Phosphate from the Phosphointermediate (EP) of the Human Red Blood Cell Na/K Pump Is Coeffluxed with Na, in the Absence of External K

REINALDO MARÍN AND JOSEPH F. HOFFMAN

From The Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06510

ABSTRACT   This study is concerned with Na/K pump-mediated phosphate efflux that occurs during uncoupled Na efflux in human red blood cells. Uncoupled Na efflux is known to be a ouabain-sensitive mode of the Na/K pump that occurs in the absence of external Na_o and K_o. Because this efflux (measured with ^{22}Na) is also inhibited by 5 mM Na_o, the efflux can be separated into a Na_o-sensitive and a Na_o-insensitive component. Previous work established that the Na_o-sensitive efflux is actually comprised of an electroneutral coefflux of Na with cellular anions, such as SO_4 (as $^{35}SO_4$). The present work focuses on the Na_o-insensitive component in which the principal finding is that orthophosphate (P_i) is coeffluxed with Na in a ouabain-sensitive manner. This P_i efflux can be seen to occur, in the absence of K_o, in both DIDS-treated intact cells and resealed red cell ghosts. This efflux of P_i was shown to be derived directly from the pump's substrate, ATP, by the use of resealed ghosts made to contain both ATP and P_i in which either the ATP or the P_i were labeled with, respectively, $[\gamma-^{32}P]ATP$ or $[^{32}P]H_3PO_4$. (These resealed ghosts also contained Na, Mg, P_i, SO_4, Ap5A, as well as an arginine kinase/creatine kinase nucleotide regenerating system for the control of ATP and ADP concentrations, and were suspended usually in $(NMG)_2SO_4$ at pH 7.4.) It was found that ^{32}P was only coeffluxed with Na when the ^{32}P was contained in $[\gamma-^{32}P]ATP$ and not in $[^{32}P]H_3PO_4$. This result implies that the ^{32}P that is released comes from ATP via the pump's phosphointermediate (EP) without commingling with the cellular pool of P_i. K_o (as K_2SO_4) inhibits this ^{32}P efflux as well as the Na_o-sensitive $^{35}SO_4$ efflux, with a $K_{0.5}$ of 0.3–0.4 mM. The $K_{0.5}$ for inhibition of P_i efflux by K_o is not influenced by Na_o, nor can Na_o act as a congenor for K_o in any of the flux reactions involving K_o. The stoichiometry of Na to SO_4 and Na to P_i efflux is $\sim$2:1 under circumstances where the stoichiometry of Na effluxed to ATP utilized is 3:1. From these and other results reported, it is suggested that there are two types of uncoupled Na efflux that differ

from each other on the basis of their sensitivity to Na_o, the source (cellular vs substrate) and kind of anion (SO_4 vs P_i) transported. Whether more than one kind of α (or β) isoform is involved to account for the pump's ability to transport P_i is not known.

INTRODUCTION

The human red cell Na/K pump while normally functioning to exchange internal Na (Na_i) for external K (K_o) also displays several other distinct modes of operation that depend upon which pump ligands are present on the two sides of the membrane (Glynn, 1985). The particular concern of this paper is the mode first described by Garrahan and Glynn (1967a,b) as "uncoupled" Na efflux, because it takes place in the absence of K_o and Na_o. This uncoupled Na efflux is known to require Na_i, Mg and ATP and to be inhibited not only by cardiotonic steroids, such as ouabain but also by low concentrations of Na_o to a maximum at 5 mM (Glynn, 1985). Because the name for this efflux stems from the idea that there were no cations in the external medium for the Na_i to exchange with, it might be expected that this process would be electrogenic. But this is not the case in red blood cells even though some preparations from other tissues, e.g., proteoliposomes containing Na,K-ATPase from shark rectal gland (Cornelius, 1989) can be electrogenic (see Dissing and Hoffman, 1990, for comparative aspects of different systems in this regard). In intact red blood cells it has been shown that the efflux of Na is directly linked to an efflux of intracellular anions and that this coupled efflux occurs electroneutrally (Dissing and Hoffman, 1990). Thus studies on uncoupled Na efflux provide new information on the molecular mechanisms underlying the functioning of the pump complex.

In the previous paper on this subject of uncoupled Na efflux (Dissing and Hoffman, 1990) anion-coupled flow was analyzed in the presence and absence of 5 mM Na_o. It was shown, in DIDS-treated red cells in which SO_4 had replaced Cl on both sides of the membrane, that the Na efflux that was inhibited by 5 mM Na_o was accompanied by an efflux of SO_4 in a stoichiometric ratio of 2 $Na^+/SO_4^=$. It was also shown that whereas Na_o and ouabain inhibited all of the SO_4 efflux, the Na efflux was only inhibited 60–80% by Na_o. In addition, replacement of SO_4 with an impermeant anion, such as tartrate (in resealed ghosts) also inhibited Na efflux by 60–80%, the inhibition of which could be relieved by the inclusion, before DIDS-treatment, of a low concentration of a permeable anion, such as Cl. Therefore, where the Na_o-sensitive component of Na efflux was shown to be coupled to a Na_o-sensitive movement of cytoplasmically-based anions (SO_4 or Cl) the nature of the Na_o-insensitive component (20–40%) was left unspecified.

Thus, the primary focus of this paper is the analysis of this Na_o-insensitive component. It was found that not only is it composed of a Na-coupled phosphate (P_i) efflux but the origin of the P_i that is coeffluxed with Na appears to be the gamma phosphate of the ATP that serves as the pump's substrate. This gamma-phosphate from ATP is thought to be transferred directly, via the pump's phosphointermediate, to the outside in concert with Na. This efflux of P_i and Na is also ouabain-sensitive, has an approximate stoichiometry of 2 Na/P_i and appears to underlie the residual Na efflux (20–40%) that occurs in ghosts containing tartrate. Other features of this Na-linked P_i-transport will be considered below together with its significance regard-

ing the pump's molecular mechanism. Preliminary accounts of this work have been previously reported (Marín and Hoffman, 1986; Hoffman, Marín, Bodemann, Callahan, and Milanick, 1991).

The above described differences in flux characteristics provide a basis for classifying ouabain-sensitive, ATP-dependent uncoupled Na efflux into two types that will be useful in the discussion that follows: type IA is comprised of a Na_o-sensitive coefflux of Na + anions, where the anion transported comes from the cytoplasm; type IB is comprised of a Na_o-insensitive coefflux of Na + P_i, where the P_i transported derives from the gamma-phosphate of ATP. There is also a third type of uncoupled Na efflux (type II) that is the subject of the companion paper (Marín and Hoffman, 1994).

MATERIALS AND METHODS

The work described here involves the use of both DIDS-treated intact red cells and resealed ghosts. The resealed ghosts are made by a procedure involving a single hypotonic hemolysis step carried out at a hemolytic ratio of 1 vol of cells to 140 vol of hemolyzing solution. The protocol used produces essentially hemoglobin-free porous ghosts (that are whitish in color) into which various desired constituents can be incorporated before the resealing of the ghost's membranes. This protocol represents the same type of ghost preparation that was used in our previous studies of uncoupled Na efflux that also involved resealed ghosts (Dissing and Hoffman, 1990). It should be emphasized that pretreatment of intact cells or resealed ghosts with DIDS essentially eliminates anion movement (i.e., SO_4 and orthophosphate) through band 3 thereby optimizing the system for detecting ouabain-sensitive effluxes of anions (cf Dissing and Hoffman, 1990). Details of the procedures follow below.

