3 ± 0.4 and 438 ± 18 μmol/liter ghosts. The ouabain-sensitive Na efflux ($^oM_{Na}^{ouab}$) was also determined as before in the presence and absence of 50 mM glucose, where sampling took place at 0, 10, 20, 30 and 40 min. The values in the table represent the means ± SEM, where n = 6.

Glynn, 1979) that Na$_o$ acts to inhibit ATP-dependent Na efflux by slowing or inhibiting dephosphorylation of the associated phosphoenzyme but it is unlikely that this explanation also applies in the type II case because the Na,K-ATPase may not be phosphorylated in the presence of Na$_o$.

The idea that Na$_o$ might act to inhibit type II uncoupled Na efflux by preventing phosphorylation can be indirectly assessed from studies concerned with the interrelationships between [Mg^{++} + P$_i$]-promoted phosphorylation (Post et al., 1973; Post, Toda, and Rogers, 1975; Askari and Huang, 1984) and the binding of cardiotonic steroids such as digitalis and ouabain (Schwartz, Matsui, and Laughter, 1968; Hansen and Skou, 1973; Bodemann and Hoffman, 1976). The studies referred to were carried out on unsided preparations of Na,K-ATPase from kidney (Post et al., 1973,

1975; Askari and Huang, 1984), heart (Schwartz et al., 1968), brain (Hansen and Skou, 1973) and porous human red cell ghosts (Bodemann and Hoffman, 1976), so it was not possible to distinguish whether the effects were due to Na_o or Na_i or both. It should also be mentioned that because the binding of ouabain is known to stabilize the phosphoenzyme, the relative rate of ouabain binding has been used as an indirect measure of the phosphorylated state of the system (see Forbush, 1983*b*). Thus, when [Mg^{++} + P_i]-promoted ouabain binding was analyzed in resealed human red cell ghosts, i.e., a sided preparation, it was found (Guerra, Steinberg, and Dunham, 1992) that the rate of ouabain binding (in the absence of K_i and K_o) was inhibited by Na_o as well as by Na_i. When this approach is now extended to the type II or [ADP + P_i] situation, it is seen (Table IV) that the ouabain binding rate (molecules bound in 30 min) is inhibited by Na_o but not by Na_i. These results imply that Na_o inhibits

TABLE III

The Effects of Na_o, K_o and K_i on Type II, or Ouabain-sensitive, [P_i + ADP]-promoted, Uncoupled Na Efflux

Na_o	K_o	K_i	${}^{o}k_{Na}$		${}^{o}M_{Na}^{ouab}$
			Alone	+Ouabain	
mM	*mM*	*mmol/liter ghosts*	h^{-1}		*mmol/liter ghosts × h*
0	0	0	0.641 ± .001	0.611 ± .001	0.75 ± .06
0	20	0	0.660 ± .003	0.615 ± .003	1.13 ± .11
0	0	120	0.623 ± .003	0.594 ± .002	0.73 ± .09
0	20	120	0.631 ± .002	0.602 ± .002	0.73 ± .07
10	0	0	0.608 ± .002	0.606 ± .003	0.05 ± .09
10	20	0	0.613 ± .001	0.613 ± .001	0.00 ± .06
10	0	120	0.634 ± .002	0.634 ± .002	0.00 ± .07
10	20	120	0.609 ± .003	0.608 ± .002	0.03 ± .09

Resealed ghosts were prepared, by the protocol described before (legend to Table I, Methods section), to contain 10 mM P_i + 500 μM ADP together with the other constituents listed. The ghosts also contained 25 mmol Na/liter ghosts and where indicated, 120 mmol K/liter ghosts (by substituting an appropriate amount of K_2SO_4 for $(NMG)_2SO_4$). The indicated concentrations of Na_o (as Na_2SO_4) and K_o (as K_2SO_4) were substituted for equivalent concentrations of $(NMG)_2SO_4$, keeping the total medium osmolarity at ~300 mosM. The ouabain-sensitive Na efflux (${}^{o}M_{Na}^{ouab}$) was also determined as described before. The results of a single experiment are shown. The values in the Table represent the means ± SEM, where $n = 4$.

[ADP + P_i]-dependent uncoupled Na efflux by preventing the formation of the phosphoenzyme; the results also imply that the presence of ADP prevents the inhibition exerted by Na_i in the [Mg^{++} + P_i]-promoted case, thereby providing for the phosphorylation of the system in order that Na transport can occur (see Discussion and legend to Fig. 2).

Effect of K_o and K_i on Type II Uncoupled Na Efflux

In contrast to Na_o, K_o acts to stimulate [ADP + P_i]-promoted Na efflux as also evidenced by the results presented in Table III and Fig. 1. The concentration of K_o that gives half-maximal activation ($K_{0.5}^{Ko}$) of Na efflux is seen (Fig. 1) to approximate 6 mM, similar to the inhibitory value of $K_{0.5}^{Nao}$ obtained for Na_o. But whether this

similarity is significant or not is not known nor, for that matter, whether Na_o and K_o interact with each other or if their respective $K_{0.5}$ values are affected by K_i. On the other hand, it is apparent that K_i prevents the stimulation of Na efflux that occurs in the presence of K_o (Table III) even though it is without effect by itself on the efflux of Na. If these effects of K_o and K_i on Na efflux are related to their respective concentration gradients then it is possible that the efflux of Na moving down its gradient, from in to out, could be stimulated by the influx of K_o moving down its gradient, from out to in, promoting the passive turnover of the pump apparatus. K_i in this situation would act to neutralize (reverse) the gradient established by K_o but would still allow for the turnover of the pump. (The presumed action of Na_o in inhibiting Na efflux is not gradient related but is based on its kinetic effects to prevent phosphorylation as mentioned above.)