Preparation of Resealed Ghosts

The ghost preparation described below is designed to optimize, by the use of procedures involving one hemolysis, the entrapment of desired intracellular constituents and to minimize interference from normal components by reducing their concentration. Blood from healthy donors was drawn into heparin (5 mg/100 ml) and the red cells were washed three times at 4°C by suspension and centrifugation (12,000 g for 1 min) with 5–10 vol of solution containing 150 mM NaCl and 10 mM Tris-HEPES (pH 7.4 at 4°C). At the end of the third washing the cells were cooled in an ice bath and then rapidly hemolysed by squirting, from a syringe, 25 ml of packed cells into 3,500 ml of vigorously stirred hemolyzing medium (1.6 mM acetic acid and 5 mM $MgSO_4$, adjusted to pH 5.0 with 1 N NaOH before hemolysis) kept at 0°C. (This procedure is similar to that suggested by Lepke and Passow (1972) for optimizing ghost integrity and recovery.) The pH was immediately adjusted to 5.8–6.0 with 1 N NaOH and stirring continued for 5 min. The hemolysis mixture was then titrated to pH 7.4 with 1 N NaOH and allowed to stand in an ice-bath (0°C) for 5 min without stirring. The mixture was then poured into 250 ml polycarbonate bottles and centrifuged for 10 min at 27,000 g (13,000 rpm) in a Dupont Co. (Wilmington, DE) GSA rotor at a temperature (refrigerator setting, −5°C) selected to keep the hemolysis mixture in the bottles from freezing but still below 0°C. The supernatant solution was rapidly (and carefully) removed by aspiration and the concentrated ghosts were washed by being resuspended in 500 ml of an iced solution (0°C) containing 12.5 mM Na_2SO_4, 5 mM $MgSO_4$, 20 mM Tris-HEPES, adjusted with TrisOH to pH 7.5 at 0°C, and centrifuged for 10 min at 27,300 g in a GSA rotor as before. The ghosts were again concentrated ready for transfer to the reversal medium. The reason it is important to keep the temperature of the ghosts no higher than 0°C is that the ghosts remain permeable in this situation, as shown by Bodemann and Passow 1972 (see Hoffman, 1992) such that constituents added at reversal can be

incorporated inside. Raising the temperature of the ghosts suspension above 0°C leads to their resealing. The concentrated ghosts were exposed in two stages to the final resealing medium. In the first stage the concentrated ghosts were resuspended in 20 vol of reversal medium (0°C) containing 12.5 mM Na_2SO_4 (or 25 mM NaCl), 10 mM $Tris_2SO_4$ (or 20 mM Tris-HCl), pH 7.5 at 0°C, 5 mM $MgSO_4$ (or $MgCl_2$), 0.25 mM Tris-EDTA, and 40 μM Ap5A. The Ap5A was incorporated in order to inhibit any residual adenylate kinase activity (see Lienhard and Secemski, 1973; Kennedy, Lunn, and Hoffman, 1986). The osmolarity of this washing solution was then brought to 300 mOsM by the addition of $(NMG)_2SO_4$ (N-methyl glucamine sulfate) or choline chloride. This suspension was then centrifuged for 15 min at 27,000 g as before. The ghosts were then resuspended with an equal volume of the resealing solution (0°C) used above but now including, in addition, either $^{22}NaCl$ (1 to 4 μCi/ml) or $Na_2^{35}SO_4$ (50 μCi/ml) or $H_3^{32}PO_4$ (50 μCi/ml) or [γ-^{32}P]ATP(2 μCi/ml) and requisite amounts of ATP and ADP together with creatine kinase and arginine kinase based nucleotide regenerating systems as specified below. The introduction of these substances at this second stage conserves the use of these materials. This final suspension was kept at 0°C for 15 min to provide 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 periods, DIDS, to a final concentration of 50 μM, was added to the resealing medium. At the end of the resealing period, the suspension was cooled in an ice bath for 5 min and then centrifuged for 5 min at 48,000 g (4°C). The supernatant solution was carefully removed and the resealed ghosts were washed twice by resuspension and centrifugation with 10 vol of a solution that contained 300 mOsmols of $(NMG)_2SO_4$ (or NaCl or choline Cl) buffered to pH 7.4 (37°C) with 10 mM $(Tris)_2SO_4$ (or 20 mM Tris Cl). The ghosts resuspended (to 50% suspension) in this latter medium were then ready to be added to the various flux media for the determination of the effluxes of either ^{22}Na or $^{35}PO_4$ as described below.

For the preparation of tartrate-loaded resealed ghosts (see Table III) tartrate replaced SO_4 in both the stage one and stage two reversal solutions. Thus these solutions contained, in addition to nucleotides, EDTA, Ap5A and either ^{22}Na or [γ-^{32}P]ATP, 12.5 mM Na_2 tartrate, 10 mM $(Tris)_2$ tartrate (pH 7.5 at 0°C), 5 mM Mg tartrate and brought to 300 mOsM with $(NMG)_2$ tartrate. The incorporation of nucleotides and the pretreatment of the second stage reversal when it contained [γ-^{32}P]ATP were carried out as described below. This procedure is equivalent to that used by Dissing and Hoffman (1990) for the preparation of similar type ghosts.

Nucleotide Regenerating Systems

The two kinds of nucleotide regenerating systems used in this study were able to hold preset ATP and ADP concentrations reasonably constant for a 30 min interval during which time the fluxes were measured (see Kennedy et al., 1986). One kind was a creatine kinase regenerating system as employed by Glynn and Karlish (1976) to maintain a given concentration of ATP in the nominal absence of ADP (see also Kennedy et al., 1986). The second kind was a combination of a creatine kinase/arginine kinase regenerating system that was used in order to manipulate independently both ATP and ADP at rather widely varying concentrations. The behavior of this mixed kinase buffering system was not studied systematically. Nevertheless buffered values of ATP and ADP that were used were determined by trial and error, on samples analyzed after the preincubation of the resealing media, by using varying concentrations of ATP, ADP, creatine, creatine phosphate, arginine and arginine phosphate as specified in the relevant legends. Examples of determined values of ATP and ADP are presented in the legends and text. The concentrations of creatine kinase and arginine kinase when used were always the same which were, per ml of reversal medium, 20 U creatine kinase and 300 U arginine kinase.

The concentrations of ATP and ADP, in mmol/liter cells or ghosts were determined by use of the methods referred to by Dissing and Hoffman (1990).

Preparation of Resealed Ghosts Containing [γ-^{32}P]ATP

Resealed ghosts containing [γ-^{32}P]ATP were prepared by adding [γ-^{32}P]ATP to the second stage reversal medium (2 μCi/ml reversal medium) together with non-radioactive ATP, a creatine kinase regenerating system and 2 mM inosine (in addition to the other constituents specified before). In specified cases an arginine kinase regenerating system was also included. Before mixing in the concentrated ghosts, the reversal medium was first preincubated for 25 min at 37°C in order to bring ATP and creatine phosphate to chemical and isotopic equilibrium (see Glynn and Karlish, 1976). At the end of this preincubation, the solution was again cooled to 0°C before the concentrated ghosts were added. Resealing was then initiated by incubation of the suspension mixture at 37°C as described above. During this resealing time inosine enters the ghosts and acts to reduce the concentration of any orthophosphate that might have been or is released during this equilibration (data not shown) by converting it to ribose phosphate presumably by the action of the ghost's residual nucleoside phosphorylase activity. At the end of resealing the suspension mixture was cooled to 0°C before the first washing of the resealed ghosts but the second wash was carried out at 37°C in order to enhance the exit of inosine.

It should be emphasized that the above protocol was changed for the experiments reported in Table II and Fig. 3. In these experiments, because orthophosphate was intentionally entrapped within the ghosts, for purposes explained in the text, inosine was omitted from the reversal medium and all washings of the resealed ghosts were carried out at 0°C.

Efflux of ^{22}Na, ^{35}SO$_4$ and ^{32}PO$_4$ from Resealed Ghosts

The efflux for each experimental condition was carried out either in triplicate or in quadruplicate in media whose composition is specified in connection with each experiment. Because the resealed and washed ghosts had been preloaded with either ^{22}Na, ^{35}SO$_4$ or ^{32}P (as [γ-^{32}P]ATP or H$_3$[^{32}P]O$_4$) as described above, the effluxes were started by adding, for each replicate, $\sim$200 μl of a 50% ghost suspension to 6 ml of flux medium, bringing the final hematocrit to between 1.5–2.0%. The flux medium had been brought to temperature equilibrium by preincubation for 5 min at 37°C. 600-μl aliquots were taken at 5, 15, 25 and 35 min. The ghosts were centrifuged, at 10,000 rpm, in a ultra-microcentrifuge for 90 s, and 500 μl samples of the supernatant were assayed for radioactivity by gamma or liquid scintillation counting in an appropriate fluor. 500 μl samples of the total suspension mixture were also counted.

The rate constant for Na efflux was estimated (cf Hoffman, 1962) 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}, t is the time, R_s is the radioactivity of each supernatant sample, 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 $\sim$15% of the total counts. Because this initial loss seemed independent of the experimental condition, it was neglected by calculating the rate constants for flux intervals between 5 and 35 min, during which time the slope of the ln transform was linear.