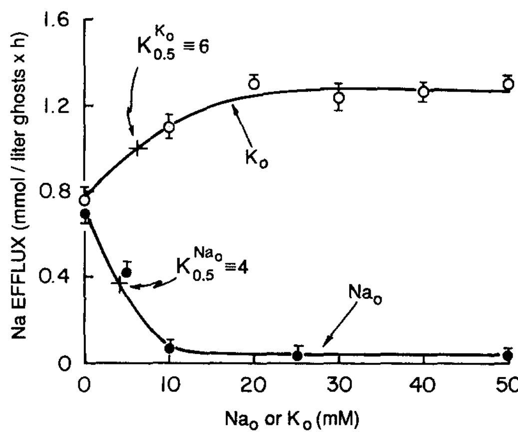


FIGURE 1. The inhibition by Na_o or the activation by K_o of ouabain-sensitive [P_i + ADP]-dependent Na efflux. Resealed ghosts were prepared, according to the protocol described before (legend to Table I, Methods section), in the presence of 10 mM P_i + 500 μM ADP together with the other constituents listed. The ouabain-sensitive efflux of Na was determined as described before by suspension of the ghosts (at 2–3% hematocrit) in media containing the indicated concentrations of Na_o (*filled circles*) or K_o (*open circles*). The concentrations of Na_o (as Na_2SO_4) and K_o (as K_2SO_4) were substituted for equivalent concentrations of $(NMG)_2SO_4$ where the osmolarity was kept constant at approximately 300 mosM. The results in which K_o and Na_o were varied were obtained in separate experiments. The data points represent the means ± SEM, where n = 4.

Regardless of the correctness of the foregoing argument (see later), it led to the measurement of K influx under the same circumstances that [ADP + P_i]-dependent Na efflux had been determined. The results are presented in Table V where it is apparent that not only is there a concomitant ouabain-sensitive influx of K but that the influx is stimulated by increasing the concentration of K_o from 1 to 10 mM. The stoichiometry of this [ADP + P_i]-promoted exchange of Na_i for K_o was estimated (see Table V) from separate measurements of ${}^oM_{Na}^{ouab}$ and ${}^iM_K^{ouab}$ on ghosts prepared from the same individual's blood in which the same protocols were followed in order to minimize variation. Thus, for the condition when K_o was 10 mM, the stoichiometric ratio of ${}^oM_{Na}^{ouab}$ to ${}^iM_K^{ouab}$ was 0.98 and indicates that the [ADP + P_i]-promoted "uncoupled" efflux of Na that is presumed to occur in the absence of K_o is converted to a 1 for 1 exchange of Na_i for K_o in the presence of K_o. Further work is needed, however, to establish not only the linkage but also the accuracy of the Na_i to K_o stoichiometry as well as the effect of K_i on ${}^iM_K^{ouab}$.

Given the fact that K_o stimulated downhill Na_i/K_o exchange, the possibility arose that ATP might be synthesized during [ADP + P_i]-dependent Na efflux. To test this notion, ghosts were prepared in the presence of 10 mM P_i + 500 μM ADP, in addition to the standard constituents, and incubated in the usual manner with 50 mM glucose and in the presence and absence of 20 mM K_o. Because the ghosts contained hexokinase, any synthesis of ATP could be followed by measuring (see Bergmeyer, 1974) the production of glucose-6-phosphate (G-6-P). However, no ouabain-sensitive synthesis of ATP was detected over an 80 min incubation period even though there was a background production of ~16 nmol G-6-P/mg ghost protein during this time that was the same regardless of the incubation conditions (data not shown). This result was not surprising in light of the fact that the [ADP + P_i]-dependent efflux of

TABLE IV

The Effect of Na_i and Na_o on the Ouabain Binding Rate to Reconstituted Ghosts Containing [ADP + P_i]

Na_i	Na_o	Ouabain molecules bound/ghost in 30 min	
		Experiment 1	Experiment 2
mmol/liter ghosts	*mM*		
25	0	161	155
25	10	3	5
0	0	154	—
0	10	7	—

Resealed ghosts were prepared, by the protocol described before (legend to Table I, Methods section), in the presence of 10 mM P_i (as H_3PO_4) + 500 μM ADP (as $Tris_2ADP$) together with the other constituents listed except for Trypan blue. (Omission of Trypan blue avoids quenching the counting of [^{3}H]-ouabain but results in about a 40% inhibition of $^{o}M_{Na}^{ouab}$.) The indicated concentration of Na_i and Na_o were obtained by adding an appropriate amount of Na_2SO_4, in substitution of $(NMG)_2SO_4$, keeping the total osmolarity at ~300 mosmol/liter. The medium contained in addition to $(NMG)_2SO_4$: 20 mM HEPES (pH 7.5 at 23°C), 50 mM glucose, 1×10^{-7} M [^{3}H]-ouabain ± 1×10^{-4} M ouabain. The ouabain binding rates were determined as described in Bodemann and Hoffman (1976). The results presented represent the average of duplicate determinations. See text for discussion.

Na would be expected to be a sensitive indicator of ATP generation since the efflux is inhibited by micromolar concentrations of ATP as discussed above.