The same methods were used to estimate the ^{35}SO$_4$ efflux and ^{32}P efflux. The rate constants for ^{35}SO$_4$ or ^{32}P efflux were also calculated as mentioned above. The ouabain-sensitive flux of Na ($^{o}M_{SO_4}^{o\,uab}$) or SO$_4$ ($^{o}M_{SO_4}^{o\,uab}$) (in mmol/liter packed ghosts × h) for each experimental condition were calculated by subtracting the mean rate constant obtained in the presence of ouabain (100 μM) from that in the absence of ouabain, and multiplying the difference by the intracellular concentration of Na or SO$_4$, respectively. The Na concentration of the resealed and packed ghosts was determined by flame photometry in ghosts previously washed in 210 mM MgSO$_4$. 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 (in

counts/mmol SO_4). Because only the total content and not the sizes of the different pools of ^{32}P within the ghosts (ATP, creatine phosphate and orthophosphate, e.g., Tables I and II) were determined, the size of the specific pool that supplied the fractional loss of ^{32}P that was effluxed though the Na/K pump remained unknown. Therefore, the ^{32}P efflux from resealed ghosts is presented as an outward rate constant $^{o}k_{32P}$, in units of reciprocal hours, and was measured in the presence and absence of 100 μM ouabain.

It should be understood that, where applicable, the statistical significance between two sets of results was determined with the two-tailed t test; if $P > 0.05$, the difference was considered not to be significant.

Efflux of ^{22}Na, $^{35}SO_4$ and P_i from Intact Cells

The intact cells that were to be used for efflux determinations were all treated in essentially the same way except for the type of radioisotope they were labeled with. The procedure used was essentially the same as that described by Dissing and Hoffman (1990). Thus, freshly drawn human red cells were washed three times at 4°C by suspension and centrifugation (at 11,000× g) with 5–10 vol of solution containing 150 mM NaCl and 10 mM HEPES (pH 7.4 at 4°C). To replace intracellular Cl with SO_4 the packed red cells were then suspended (10% hematocrit) in 95 mM Na_2SO_4, 5 mM NaH_2PO_4, 5 mM glucose and 5 mM adenosine (pH 7.4 with 1 N NaOH) and incubated at 37°C for 30 min. This procedure was repeated twice and the cells were then suspended in the same solution which included either $^{35}SO_4$ (50 μCi/ml suspension) or ^{22}Na (5 μCi/ml suspension) and incubated for 3 h at 37°C. 30 min before the end of this incubation period, 50 μM DIDS (final concentration) was added to the suspension to minimize anion transport via Band 3 and to trap $^{35}SO_4$ (or P_i, when desired) inside the cells. Then, the cells were washed three times with a solution of 210 mM $MgSO_4$ and 10 mM $Tris_2SO_4$. The labeled cells were assayed (at 1 to 2% hematocrit) for either $^{35}SO_4$ efflux or ^{22}Na efflux following the method described above for resealed ghosts. All the solutions used during the preparation steps as well as during the flux measurements (the composition of which are detailed in the relevant legends) were pre-equilibrated (gassed) with N_2 for 30 min at pH 5.0 in order to reduce contamination from traces of CO_2/bicarbonate. At the end of this pre-equilibration with N_2, the solutions were adjusted to the stated pH. Note that the solutions used in the preparative loading steps were free of K_o. This was done in order to raise Na_i (to the levels indicated in the relevant legends) to increase the magnitude of uncoupled Na efflux in these cells (see Dissing and Hoffman, 1990).

For measurement of orthophosphate (P_i) efflux from intact cells the above procedure for cell preparation was followed except that the cells were unlabeled. In addition, the efflux procedure was modified in that at the conclusion of the third wash, following the 3-h incubation period, the packed cells were added to the efflux medium (as specified in the relevant legends) to a final hematocrit of 15–25%. This cell suspension was then incubated at 37°C with aliquots being removed for P_i analysis at 0, 30, and 60 min. The samples were centrifuged in an ultra-microcentrifuge for 90 s and the supernatants were assayed for P_i following the method of Forbush (1983). The ouabain-sensitive P_i efflux, $^{o}M_{P_i}^{ouab}$, in mmol/liter cells × h, was taken as the difference in the efflux of P_i in the absence and presence of 100 μM ouabain.

Influx of Na and SO_4 into Resealed Ghosts

As described by Dissing and Hoffman (1990) the influxes were begun by adding resealed red cell ghosts, to a final hematocrit of ~1.5–2%, into media that contained either 6 μCi/ml ^{22}Na or 10 μCi/ml $^{35}SO_4$. The ghost preparation and the composition of the different media used are specified in the relevant legends. The suspension was incubated at 37°C and samples were removed at 5, 15, 25, and 35 min. The samples were immediately centrifuged (11,000 g for 1

min) and then washed twice at 4°C by centrifugation and resuspension in 220 mM MgSO$_4$. The packed ghosts were rehemolyzed with distilled water and the radioactivity determined by appropriate counting. The specific activity of the relevant isotope of each suspension mixture was also determined. The influxes were calculated as described in Dissing and Hoffman (1990). The ouabain-sensitive influx of either Na, $^iM_{Na}^{o\,uab}$, or SO$_4$, $^iM_{SO_4}^{o\,uab}$ (both in mmol/liter ghosts × h) was taken as the difference in the influx rates in the absence and presence of 100 μM ouabain.

ATPase Activity of Resealed Ghosts

The ATPase activity was determined on resealed ghosts containing [γ^{32}-P]ATP in combination with a creatine kinase/arginine kinase regenerating system as described before. The procedure used was based on that described by Glynn and Karlish (1976). Thus, the [γ^{32}-P]ATP was preincubated in the presence of the nucleotide regenerating system constituents in order to reach chemical and isotopic equilibrium between ATP, creatine phosphate and arginine phosphate. In addition, inosine was included in the reversal medium so that ^{32}P$_i$ released during resealing was converted to ribose-PO$_4$ thereby lowering the background level of ^{32}P$_i$ inside the ghosts. Excess inosine was removed from the ghosts by subsequent washing before the final incubation at which time the ATPase activity, that is the rate of liberation of ^{32}P$_i$ inside the ghosts, was determined. It should be noted that because the resealed ghosts had been treated with DIDS almost all of the liberated ^{32}P$_i$ was entrapped inside but some of the ^{32}P was in the supernatant due to its export via Na uncoupled Na efflux (type IB) as shown below. The incubation was carried out, at 1.5–2% hematocrit, in a manner completely comparable to that described for Na efflux except that the total suspension was analyzed for ^{32}P. Thus, 1-ml samples of the incubation mixture, containing 20–25 μl of ghosts, were removed at various time intervals from 5 to 35 min and treated (vortexed) with 1 ml of a suspension of activated charcoal (20 ml ice-cold 6% trichloroacetic acid + 1 g charcoal). The charcoal/ghost mixture was centrifuged and the supernatant, containing only P$_i$, was counted for ^{32}P activity. The ouabain-sensitive ATPase activity was calculated from the difference in the ATPase rates obtained in the absence and presence of 100 μM ouabain.

Reagent Sources

Hexokinase, Ap5A, DIDS, ouabain, (NMG)$_2$SO$_4$, arginine, creatine, arginine phosphate, creatine phosphate, arginine kinase, creatine kinase and luciferin/luciferase were all purchased from Sigma Chemical Co. (St. Louis, MO). Radioactive isotopes were purchased either from New England Nuclear Corp. (Boston, MA) (^{22}Na and ^{35}SO$_4$) or from ICN Biomedicals, Inc. (Costa Mesa, CA) (^{35}SO$_4$, H$_3^{32}$PO$_4$ and [γ-^{32}P]ATP). It should be noted that all batches of H$_3^{32}$PO$_4$ that were used were filtered twice through a Millipore 0.45 μm filter (Millipore Corp., Bedford, MA) to remove traces of impurities before being neutralized with Trishydroxide.