[ADP + P_i^+] Promotes Ouabain-sensitive P_i Efflux

Because cellular anions were found to be cotransported with Na in type I or ATP-dependent uncoupled Na efflux (Dissing and Hoffman, 1990; Marín and Hoffman, 1994) it was of interest to study the extent to which anion transport may occur in type II or [ADP + P_i]-promoted uncoupled Na efflux. Ghosts were thus labeled with either $^{32}P_i$ or $^{35}SO_4$ during entrapment of 10 mM P_i and 50 mM SO_4 in their normal preparation before exposure to DIDS and before resealing at 37°C. The results of these studies are presented in Table VI where it is clear that, in the absence

TABLE V

[ADP + P$_i$] Promotes an Ouabain-sensitive Influx of K into Red Cell Ghosts

K_o	^iM_K		$^iM_K^{ouab}$	$^oM_{Na}^{ouab}$
	Alone	+Ouabain		
mM	*mmol/liter ghosts × h*		*mmol/liter ghosts × h*	
1.0	1.46 ± .013	0.60 ± .002	0.86 ± .01	—
10	1.72 ± .003	0.60 ± .002	1.12 ± .04	1.10 ± .11

Resealed ghosts were prepared in accordance with the protocol described before (legend to Table I, Methods section), in the presence of 10 mM P$_i$ + 500 μM ADP together with the other constituents listed. The K influx was measured, with ^{42}K as described in Methods, by suspension of the ghosts, at 2.5% hematocrit, in media that contained the indicated concentrations of K_o. K_o (as K_2SO_4) was added to the medium in substitution for an osmotically equivalent concentration of $(NMG)_2SO_4$. $^oM_{Na}^{ouab}$ was measured as described in the legend to Table III under the same circumstance as K influx (see text). The values for the ouabain-sensitive K influx, $^iM_K^{ouab}$, as well as $^oM_{Na}^{ouab}$ represent the means ± SEM, where $n = 4$.

of K_o or Na_o, there is a ouabain-sensitive efflux of P$_i$ but not of SO_4. It is also clear that the efflux of P$_i$ is inhibited by K_o and by Na_o. Na_o, it will be recalled (see Table III) also shuts down Na efflux but this was not so for K_o where Na efflux was actually stimulated. Together, the foregoing results make it evident that in the absence of K_o, P$_i$ is coeffluxed with Na (Table VI), whereas in its presence, the cotransport is converted to a one for one exchange of Na_i for K_o (Tables III, V). This effect of K_o in type II uncoupled Na efflux is analogous to its actions in type I or ATP-dependent uncoupled Na efflux. In type I, without K_o, cellular anions are also cotransported

TABLE VI

The Efflux of P$_i$ and SO$_4$ that Occurs during Type II, or [ADP + P$_i$]-promoted Uncoupled Na Efflux

Anion measured	Na_o	K_o	$^ok_{Anion}$		$^oM_{Anion}^{ouab}$
			Alone	+Ouabain	
	mM	*mM*	*h^{-1}*		*mmol/liter ghosts × h*
$^{32}P_i$	0	0	0.521 ± .005	0.481 ± .004	0.40 ± .06
	10	0	0.477 ± .003	0.474 ± .003	0.03 ± .04
	0	0	0.492 ± .003	0.453 ± .002	0.39 ± .04
	10	0	0.458 ± .002	0.456 ± .002	0.02 ± .03
	0	20	0.458 ± .002	0.457 ± .002	0.01 ± .03
$^{35}SO_4$	0	0	0.056 ± .002	0.058 ± .002	0 ± .03
	10	0	0.056 ± .002	0.057 ± .002	0 ± .03

Resealed ghosts were prepared in accordance with the protocol described before (legend to Table I, Methods section) in the presence of 10 mM P$_i$ + 500 μM ADP together with the other constituents listed. The ghosts also contained 25 mM Na/liter ghosts as well as 40 mM SO_4/liter ghosts. Before resealing and exposure to DIDS the ghosts were loaded with either $^{32}PO_4$ or $^{35}SO_4$ after splitting the batch of ghosts into two subgroups. The efflux of $^{32}P_i$ and $^{35}SO_4$ were measured, respectively, into media containing the indicated concentrations of Na_o (as Na_2SO_4) and K_o (as K_2SO_4) that were substituted for osmotically equivalent concentrations of $(NMG)_2SO_4$. The efflux of $^{32}P_i$ and $^{32}SO_4$ were measured in the presence and absence of 100 μM ouabain with the ouabain-sensitive efflux of anions ($^oM_{Anion}^{ouab}$) representing either $^oM_{Pi}^{ouab}$ or $^oM_{SO4}^{ouab}$, and calculated as described before. The values in the table represent the means ± SEM, where $n = 4$.

with Na out of the cell, but the addition of K_o activates in this instance the pumped exchange of Na_i for K_o, which pari passu inhibits completely the efflux of anions (Dissing and Hoffman, 1990; Marín and Hoffman, 1994.)

The stoichiometry of Na/P_i cotransport can be estimated from experiments where ${}^oM_{Na}^{ouab}$ and ${}^oM_{Pi}^{ouab}$ were measured on ghosts prepared to contain the same concentrations of P_i (10 mM) and ADP (500 μM) and incubated in media free of Na_o and K_o. The mean value (mmol/liter ghosts × h ± variance) of ${}^oM_{Na}^{ouab}$, averaged from eight different experiments reported in this paper, was 0.71 Na ± .03; while for ${}^oM_{Pi}^{ouab}$, the comparable value, averaged from the two experiments reported in Table VI (first and third rows), was 0.40 P_i ± 0.04. Thus, the stoichiometric ratio for the cotransport of Na to P_i is 0.71 to 0.40 or 1.79 ± 0.19. This result implies that the transported P_i is divalent (2 Na per P_i) and if so is similar to values reported for other types of Na/P_i

TABLE VII

The Effect of AsO_4 on Type II, or [ADP + P_i]-promoted, Uncoupled Na Efflux

P_i	AsO_4	${}^ok_{Na}^{ouab}$	${}^oM_{Na}^{ouab}$
mM	*mM*	h^{-1}	*mm/liter ghosts × h*
10	0	0.027 ± .004	0.68 ± .10
0	0.05	0.001 ± .003	0.03 ± .08
10	0	0.028 ± .004	0.70 ± .10
0	2	0.002 ± .008	0.05 ± .20
0	5	0.001 ± .007	0.03 ± .18
0	10	−0.002 ± .005	−0.02 ± .13

Resealed ghosts were prepared, according to the protocol described before (legend to Table I, Methods section) in the presence of 500 μM ADP together with the other constituents listed. In addition either 10 mM P_i, or AsO_4, at the indicated concentrations, were also loaded into ghosts before resealing and exposure to DIDS. AsO_4 (as Na_2HAsO_4) when added was substituted for an osmotically equivalent concentration of Na_2SO_4 so that the ghosts, in all instances, were prepared to contain 25 mmol Na/liter ghosts. The efflux of Na was measured in the presence and absence of 100 μM ouabain and the ${}^oM_{Na}^{ouab}$ calculated as described before. The results of two different experiments are shown. The values in the table represent the means ± SEM, where $n = 3$.

cotransporters (ouabain-insensitive) one of which occurs in human red cells (Shoemaker, Bender, and Gunn, 1988; Wehrle and Pedersen, 1989; Murer, 1992).

Effect of AsO_4 on Type II Uncoupled Na Efflux

The question arose concerning the extent to which AsO_4 could substitute for P_i in [ADP + P_i]-dependent uncoupled Na efflux. This was based first on the finding that AsO_4 (but not SO_4) could substitute for P_i in [Mg^{++} + P_i]-promoted ouabain binding, implying that the pump could be arsenylated in analogy with its phosphorylated counterpart (Schwartz et al., 1968). And second, that AsO_4 in place of P_i could support Rb deocclusion (Forbush, 1988) as well as Rb_i/Rb_o (that is, K_i/K_o) exchange, another mode of transport mediated by the Na/K pump (Kenney and Kaplan, 1988b). While K_i/K_o exchange is known to be dependent on the presence of nucleotides as well as P_i (Glynn, Lew and Luthi, 1970; Simons, 1974, 1975) the case

studied by Kenney and Kaplan (1988*b*) utilized a system that involved ADP as the nucleotide of choice in addition to P_i or AsO_4, conditions similar to those used in the present work. Given this background, it is surprising, as shown by the results presented in Table VII, that AsO_4 is unable to substitute for P_i in supporting type II uncoupled Na efflux. Thus, AsO_4, in the presence of ADP, was essentially without effect on activating ${}^{o}M_{Na}^{ouab}$, in contrast to the flux observed in [ADP + P_i]. (The concentrations of AsO_4 studied here bridged the biphasic effects of AsO_4 on Rb_i/Rb_o seen by Kenney and Kaplan, 1988*b*.) The results presented in Table I (Experiment A) and Table VII also point to differences in the selectivity of anions (P_i > > > AsO_4, SO_4) that support type II uncoupled Na efflux (see later). In addition, these

TABLE VIII

The Effects of Vanadate and Oligomycin on Type II, or [ADP + P_i]-Promoted, Uncoupled Na Efflux

P_i	K_o	Vanadate	Oligomycin	${}^{o}k_{Na}^{ouab}$	${}^{o}M_{Na}^{ouab}$
mM	*mM*	*μM*	*μg/ml*	h^{-1}	*mmol/liter ghosts × h*
10	0	0	—	0.025 ± .002	0.63 ± .05
10	0	2	—	0.004 ± .001	0.10 ± .03
10	0	0	—	0.026 ± .005	0.65 ± .13
10	0	100	—	0.002 ± .005	0.05 ± .13
10	5	0	—	0.043 ± .003	1.08 ± .08
10	5	100	—	0.001 ± .004	0.03 ± .10
10	20	0	—	0.041 ± .006	1.03 ± .15
10	20	100	—	0.027 ± .004	0.68 ± .10
10	—	—	0	0.026 ± .002	0.65 ± .05
10	—	—	5	0.005 ± .002	0.13 ± .05

Resealed ghosts were prepared, according to the protocol described before (legend to Table I, Methods section) in the presence of 500 μM ADP together with the other constituents listed. In addition to 10 mM P_i, vanadate, at the indicated concentrations, was also loaded into ghosts before resealing and exposure to DIDS. Oligomycin as well as vanadate at the indicated concentrations was also present in the medium. All media used in connection with the testing of oligomycin also contained 0.25% ethanol (the solvent for oligomycin). The efflux of Na was measured in the presence and absence of 100 μM ouabain and the ${}^{o}M_{Na}^{ouab}$ calculated as described before. In all instances the ghosts were prepared to contain 25 mmol Na/liter ghosts. The results of three different experiments are shown. The values in the table represent the means ± SEM, where n = 4.

differences between the effects of AsO_4 and P_i on the two types of transport modes emphasize differences in the conformational transitions that the pump can make in response to different ligands (e.g., ±K) when differentiated by the types of unidirectional fluxes (Na vs K or Rb) that are measured (see later).