Notations and Abbreviations Used

The subscripts of i and o are used to refer, respectively, to internal and external concentrations of ions. Inorganic phosphate (orthophosphate) is symbolized, by convention, as P$_i$ or ^{32}P$_i$ and when inside ghosts or cells is represented as (P$_i$)$_i$ or (^{32}P$_i$)$_i$. Gamma-^{32}P labeled ATP is [γ-^{32}P]ATP and when ^{32}P is released to the cell's outside it is referred to either as ^{32}P, ^{32}P$_i$ or (^{32}P$_i$)$_o$. DIDS is 4,4-diisothiocyanostilbene-2,2'-disulfonic acid. PIPES is 1,4-Piperazinediethane-sulfonic acid. Ap5A is P^1,P^4-di(adenosine-5')pentaphosphate. Cr is creatine, CrP is phosphocreatine, Arg is arginine and ArgP is arginine phosphate. The efflux and influx of ions, such as Na, is symbolized as $^oM_{Na}$ and $^iM_{Na}$, respectively, in units of mmol/liter cells (or ghosts) × h. Ouabain-sensitive fluxes are indicated as a superscript, abbreviated, ouab. The outward rate constant for ^{32}P (in h^{-1}) is indicated as $^ok_{32p}$.

RESULTS

Effect of External Na

As pointed out above uncoupled Na efflux can be operationally divided into a Na_o-sensitive (type IA) and a Na_o-insensitive (type IB) component. This is illustrated in Fig. 1 where the Na efflux from SO_4-loaded DIDS-treated red cell ghosts is plotted against three different concentrations of Na_o. Compared to its value at 0 Na_o, Na
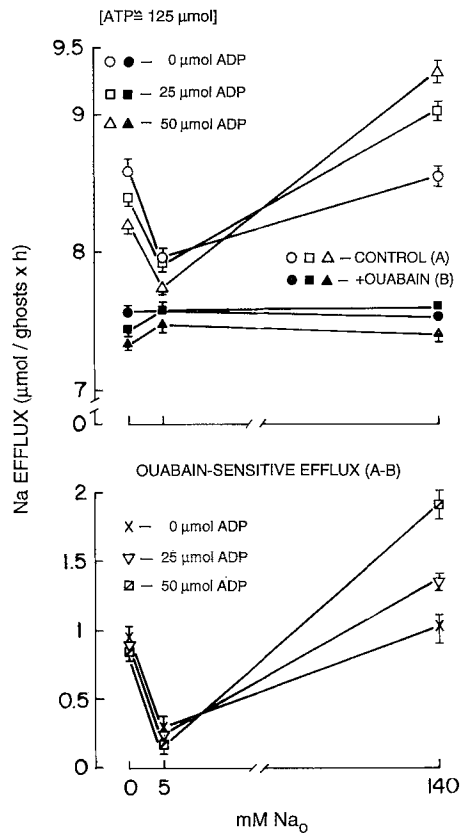

FIGURE 1. The effect of Na_o on uncoupled Na efflux from DIDS-treated resealed ghosts and the influence of varying concentrations of ADP at a preset level of ATP. The ghosts had been prepared to contain (in mM): 12 Na_2SO_4 + 10 $Tris_2SO_4$ + 5 $MgSO_4$ + 0.25 Tris-EDTA + 0.04 Ap5A together with a creatine kinase/arginine kinase nucleoside regenerating system (see Materials and Methods). The regenerating system consisted of (in micromolar): 125 ATP + 10 Cr + 500 CrP + 800 Arg + 1 ArgP together with either 0 or 25 ADP as indicated. When ADP was 50 μM, Cr was 50 μM, Arg was 100 μM and ArgP was 10 μM. The efflux of ^{22}Na was measured at 37°C (pH 7.4) from ghosts suspended in 300 mosmol $(NMG)_2SO_4$ containing 10 mM $(Tris)_2SO_4$ with Na_2SO_4 being substituted for $(NMG)_2SO_4$ at the indicated concentrations of Na_o. The values of ATP and ADP given in the figure are approximate—the measured values are listed in experiment 2 of Table IX. The upper panel shows the efflux of Na (in mmol/liter ghosts × h) from ghosts in the absence (*open symbols*) and presence (*filled symbols*) of 100 μM ouabain. The ouabain-sensitive difference in Na efflux, shown in the lower panel, represents the difference in their respective effluxes at each value of Na_o relative to the concentration of ADP. Na_i was ~25 mmol/liter ghosts. Each point graphed is the mean ± SEM (*half-bars*) where n = 3. See text for discussion.

efflux is inhibited at 5 mM Na_o and is stimulated at higher concentrations of Na_o such as 140 mM. The inhibition that occurs at 5 mM Na_o has been shown to be maximal (Dissing and Hoffman, 1990). It is also important to note that ouabain inhibits the Na efflux to approximately the same extent independent of Na_o. Uncoupled Na efflux is thus represented by the Na efflux that takes place between 0 and 5 mM Na_o whereas the efflux that is stimulated by Na_o reflects a different kind of transport mode of the Na/K pump, namely Na_i/Na_o exchange (Garrahan and Glynn,

1967a). This biphasic action of Na_o on both the Na_o-sensitive and ouabain-sensitive effluxes of Na has been noted before (Garrahan and Glynn, 1967b; Sachs, 1970; Lew, Hardy, and Ellory, 1973; Karlish and Glynn, 1974; Glynn and Karlish, 1976; Dissing and Hoffman, 1990). Thus, type IA uncoupled Na efflux is identified as the 5 mM Na_o-sensitive component of Na efflux that is also ouabain-sensitive. It was previously established (Dissing and Hoffman, 1990) that this efflux of Na was actually comprised of a coefflux of Na together with anions that come from the cell's cytoplasm, such as SO_4 or Cl. In contrast, the type IB component is defined as being that portion of the uncoupled Na efflux that is ouabain-sensitive but Na_o-insensitive. The results reported in the next section show that the Na efflux that is associated with type IB is also coupled to a coefflux of anions; it differs from type IA in that instead of being linked to cytoplasmic anions, it is coupled to an efflux of P_i that in turn is donated to the pump complex directly from ATP.

Because the ghosts used in the experiments, as presented in Fig. 1, were made to contain varying concentrations of ADP at constant ATP, the results also show that the Na_i/Na_o exchange flux that is stimulated by increasing Na_o from 5 to 140 mM is directly related to the concentration of ADP. While these results confirm that Na_i/Na_o exchange can occur with (Garrahan and Glynn, 1967c; Glynn and Hoffman, 1971) and without (Glynn and Karlish, 1976; Blostein, 1983) ADP, they also show that the uncoupled Na efflux that is ongoing between 0 and 5 mM Na_o is insensitive to the concentration of ADP (Fig. 1, ouabain-sensitive flux). The former authors found that with ADP present Na_i/Na_o exchange could take place without net hydrolysis of ATP whereas the latter showed that without ADP the exchange utilized ATP. The results presented in Fig. 1 will be referred to again when the stoichiometry of Na efflux to ATPase activity is considered (Table IX) as well as the question as to what extent the processes identified as uncoupled Na efflux take place in the presence of 140 mM Na_o.

Phosphate (^{32}P) Is Coeffluxed with Na

It was previously shown (Dissing and Hoffman, 1990) that in DIDS-treated red cells, where SO_4 had replaced Cl on both sides of the membrane, the Na_o-sensitive component of uncoupled Na efflux (type IA) was in fact comprised of a coefflux of Na_i and SO_4. And this is again confirmed in the results of the two experiments presented in Table I where it is evident that, with DIDS-treated, SO_4-loaded resealed ghosts (see Materials and Methods), the efflux of both Na_i and SO_4 is inhibited by 5 mM Na_o. The important addition to these results is that there is also an accompanying efflux of ^{32}P that is Na_o-insensitive, because it continues to take place in the presence of 5 mM Na_o in a ouabain-sensitive manner. This efflux of ^{32}P is the same whether or not Na_o is present and is taken to represent type IB uncoupled Na efflux as defined before. Although the stoichiometry of Na to SO_4 efflux can be estimated from the results given in Table I, this cannot be done for the ^{32}P efflux. This is because the relative concentrations (and their turnover rates) of the various constituents comprising the total intracellular pool of ^{32}P was not determined. This information would be needed, along with the identity of the source that supplies the

^{32}P for transport, in order to convert the outward rate constant, $^o k^{ou\ ab}_{32P}$ into a flux with the appropriate units. As considered later, use of a different approach has provided a reasonable estimate.