Effect of Vanadate and Oligomycin on Type II Uncoupled Na Efflux

The use of vanadate offers another approach to studying the reaction mechanism associated with [ADP + P_i]-dependent uncoupled Na efflux. This is because vanadate is thought not only to bind to the Na/K pump with high affinity, presumably at the P_i binding site (Cantley, Cantley, and Josephson, 1978) but also because it inhibits type

I uncoupled Na efflux and the Na-ATPase associated with it (Beaugé, Cavieres, Glynn, and Grantham, 1980; Blostein, 1983; Sachs, 1986*a*). It is clear from the results presented in Table VIII that vanadate is a potent inhibitor of type II uncoupled Na efflux because 2 and 100 μM vanadate are equally effective. There is evidence that E_2 is the form of the enzyme that is stabilized by vanadate (Karlish, Beaugé, and Glynn, 1979; Robinson and Mercer, 1981) and it is attractive to think that this interpretation applies in the present case (with or without bound ADP). But vanadate inhibition in the presence of K_o is more complicated since the stimulation of Na efflux that occurs with either 5 or 20 mM K_o (see also Table III) is only partially inhibited at 20 mM K_o. Perhaps when the basis for the stimulation of Na efflux by K_o is understood, an explanation for vanadate's inhibition of just the extra or K_o-stimulated component seen at 20 mM K_o will be forthcoming.

The results presented in Table VIII also show that type II uncoupled Na efflux is inhibited by oligomycin. Oligomycin is known to inhibit the red cell Na/K pump as well as other transport modes of the pump including type I uncoupled Na efflux (Glynn, 1985; Blostein, 1970, 1983; Sachs, 1980). Sachs (1980) has provided convincing evidence that oligomycin combines preferentially with the Na forms of E_1 or E_1P of the pump, preventing the transformation of these forms to their E_2 or E_2P counterparts. This is important for it can be taken to mean that Na in its type II uncoupled mode acts the same as Na in its type I uncoupled mode. Thus, as discussed more fully later, it is unlikely that Na in type II uncoupled efflux is acting as a surrogate K being transported via an uncoupled K efflux or K_i/K_o exchange pathway (see Glynn and Lüthi, 1968; Glynn et al., 1970; Simons, 1974; Sachs, 1986*b*). This is so even though the presence of [ADP + P_i] can also set the stage for the occurrence of K_i/K_o exchange (Glynn, 1985; Kaplan and Kenney, 1982; Kenney and Kaplan, 1988*a*).

DISCUSSION

This paper concerns a transport mode of the red cell Na/K pump that is known as uncoupled Na efflux because it is inhibited by ouabain and occurs in the absence of an exchangeable cation, such as K_o or Na_o (Garrahan and Glynn, 1967*a*). The primary finding reported here is that the combination of the ligands, ADP + P_i, will not only interact with the pump to drive uncoupled Na_i efflux (Table I) but that the P_i that is required for activation is extruded via the pump as well (Table VI). Both the Na_i and P_i effluxes were shown to be ouabain-sensitive and that the stoichiometry of their cotransport was close to 2 Na_i to 1 P_i. Prior to being effluxed, P_i appeared to phosphorylate the pump as deduced from the effects of Na_o on ouabain binding (Tables III and IV) and of vanadate (Table VIII). Thus, this is the second instance (see below) where the Na/K pump can be made to transfer P_i to the *outside* of the cell during its phosphorylation/dephosphorylation cycle. This is in contrast to the normal operation of the pump where it is known that the breakdown of the pump's phosphointermediate releases P_i to the inside of the cell even though the P_i so released may subsequently appear outside via some other route (Whittam and Ager, 1964; Schatzmann, 1964; Sen and Post, 1964). The addition of K_o, in the [ADP + P_i] situation, prevents the extrusion of P_i to the outside (Table VI) while stimulating Na_i efflux (Table III) and promoting a 1 for 1 exchange of Na_i for K_o (Table V). Evidence

for a direct linkage between Na_i and K_o is implied by the apparent constancy in their 1 to 1 stoichiometry when K_o is raised from 1 to 10 mM but would be strengthened by measurements of ${}^{i}M_K^{ouab}$ and its dependency on Na_i. In addition, more work will be necessary to clarify the molecular basis not only for the stimulation of Na_i efflux by K_o and the latter's inhibition by K_i (Table III) but also for the conversion of the pump apparatus by [ADP + P_i] to liberate P_i to the outside and the regulatory role played by K_o in this situation.

Reaction Scheme for [ADP + P$_i$]-dependent Uncoupled Na Efflux

The Albers-Post type reaction scheme (Glynn, 1985) presented in Fig. 2 depicts possible transphosphorylation steps of the pump that might underlie the transloca-