That ATP serves as the proximate source for the ^{32}P that is coeffluxed with Na is established by the results presented in Table II. The experimental design involved the use of two identically prepared batches of resealed red cell ghosts that while containing the same concentrations of both ATP and orthophosphate (H_3PO_4), differed from each other in that in one batch it was the ATP that was radio-labeled ([γ^{32}-P]ATP) whereas in the other, the orthophosphate was labeled ([^{32}P]H_3PO_4). It should also be understood that in these kinds of experiments, the pool size and the

TABLE I

The Coefflux of Na, SO_4 and ^{32}P from DIDS-treated Resealed Ghosts during Uncoupled Na Efflux

Experiment	ATP	Na$_o$	$^oM^{o\ uab}_{Na}$	$^oM^{o\ uab}_{SO4}$	$^ok^{o\ uab}_{32P}$
	mmol/liter ghosts	*mM*	mmol/liter ghosts × h		h^{-1}
1	0.2	0	0.975 ± .060	0.360 ± .078	0.472 ± .005
(*n* = 4)		5	0.200 ± .035	0.000 ± .065	0.462 ± .003
2	0.6	0	1.150 ± .060	0.480 ± .120	0.294 ± .009
(*n* = 4)		5	0.375 ± .090	0.040 ± .120	0.315 ± .005

The results of two experiments are presented. In each case, concentrated ghosts, prepared as described in Materials and Methods, were divided into three groups and loaded with either ^{22}Na, $^{35}SO_4$ or [γ-^{32}P]ATP before resealing that were otherwise treated in the same way. The ghosts were also made to contain (in mM): 12 Na$_2$SO$_4$ + 10 (Tris)$_2$SO$_4$ + 5 MgSO$_4$ + 0.25 mM Tris-EDTA + 0.04 Ap5A together with ATP and a nucleotide regenerating system and brought to 300 mosmol with (NMG)$_2$SO$_4$. In experiment 1, the nucleotide regenerating system consisted of arginine kinase and creatine kinase together with (in μM): 1000 Arg, 1 ArgP, 10 Cr, 500 CrP and 200 ATP; in experiment 2, the system consisted of creatine kinase together with (in μM): 100 Cr + 2500 CrP and 600 ATP. As described in Materials and Methods the resealing medium was preincubated in the presence of inosine before the addition of the ghosts and their subsequent resealing. The efflux was carried out at 37°C at an hematocrit of 1.5–2% in a medium that contained 10 mM (Tris)$_2$SO$_4$ (pH 7.4) and brought to 300 mosmol with (NMG)$_2$SO$_4$. The measured ghost concentration of ATP in samples taken before the flux assay in experiments 1 and 2 was, respectively, 260 and 580 μmol/liter ghosts. Na$_i$ and (SO$_4$)$_i$ were, respectively, ~25 and 43 mmols/liter ghosts in both experiments. 2.5 mM Na$_2$SO$_4$, when present, was substituted for an osmotically equivalent concentration of (NMG)$_2$SO$_4$. The effluxes of ^{22}Na, $^{35}SO_4$ and ^{32}P were measured in the absence and presence of 100 μM ouabain. The ouabain-sensitive differences and their respective effluxes are given in the Table. The values presented represent the means ± SEM. See text for discussion.

specific activity of the entrapped [γ-^{32}P] labeled ATP together with unlabeled ATP was maintained essentially constant at the indicated concentrations, over the flux periods used, by an incorporated regenerating system (see Materials and Methods and Table II, legend). In addition, the specific activity of the ^{32}P contained in the γ-phosphate of ATP was made to be approximately the same as that of the [^{32}P]H_3PO_4 in order to have equivalent sensitivities in evaluating their relative effluxes.

It is apparent in Table II, in which the results of two separate experiments are shown, that a ouabain-sensitive efflux of ^{32}P is only seen when the label's source is in [γ-^{32}P]ATP. The results also indicate that the ^{32}P that is effluxed cannot have mixed

with the unlabeled intracellular orthophosphate pool for otherwise its specific activity would have decreased to levels that would have made its efflux undetectable. In addition, it was independently established by paper chromatography that the form of the ^{32}P that is effluxed is orthophosphate. It follows from these results that the presumed pathway for ^{32}P efflux from its intracellular source, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, to its release outside directly involves the formation and subsequent breakdown of the pump's ^{32}P-phosphointermediate (EP). This efflux of ^{32}P is in sharp contrast to what

TABLE II

Substrate ATP and Not the Cellular Pool of Orthophosphate Supplies the Phosphate
that Is Coeffluxed with Na during Uncoupled Na Efflux in DIDS-treated
Resealed Ghosts

| Entrapped ^{32}P source | ^{32}P efflux rate constant | | |
| | h^{-1} | | |
	Control (A)	+ Ouabain (B)	ΔOuabain (A–B)
100 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ + 1.0 mM $H_3 PO_4$	0.582 ± .002	0.130 ± .001	0.452 ± .002
100 μM ATP + 1.0 mM $[^{32}\text{P}]H_3 PO_4$	0.175 ± .002	0.169 ± .003	0.007 ± .004
200 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ + 200 μM H_3PO_4	0.578 ± .005	0.112 ± .003	0.466 ± .006
200 μM ATP + 200 μM $[^{32}\text{P}]H_3 PO_4$	0.106 ± .005	0.104 ± .003	0.002 ± .006

The results of two experiments are shown. Details of the ghost preparation used are described in Materials and Methods. The basic protocol is that for the two batches of ghosts studied in each experiment, the ghosts were prepared in an identical fashion to contain the indicated concentrations of ATP and orthophosphate (H_3PO_4), the only difference being whether the entrapped radioactivity (^{32}P) was contained in ($\gamma\text{-}^{32}$P]ATP or in [^{32}P]H_3PO_4. Except for the ^{32}P source, the presence of H_3PO_4 and the nucleotide regenerating system incorporated, the ghosts' contents were the same as described in the legend to Table I. In the first (upper) experiment, the ghosts were prepared to contain (in μM): 100 Cr, 5,000 CrP and 100 ATP together with 1 mM H_3PO_4. The concentration of ATP, ADP, and P_i was determined on samples of ghosts taken at the time of the flux assay. ATP was found to be 198 μmol/liter ghosts, ADP was < 1 μmol/liter ghosts and P_i was 1.3 mmol/liter ghosts. In the second (lower) experiment, the ghosts were prepared to contain, in addition to 200 μM H_3PO_4, the creatine kinase and arginine regenerating system that had the same composition as that described in the legend to Table I (Experiment 1) where the final ATP concentration was determined to be 260 μmol/liter ghosts. It should be understood that the preincubation of the resealing medium was carried out in the absence of added inosine in order to preserve the presence of H_3PO_4 or [$\gamma\text{-}^{32}$P]H_3PO_4. The efflux was carried out at 37°C at an hematocrit of 1.5 to 2 percent in a medium that contained 10 mM (Tris)$_2$SO$_4$ (pH 7.4) and brought to 300 mosmol with (NMG)$_2$SO$_4$. The concentration of ouabain, when present was 100 μM. The values of the efflux rate constants represent the means ± SEM where n = 4 in both experiments. Na$_i$ was ∼25 mmol/liter ghosts. See text for discussion.

happens during the pump's normal operation, when it is exchanging Na$_i$ for K$_o$, where EP is known to yield its P_i to the inside (see later).

It was reported by Dissing and Hoffman (1990) that the uncoupled efflux of Na from human red cell ghosts, carried out in the absence of Na$_o$ and K$_o$, was inhibited by ∼60–80% when an impermeant anion, such as tartrate, was substituted for a permeant anion, such as SO$_4$ or Cl, on both sides of the membrane. These results supported the idea that the uncoupled Na efflux that was so inhibited (type IA)

actually involved a coefflux of Na with anions that were cytoplasmically derived. But the results raised the question concerning the nature of the residual ouabain-sensitive Na efflux that persisted in the tartrate-loaded ghosts. This problem was partially resolved when it was found (Table III) that not only was there a ouabain-sensitive efflux of ^{32}P from ghosts that contained [γ-^{32}P]ATP but also that the $^{o}k_{32_P}^{ou\,ab}$ was unaffected by Na_o (range 0 to 140 mM). The partial inhibition of $^{o}M_{Na}^{ouab}$ exerted by 5

TABLE III

The Coefflux of Na and ^{32}P from Tartrate-loaded DIDS-treated Resealed Ghosts During Uncoupled Na Efflux

Na_o	K_o	$^{o}M_{Na}^{o\,uab}$ mmol/liter ghosts × h / μmol ATP/liter ghosts		^{32}P efflux rate constant h^{-1}		
		1500	200	Control	+ Ouabain	ΔOuabain
mM				*A*	*B*	*A–B*
0	0	0.48 ± .07	0.53 ± .06	0.472 ± .001	0.232 ± .002	0.240 ± .002
5	0	0.25 ± .06	0.20 ± .04	0.483 ± .002	0.235 ± .001	0.248 ± .002
140	0	—	—	0.475 ± .001	0.228 ± .002	0.247 ± .002
0	10	4.98 ± 0.09	1.67 ± .06	0.242 ± .001	0.231 ± .002	0.011 ± .002

The results of two different experiments are presented. In the first, in which 1,500 μmol ATP/liter ghosts was incorporated, only the efflux of Na was measured at the indicated concentrations of Na_o and K_o. In the second experiment, in which 200 μmol ATP/liter ghosts was entrapped, both the Na and ^{32}P efflux were measured with or without Na_o and K_o. In both experiments, the ghosts were prepared to contain tartrate instead of Cl as the principal anion as detailed in Materials and Methods. The ghosts contained (in mM): 12.5 Na_2 tartrate, 5 Mg tartrate, 10 (Tris)$_2$ tartrate (pH 7.4), 0.25 Tris-EDTA and 0.04 Ap5A. This solution was brought to 300 mosmol with (NMG)$_2$ tartrate. In the first experiment, 1,500 μM ATP was incorporated into ghosts without a regenerating system. In the second experiment, 200 μM ATP was incorporated together with an arginine kinase/creatine kinase regenerating system that consisted of (in μM): 1,000 Arg, 1 ArgP, 10 Cr and 500 CrP. The ghosts in the second experiment were split into two batches so that ^{22}Na could be loaded into one batch and [γ-^{32}P]ATP into the other, otherwise the preparative procedures were the same. The resealing medium was preincubated with inosine as described in Materials and Methods. Analysis of the ghosts at the beginning of the efflux measurement indicated that the content of ATP was 189 μmol/liter ghosts and ADP was <0.4 μmol/liter ghosts. The efflux medium contained 10 mM (Tris)$_2$ tartrate at pH 7.4 brought to 300 mosmol with (NMG)$_2$ tartrate. When present, Na_2 tartrate or K_2 tartrate was substituted for an osmotically equivalent concentration of (NMG)$_2$ tartrate. All flux solutions were gassed with N_2 before the addition of ghosts, in order to reduce contamination from ambient CO_2. The effluxes of ^{22}Na and ^{32}P were measured at 37°C at an hematocrit of 1.5–2% in the absence (*A*) and presence (*B*) of 100 μM ouabain, with the ouabain-sensitive effluxes indicated (*A–B*). Na_i was ~25 mmol/liter ghosts. All flux values presented represent the means ± SEM where for the Na and ^{32}P efflux measurements n = 3 and 4, respectively.

mM Na_o (Table III) is presumably due to traces of HCO_3^- even though the suspension media before the flux measurements were gassed with N_2. (The level of Na_o-sensitive Na efflux seen here is just slightly lower than that observed in ungassed solutions or when 0.1 mM $NaHCO_3$ was intentionally added to the suspension media but the $^{o}M_{Na}^{ouab}$ measured in the presence of 5 mM Na_o is essentially the same in all instances as presented in Table III [data not shown]). Note that $^{o}k_{32_P}^{ou\,ab}$ is inhibited by

10 mM K_o under circumstances where the $^oM_{Na}^{ouab}$ is markedly stimulated because of activation of the pump's exchange of Na_i for K_o. This inhibition by K_o will be considered in more detail below. It should further be noted that for the two ATP concentrations used no differences in the effects would be expected for the pump when it is engaged in uncoupled Na efflux since the pump's apparent affinity for ATP in this mode is ~1 μmol/liter ghosts (Glynn and Karlish, 1976); but the differences in pump rates during Na_i/K_o exchange are consistent with the pump's lower apparent ATP affinity that approximates 250 μmols/l ghosts (Kennedy, et al., 1986).[1]

Orthophosphate P_i Efflux from Intact Cells

Having established, as described above, that during uncoupled Na efflux the pump extrudes ^{32}P from $[\gamma\text{-}^{32}P]ATP$ in conjunction with Na from resealed ghosts, it was natural to wonder if a similarly coupled efflux of P_i could be seen in intact red blood cells. But the problem is different in intact cells than in ghosts because what is measured in the experiments with intact cells is the rate of appearance of P_i in the medium without regard to its source, in contrast to the results discussed above on ^{32}P efflux. If P_i is extruded by the pump in uncoupled Na efflux it is necessary to distinguish this pathway from any others by which P_i from the inside of the cell can appear outside. This is an important consideration because it is known that the ATP hydrolysis that accompanies the pumped exchange of Na_i for K_o occurs on the inside of the cell (Whittam and Ager, 1964; Schatzmann, 1964). Thus, the reason for using DIDS-treated cells was to eliminate the loss of P_i from the cell that could occur through band 3. The use of DIDS increases the sensitivity of the measurement by reducing the background efflux of P_i to levels that pump mediated P_i extrusion can be detected. The results (Table IV) show that not only is there a ouabain-sensitive efflux of P_i but that this efflux is increased by increasing Na_i and is the same whether or not Na_o is present. Increasing Na_i in the range shown is known to activate uncoupled Na_i efflux (Dissing and Hoffman, 1990) and the increase in the Na_o-insensitive efflux of P_i is consistent with this effect. The insensitivity of the P_i efflux to Na_o is also consistent with expectations based on the ^{32}P efflux results presented in Table III.

It should be noted that because of the small size of the P_i efflux (Table IV) the hematocrit of the cell suspension was increased to improve the accuracy of the determination. Because the cells contained relatively high levels of K_i (75 to 90 mmol/liter cells), small amounts of K_i could leak out during the course of the measurement (30 to 60 min) to raise K_o to where there was partial activation of Na_i/K_o exchange. It was therefore important to test the effect of added K_o on the ouabain-sensitive efflux of P_i in the uncoupled Na efflux condition, that is, in the absence of Na_o. The results of such an experiment are shown in Fig. 2 where it is clear that K_o inhibits not only the efflux of P_i but also, under the same circumstances,

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[1] It should be noted that in the discussion that attends Fig. 4 of Kennedy et al. (1986) the symbol, $K_{0.5}^{ADP}$, should be $K_{0.5}^{ATP}$.

the efflux of SO_4. (Because the efflux of both P_i and SO_4 were linear, over the time intervals during which they were measured, the K_o values plotted in Fig. 2 represent the average of the initial and final concentrations of K_o.) It is evident that the concentration of K_o that reduces by one-half ($K_{0.5}$) the efflux of both P_i and SO_4 approximates 0.3 to 0.4 mM K_o. These results support the view that under circumstances where K_o is nominally zero (that is, on the average, less than 0.13 mM), as in the experiments presented in connection with Table IV, that there is no appreciable Na_i/K_o exchange. This is also consistent with the finding of Dissing and Hoffman (1990) that the uncoupled efflux of Na was electroneutral in this circum-

TABLE IV

Ouabain-sensitive Efflux of P_i from DIDS-treated Intact Red Blood Cells during Uncoupled Na Efflux

Experiment	Na_i	Na_o	$^oM_{P_i}$		$^oM_{P_i}^{o\,uab}$
			μmol/liter cells × h		
			Control	+ Ouabain	ΔOuabain
	mmol/liter cells	*mM*	*A*	*B*	*A–B*
1	2	5	43.5 ± 1.3	38.8 ± 1.2	4.8 ± 1.8
(n = 3)	15	5	85.0 ± 2.2	38.5 ± 1.4	46.5 ± 2.6
2	20	0	76.2 ± .5	30.9 ± 0.2	45.4 ± 0.5
(n = 3)	20	5	78.9 ± .3	33.6 ± 0.6	45.3 ± 0.7
	20	140	55.9 ± .3	11.4 ± 0.3	44.5 ± 0.4