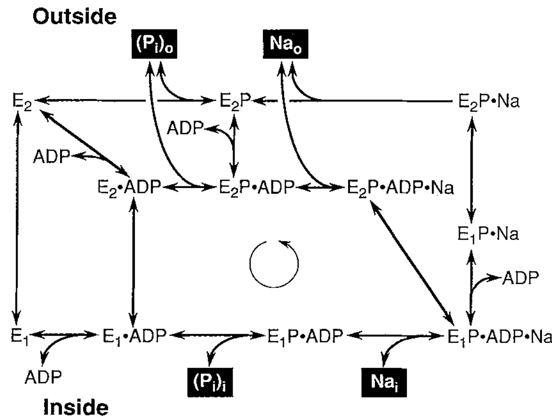


FIGURE 2. Reaction scheme of the red cell Na/K pump that depicts the coupled release of orthophosphate [(P_i)] from the inside (i) to the outside (o) of the ghost during Type II uncoupled Na efflux. The symbols E_1, E_2, E_1P, and E_2P refer to different conformations of the unphosphorylated and phosphorylated forms of the Na,K-ATPase (E), respectively. As discussed in the text the Albers-Post type model shown here (cf, Glynn, 1985) is divided into two cycles that operate in a counter-clockwise direction. In the lowest line, common to both cycles, ADP is shown to form E_1-ADP before taking on $(P_i)_i$ and Na_i to form E_1P-ADP-Na. For reasons given in the text (see Table IV), ADP is shown to bind before either $(P_i)_i$ or Na_i in order to prevent Na_i, in ADP's absence, from inhibiting the formation of E_1P (see Post et al., 1973; Bodemann and Hoffman, 1976). Thus the order of binding of $(P_i)_i$ and ADP is not critical as long as both are bound before Na_i; similarly, if ADP is bound first then $(P_i)_i$ and Na_i could presumably bind in either order. The two cycles differ in their respective intermediates depending upon whether or not ADP stays bound (inner cycle) or is released (outer cycle) during the E_1 to E_2 transitions of the intermediates as $(P_i)_i$ and Na_i are translocated releasing, respectively, $(P_i)_o$ and Na_o to the outside. Note that neither the stoichiometry of Na binding and its occluded forms (Glynn and Karlish, 1990) nor the involvement of Mg are specified in this scheme. See text for further discussion.

tion of Na_i and $(P_i)_i$ during type II uncoupled Na efflux. Two schemes are presented here that have in common the association of the pump (E) with ADP, P_i and Na_i to form $E_1P{\cdot}ADP{\cdot}Na$ (bottom line). For reasons already discussed (see also the legend to Fig. 2) ADP is assumed to bind to E before either P_i or Na_i. The subsequent intermediates differ depending upon whether ADP remains bound (inner cycle) or is released (outer or peripheral cycle), in recognition of the fact that there is no evidence as yet to distinguish between these two possibilities. This is so even though there is convincing evidence that E can have bound at the same time both a

nucleotide (e.g., ATP) and P_i, the latter being bound at its catalytic site (Sachs, 1981; Askari and Huang, 1982, 1984; Forbush, 1987; Buxbaum and Schoner, 1991). Note that the $(P_i)_i$ that E binds is the P of the phosphorylated intermediate that will be released to the outside, together with deoccluded Na, as E makes its transition from the E_1 to the E_2 forms. It should also be understood that while the binding of Na and P_i is presented as ordered in the scheme, there is no basis for knowing the sequence in either instance or whether or not the overall process operates electroneutrally or electrogenically. Attempts to measure the ouabain-sensitive electrical properties of the process by means utilized before (Dissing and Hoffman, 1990) were encumbered by the increased leakage of the ghosts to ions as mentioned in connection with the results presented in Table I.

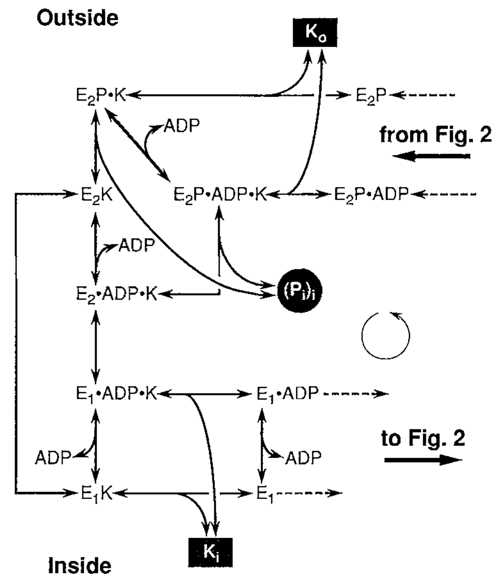


FIGURE 3. Reaction scheme that depicts the effect of K_o on converting type II, or $[P_i + ADP]$-dependent, uncoupled Na_i efflux to Na_i/K_o exchange. The intermediates indicated in the upper right of the figure represent extensions of the outer (E_2P) and inner ($E_2P{\cdot}ADP$) cycles presented in Fig. 2. The K_o that adds to either of these two intermediates prevents the release of P_i to the outside and presumably promotes the release of P_i to the inside $[(P_i)_i]$. The two forms of the dephosphorylated enzyme (E_2K or $E_2ADP{\cdot}K$) in converting to their respective E_1 forms, release their bound (occluded) K to the inside (K_i). The resultant forms (E_1 or $E_1{\cdot}ADP$) are then available for recycling as depicted in Fig. 2. Thus, in the presence of K_o, the translocation of Na_i to Na_o seen in Fig. 2 results in the translocation of K_o to K_i. The two schemes taken together (Figs. 2 and 3) indicate possible phosphointermediates that underlie Na_i/K_o exchange and a mechanism for P_i retention that occurs in the presence of K_o. See text for further discussion.