The results of two experiments are shown in which the preparation of SO_4-loaded cells for efflux and the assay for $(P_i)_o$ are the same as that described in Materials and Methods except for the following modifications. To change Na_i, as in experiment 1, the cells were split into two groups before being preincubated for 3 h at 37°C, at 10% hematocrit, in a medium that contained either 95 mM Na_2SO_4 + 5 mM NaH_2PO_4 or where the Na_2SO_4 had been replaced by an isosmolar concentration of $(NMG)_2SO_4$. The cells sampled at the time of the efflux assay were found to contain the indicated concentration of Na_i. In addition, the ATP content of the low and high Na_i cells of experiment 1 was 0.9 and 1.4 mmol/liter cells, respectively. The cells in experiment 2 were preincubated in the same way as the high Na_i cells in experiment 1. The efflux was carried out at 37°C, where the hematocrit for experiments 1 and 2 were ~9 and 25% respectively, in media that contained 220 mM $MgSO_4$ + 10 mM $(Tris)_2SO_4$ (pH 7.4). When Na_o was present, the indicated concentrations were obtained by substituting Na_2SO_4 for $MgSO_4$ in isosmolar amounts. The efflux of P_i was measured in the absence and presence of 100 μM ouabain with the ouabain-sensitive difference, $^oM_{P_i}^{o\,uab}$, noted. The values presented represent the means ± SEM where $n = 3$.

stance. The results also indicate that at less than saturating concentrations of K_o, both P_i and SO_4 efflux continue to occur. A direct test, of course, of whether K_i leakage to the outside could be involved in this way is to estimate the $K_{0.5}$ value for K_o on the inhibition of ^{32}P efflux from K_i-free ghosts. The results of this type experiment, which are presented in Fig. 3, show that the $K_{0.5}$ for K_o inhibition of ^{32}P efflux is essentially the same as that obtained in the aforementioned case with intact K-containing cells (Fig. 2). It is also evident in the results shown in Fig. 3 that the inclusion of 1 mmol P_i/liter ghosts was without influence on the efflux of ^{32}P, similar to the findings presented before (Table II).

Thus, while the evidence presented above indicates that the ouabain-sensitive P_i released from intact cells is effluxed through the pump, presumably via EP, in analogy with the ^{32}P efflux, in the uncoupled efflux mode, mention should be made of the possible involvement of the intracellular pool of P_i, i.e., $(P_i)_i$, in this efflux. The results presented in Table IV could be interpreted as indicating that if the mechanism of P_i release was not pump-mediated but occurred by passive diffusion, it would be dependent on the size of the $(P_i)_i$ pool, the latter being dependent on the ouabain-sensitive hydrolysis rate of ATP. But this interpretation would be misleading because the hydrolysis rate of ATP is markedly stimulated by K_o, in turning on the pumped exchange of Na_i for K_o, but the P_i efflux in this situation is inhibited by K_o
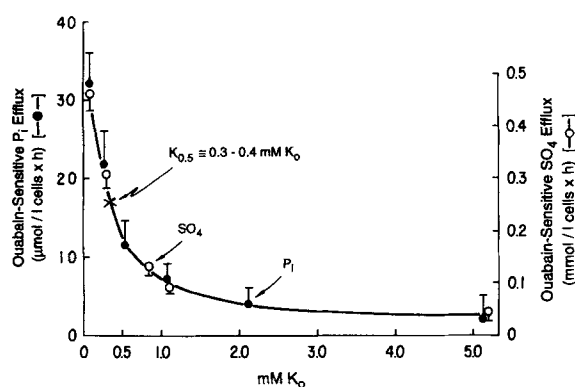

FIGURE 2. The inhibition by K_o of P_i and SO_4 efflux during uncoupled Na efflux from DIDS-treated intact red cells. Two different batches of the same cells were used whose preparation differed only in that one batch was loaded with $^{35}SO_4$ during the preparation procedure (see Materials and Methods) where $Cl_{i,o}$ had been replaced with $(SO_4)_{i,o}$. The efflux of either P_i or $^{35}SO_4$ was measured from cells suspended in a medium containing 10 mM $(Tris)_2SO_4$ at pH 7.4 (37°C), 5 mM glucose and made up to 300 mosmol with $(NMG)_2SO_4$. The indicated values of K_o were added as K_2SO_4 substituting for an osmotic equivalent concentration of $(NMG)_2SO_4$. The approximate hematocrit for cells from which P_i and $^{35}SO_4$ efflux was determined was, respectively, 10 and 1.5 to 2.0%. The values of K_o graphed represent the average of their initial and final values during the flux periods (the value at no added K_o was <0.06 mM in both experiments). Initial cellular contents of Na_i, K_i, $(SO_4)_i$ and ATP were approximately (in mmol/liter cells) 10, 94, 43, and 1 ± 0.1, respectively. The efflux of $(P_i)_i$ and SO_4 were measured in the absence and presence of 100 µM ouabain. The difference between these two curves represents the ouabain-sensitive component for each type of efflux, which is plotted in this figure. Each point graphed represents the mean ± SEM (half-bars) where $n = 4$.

(Fig. 2). In addition, separate experiments (data not shown) indicate that when up to 5 mmol $(P_i)_i$/liter cells is entrapped in intact cells, before DIDS treatment, that the subsequent P_i efflux is unaffected (that is, it remains the same), again consistent with the results found on ^{32}P efflux (Table II, Fig. 3) that the origin of P_i is from the gamma phosphate of ATP and that this phosphate is transferred directly to EP without commingling with $(P_i)_i$.

Effect of K_o on P_i Efflux Is Independent of Na_o

Even though Na_o is without influence on the P_i efflux in the uncoupled mode, it was of interest to test if there was any interaction between Na_o and K_o on the latter's

inhibition of the P_i efflux. This is because Na_o is known to competitively shift the apparent affinity ($K_{0.5}^{APP}$) of the pump for K_o without having any effect on the pump's V_{max} (Post, Merritt, Kinsolving, and Albright, 1960; Whittam and Ager, 1964; Garrahan and Glynn, 1967b; Sachs, 1967, 1977). Thus, in Na_o-free situations where the apparent inhibitory constant, K_I^{APP}, for ouabain-sensitive K_o uptake (or Na efflux) is ~0.15 to 0.20 mM K_o, it is increased to ~1.5 to 2.0 mM K_o in solutions that
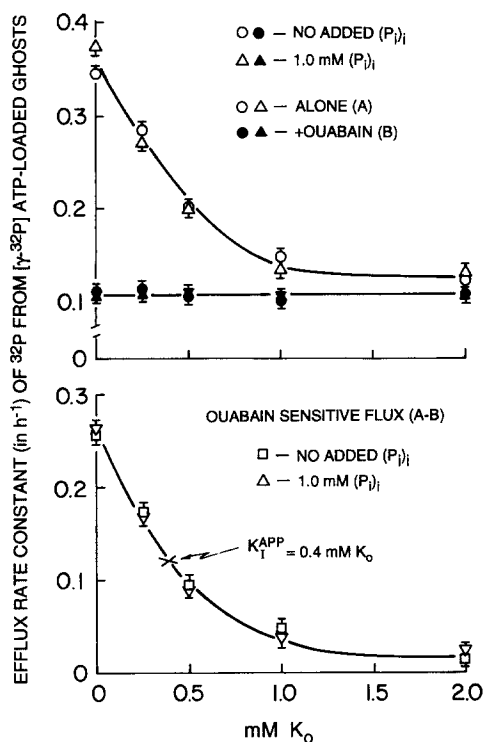

FIGURE 3. The effect of K_o and $(P_i)_i$ on the efflux of ^{32}P, during uncoupled Na efflux, from DIDS-treated resealed ghosts containing $[\gamma^{-32}P]ATP$. Ghosts were prepared to contain (in addition to Na_2SO_4, $(NMG)_2SO_4$, $MgSO_4$, EDTA, Ap5A) an arginine kinase/creatine kinase regenerating system with and without 1 mM P_i (as H_3PO_4) as described in Materials and Methods. Because the effect of the presence of $(P_i)_i$ was to be tested on the efflux of ^{32}P the equilibration of the second resealing medium was carried out in the absence of inosine. The composition of the regenerating system was (in μM): 1000 Arg + 1 ArgP + 10 Cr + 500 CrP and 200 ATP. The measured concentration of ATP at the beginning of the flux assay was 189 $\mu mol/liter$ ghosts; the concentration of $(P_i)_i$ when added was 1.3 mmol/liter ghosts otherwise it was <0.1 mmol/liter ghosts. The efflux was carried out at 37°C at an hematocrit of 1.5–2% in a medium that contained 10 mM $(Tris)_2SO_4$ (pH 7.4), the indicated concentrations of K_o (added as K_2SO_4) and brought to 300 mosmols with $(NMG)_2SO_4$. The values of K_o graphed represent the average of their initial and final values during the 5 to 35 min flux period. Na_i was 25 mmol/liter ghosts. The upper panel shows the efflux rate constant ($^o k_{32P}$) of ^{32}P in the absence (open symbols) and presence (closed symbols) of 100 μM ouabain. The ouabain-sensitive difference in the paired curves is shown in the lower panel. Each point graphed represents the mean ± SEM (half-bars) where n = 3.

contain high Na_o (~140 mM). But, surprisingly, this is not the case for the ouabain-sensitive P_i efflux, where, as shown by the results presented in Fig. 4 (lower graph), the K_I^{APP} for K_o was ~0.25 mM and remained unchanged when Na_o was increased from zero to 140 mM. It is surprising because this appears to be the only instance for the known parameters of the pump in which there is no interaction between Na and K (e.g., Hoffman et al., 1991).