Reaction Scheme for Conversion of [ADP +P_i]-dependent Uncoupled Na Efflux to Na_i Exchange for K_o

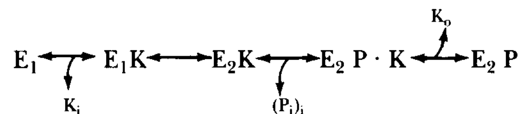
The reaction scheme presented in Fig. 2 is extended in Fig. 3 to include the effects of K_o and K_i. Again the inner and outer cycles differ, respectively, only with regard to whether or not ADP remains bound to an E form of the pump during a single turnover. Note that there are two consequences, common to both cycles, that result from the binding of K_o either to E_2P or to $E_2P{\cdot}ADP$. The first is that K_o inhibits the release of P_i to the outside, as evidenced by the results presented in Table VI. This effect of K_o represents the same action K_o has on the release of P_i in type I or ATP-dependent uncoupled Na efflux (Marín and Hoffman, 1994). Although K_o could

act to prevent the dephosphorylation of the EP forms, the more likely effect is that K_o binding catalyzes dephosphorylation with consequent release of $(P_i)_i$ to the inside as depicted in Fig. 3. This action would be consistent with K_o's effects in ATP-driven Na_i/K_o exchange (Whittam and Ager, 1964; Schatzmann, 1964; Glynn, 1985; Glynn and Karlish, 1990). Equally likely is that K_o favors the dissociation of ADP from its E_2 forms, again in analogy to the situation that obtains in ATP-dependent Na_i/K_o exchange (Eisner and Richards, 1981; Beaugé and DiPolo, 1981). The second consequence is that K_o binding to the E_2 forms on the outside results in its translocation across the membrane and its subsequent release on the inside (K_i). This transport of K stimulates the efflux of Na_i (Table III) and occurs as a one for one exchange of Na_i for K_o as discussed before (Table V). Although the model presented in Fig. 3 would appear to accommodate K_o/K_i exchange, this has yet to be tested in the present context (see later).

Na_i as a Surrogate for K_i

It is instructive to ask the extent to which Na_i is acting as a surrogate K in the types of Na effluxes as characterized in this paper. This is of interest because the alkali cation selectivity of the Na_i/K_o exchange pump can be altered depending upon the prevailing circumstances. Thus, Li_i (Dunham and Senyk, 1977) and H_i^+ (Polvani and Blostein, 1988) can be shown to substitute for Na_i; in addition, Li_o (McConaghey and Maizels, 1962; Sachs and Welt, 1967) and H_o^+ (Polvani and Blostein, 1988) can substitute for K_o on the pump in a ouabain-sensitive manner. More to the point is that Na_o, in the absence of K_o, can act as a congener for K_o in promoting two types of Na_i/Na_o exchange. One type is ATP and ADP dependent (Garrahan and Glynn, 1967*b;* Glynn and Hoffman, 1971) and occurs as a one for one exchange of Na_i for Na_o without any net hydrolysis of ATP. In contrast, the second type utilizes ATP in an ADP-independent manner and is thought to mediate an exchange of 3 Na_i for 2 to 3 Na_o (Glynn and Karlish, 1976; Blostein, 1983). Given this pliancy in the pump's ion selectivity, the question can be raised concerning the resemblance of Na_i and K_i in the relationship of type II uncoupled Na_i efflux to uncoupled K_i efflux (Sachs, 1986*b*), and in the relationship of [ADP + P_i]-dependent Na_i/K_o exchange to K_i/K_o exchange (Glynn et al., 1970; Simons, 1974). The case of K_i/K_o exchange is especially relevant because this exchange is thought to represent a reversal of the K entry mechanism involving $(P_i)_i$ and ATP (see Glynn, 1985) or $(P_i)_i$ and ADP (Kaplan and Kenney, 1982).

If Na_i were acting as a surrogate K_i in ouabain-sensitive uncoupled K efflux, then, as suggested for K_i by Sachs (1986*b*), type II uncoupled Na efflux could analogously be mediated through a pathway depicted on the left-hand side of Fig. 3 (in the absence of K_o) either as



or a comparable scheme (inner cycle) where the intermediates have ADP bound. The characteristics of uncoupled K efflux, as evidenced by Sachs (1986*b*), that make it unlikely that Na_i is substituting for K_i in this manner are that uncoupled K_i efflux (*a*)

is inhibited by Na_i, (*b*) is not inhibited by oligomycin in the absence of Na_i, and (*c*) can take place in the absence of ATP and in the absence of $(P_i)_i$ as well. In contrast, type II uncoupled Na_i efflux is not affected by K_i (Table III), is inhibited by oligomycin (Table VIII), is inhibited by ATP (Table II) and, as shown in Table I, is obligatorily dependent upon the combined presence of [ADP + P_i].

There are several reasons for thinking that the involvement of Na_i in type II uncoupled Na efflux is also distinct from any K_i-like action in ouabain-sensitive K_i/K_o exchange. One reason is that ^{42}K efflux in K_i/K_o is known to be unaffected by Na_o (Glynn and Lüthi, 1968; Glynn et al., 1970), yet type II uncoupled Na_i efflux is inhibited by Na_o whether or not K_i or K_i/K_o is present (Table III). Another reason is that Na_i has been shown to be competitive with K_i in K_i/K_o exchange in reducing the efflux of K (Simons, 1974; Kaplan and Kenney, 1982; Sachs, 1986*a*) but type II uncoupled Na efflux is the same whether or not K_i or K_i/K_o is present (Table III).

Two other aspects of K_i/K_o exchange should be noted since they also bear on the distinction between these two types of fluxes. The first is that although K_i/K_o exchange can take place in the presence of [ADP + P_i], K_i/K_o exchange can also be activated, separately, by either ADP or P_i alone (Kaplan and Kenney, 1982; Kenney and Kaplan, 1988*a*, *b*). This contrasts sharply with the requirement of type II uncoupled Na efflux and its dependence on both ADP + P_i (Table I). The second and more dramatic difference between the two types of fluxes is that AsO_4 cannot substitute for P_i in type II uncoupled Na efflux (Table VII) but can replace P_i in K_i/K_o exchange (Kenney and Kaplan, 1988*b*). Thus, type II uncoupled Na efflux is inhibited (or inactive in the presence of [ADP + AsO_4]) whereas K_i/K_o exchange is stimulated.

Inability of AsO_4 to Substitute for P_i

It is not clear in the latter results what the basis is that allows AsO_4 to support K_i/K_o exchange but not type II uncoupled Na efflux. Because the ghosts in both instances contain ADP + AsO_4, the difference in AsO_4's action must reside with the type of flux being measured (K vs Na) and the presence or absence of K. Thus, it would be interesting to know whether or not AsO_4 could, in the presence of K_o, substitute for P_i in stimulating a Na efflux (Table III) and mediate a one for one exchange of Na_i for K_o (Table V). Perhaps the failure of AsO_4 to substitute for P_i in the absence of K_o is connected, not with the arsenylation of the pump, but with its inability to be transported out with Na as P_i is (Table VI). Thus where K_o inhibits P_i efflux but not Na efflux, the incoming K could stimulate dearsenylation, resulting in its own deocclusion (see Forbush, 1988). The effects of K_i and whether K_i is also required in this situation would need to be tested. A related question concerns the effect of K_i alone (in the absence of K_o) relative to its possible support of Na efflux in the presence of AsO_4.

Types of Uncoupled Na Efflux

Table IX summarizes the different types of ouabain-sensitive uncoupled Na efflux that occur in human red blood cells. This extends the table presented in the companion paper (Marín and Hoffman, 1994) to include Type II uncoupled Na efflux as characterized in the present paper. It is now clear that there are three

different types of uncoupled Na efflux. Although all three types have in common coupled anion fluxes, each type displays several features that make them easily distinguishable from each other. Thus, type I is driven by ATP in contrast to type II, which depends on the combination [ADP + P_i]. In type IA, cytoplasmic SO_4 or Cl is transported in conjunction with Na_i and both types of fluxes are inhibited by Na_o. In type IB, the P_i that is effluxed (via EP) with Na_i comes directly from the pump's substrate (ATP) and, in contrast to type IA, neither flux is inhibited by Na_o. But in type II, uncoupled Na_i efflux, in representing a sort of cross between types IA and IB, it is cytoplasmic P_i [as in type IA) that is exported via EP (as in type IB) with Na_i, where again both the P_i and Na_i effluxes are inhibited by Na_o. In all three cases K_o inhibits the efflux of anions and converts uncoupled Na efflux to an exchange flux of Na_i for K_o.

The most important aspect of the results summarized in Table IX concerns types IB and II uncoupled Na efflux. Thus, not only does P_i efflux occur in these two different circumstances, it appears to do so by formation and breakdown of the pump's phosphointermediate, EP. While this type of Na-coupled, phosphate transport is completely new, it is not clear how the pump's subunit and/or charge structure is subverted, by the changes made in ligand specification or availability, to accommodate this type of transfer mechanism. In contrast, anion coupling in type IA would appear to be handled by a process distinct from that associated with P_i (see Dissing and Hoffman, 1990).

TABLE IX

Types of Anion-coupled Transport that Occur in Uncoupled Na Efflux in Human Red Blood Cells

Type	Substrate	Anion cotransported	Source of anion	Anion transport inhibited by		Na efflux inhibited by	
				Na_o	K_o	Na_o	K_o
IA*	ATP	SO_4 or Cl	Cytoplasm	Yes	Yes	Yes	No
IB‡	ATP	P_i from E-P	Substrate (γ-P of ATP)	No	Yes	No	No
II§	ADP + P_i	P_i from E-P	Cytoplasm	Yes	Yes	Yes	No

*Dissing and Hoffman (1990); ‡Marin and Hoffman (1994); §This paper.

Anion Selectivity in Uncoupled Na Efflux

It is known that different SO_4 and PO_4 binding proteins purified from microorganisms show remarkable specificity/selectivity for different oxyanions (Jacobson and Quiocho, 1988; Luecke and Quiocho, 1990). Thus, a PO_4-binding protein binds PO_4 and AsO_4 at least five orders of magnitude tighter than SO_4 and a SO_4-binding protein shows a similar selectivity over PO_4. The specificity of the PO_4-binding protein is known, from its x-ray structure at 1.7 Å resolution, to be conferred by hydrogen bonds (Luecke and Quiocho, 1990). While we do not know the relationship of these results to the proteins comprising the Na/K pump, we have found that with 50 mmol $(SO_4)_i$/liter ghosts, the SO_4 efflux component of type IA uncoupled Na

efflux is not influenced by 10 mmol $(P_i)_i$/liter cytoplasmic P_i (our unpublished results). In addition, as discussed before, the pump in type II uncoupled Na efflux is specific for P_i in contrast to both AsO_4 and SO_4. As a result, the pump's protein complex (presumably its α-subunit) must be considered to be remarkable not only because its oxyanion specificity appears to be conferred on the same protein by selective alteration of the ligands available on the two sides of the membrane but also in the action of Na_o and K_o to control anion extrusion. On the other hand, too little is known to provide any insight into the mechanisms that might be involved, including different coordinated conformational states of the pump protein(s) that underlie the cotransport of anions with Na.

Finally, as the discussion above in connection with Table IX should have made clear, the term uncoupled Na efflux is an oxymoron. Nevertheless no recommendation is made to substitute a different term because the usage of uncoupled seems to be well-entrenched and no alluring (and valid) alternative has been forthcoming.

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