The above results raise the question concerning the inherent nature of the Na_o-insensitive component of uncoupled Na efflux that is coupled to P_i efflux (type IB) compared to its type IA or Na_o-sensitive cytoplasmically-linked anion (SO_4) component. Are these types two aspects of a single kind of Na/K pump or do they represent two kinds of pumps with overlapping or common properties? It should be remembered that these different aspects only become evident in the uncoupled mode
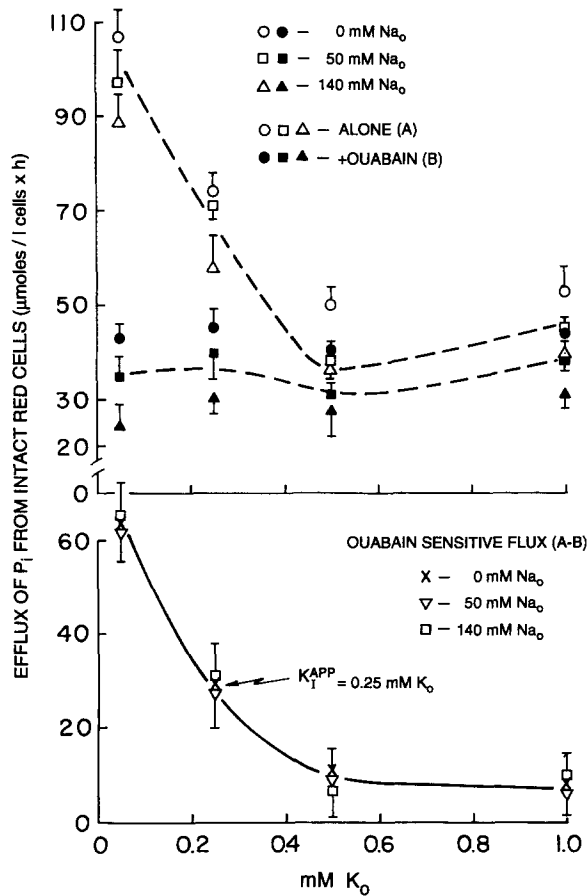

FIGURE 4. Na_o does not compete with K_o for inhibition of P_i efflux during uncoupled Na efflux from DIDS-treated intact cells. The cells were preincubated (at 10% hematocrit) for 3 h at 37°C in a solution that contained (in millimolar): 95 Na_2SO_4, 5 NaH_2PO_4, 5 glucose and 5 adenosine (pH 7.4) in order to substitute $(SO_4)_{i,o}$ for $(Cl)_{i,o}$ as described in Materials and Methods. DIDS was added to a final concentration of 50 μM 30 min before the end of incubation. The cells were then washed three times and resuspended, at 10% hematocrit, in a flux medium that contained 220 mM $MgSO_4$ and 10 mM $(Tris)_2SO_4$ at pH 7.4 (see Dissing and Hoffman, 1990). Equivalent osmotic amounts of K_2SO_4 and/or Na_2SO_4 were substituted for $MgSO_4$ as indicated. The nominally zero K_o was measured at the beginning of the flux period and found to be <0.05 mM. The upper panel shows the efflux of $(P_i)_i$ in the absence (*open symbols*) and presence (*closed symbols*) of 100 μM ouabain. The ouabain-sensitive difference between each of the paired curves is shown in the lower panel. The K_I^{APP} indicates the concentration of K_o that reduces the P_i efflux by 50%. Na_i and ATP were ~20 and 1 to 1.2 mmol/liter ghosts, respectively. Each point graphed represents the mean ± SEM (*half-bars*) where $n = 4$.

whereas the pump properties appear to be uniform in the presence of K_o, whether or not the pump is engaged in Na_i/K_o or K_i/K_o exchange. Thus, it would be important to know if the K_I^{APP} for the pumped uptake of K_o, during Na_i/K_o exchange, by the type IB component was insensitive to Na_o as was its P_i efflux in the absence of K_o (Fig. 4). But whether or not this characteristic of type IB in the absence of K_o persists in the presence of K_o is not known. Until a method is devised to operationally separate

type IA and IB components from each other in the presence of K_o, it will not be possible to study the comparative effects of Na_o on the pump's activation by K_o.[2]

The results on the inhibition by K_o of the efflux of P_i during uncoupled Na efflux (whereby K_o converts uncoupled Na efflux to Na_i/K_o exchange) also raises the question concerning the mechanism of this effect. While this is considered in more detail in the discussion, it would appear that the binding of K_o to the E_2P form of the pump forces a change in the pump's conformation such that the consequent release of P_i is to the inside.

Effect of Protons$_o$ on SO$_4$ and P$_i$ Efflux

Polvani and Blostein (1988) have reported that external protons (protons$_o$) can activate, in human red blood cells, a pump-mediated exchange of Na_i for protons$_o$ in analogy with Na_i/K_o exchange that was ATP-dependent and strophanthidin-sensitive. This Na_i for proton$_o$ exchange only occurs in the absence of Na_o and K_o (that is, in the uncoupled Na efflux mode) and when the pH_o is less than 6.8 (this was confirmed by Dissing and Hoffman, 1990; see their Fig. 7). The results presented in Table V show that, in intact cells, when the pH_i is clamped at ~7.3 (by the use of DIDS treatment [see Heinz and Hoffman, 1990]), lowering the pH_o to 6.2 inhibits the effluxes of both P_i and SO$_4$. (In the two instances shown, the SO$_4$ efflux is not significantly different from zero.) Both anion effluxes take place at their usual rates when the pH_o is 7.4. Thus, these results mirror those of Polvani and Blostein (1988) in showing that when protons$_o$ are transported to the inside below pH_o 6.8, that SO$_4$ and P_i are transported out above but not below pH_o 6.8. These results also imply that protons$_o$ might be acting in an analogous way to K_o (e.g., Figs. 2 and 3) in preventing the release of anions to the outside as discussed before.

It should also be noted (Table V) that when the pH_o is raised to 8.2 that, although the fluxes are reduced compared to their values when the pH_o is 7.4, the effluxes of SO$_4$ and P_i continue to occur in concert with the efflux of Na. These results are in contrast to those obtained on kidney Na,K-ATPase inserted into liposomes (Gold-shleger, Shahak, and Karlish, 1990). These authors found that "uncoupled" Na efflux in this preparation occurred electroneutrally in the pH_o range 6.5–7.0 becoming electrogenic as the pH_o was increased to pH 8.5. They interpreted their results on Na efflux at pH 6.5–7.0 as representing $Na_i/proton_o$ exchange (proton movement was not measured) with the proton$_o$ influx being lost at the higher pH_o. Because anion efflux in red cells continues to occur at pH_o 8.2, it would be of interest to know the extent to which these types of fluxes might be displayed by the kidney vesicle

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[2] It is possible to estimate the magnitude of this effect by assuming that the two types of uncoupled Na efflux are different with regard to the competitive effects of Na_o on K_o activation. If it is also assumed that on average type IB comprises 25% of the total pumped exchange of Na_i for K_o (because all of the pumps are functional in both the uncoupled and Na_i/K_o exchange modes even though the V_{max} of their operation is different [see Dissing and Hoffman, 1990]) and that the K_o activation of the pump's uptake of K_o by this component is insensitive to Na_o. By using the results of Sachs (1977) on the K_o activation of the pump's uptake of K_o in the presence of 160 mM Na_o and his rate equation (Eq. 3) it was found that the apparent $K_{0.5}^{APP}$ was ~1.03 mM K_o when only 75% of the uptake was Na_o-sensitive compared to 1.32 mM K_o, when all of it was. In addition, the sigmoidicity of the activation curve seen in the latter instance becomes antisigmoidal in the former case.

preparation. But considering that other properties of what is called uncoupled Na efflux in the two types of preparations are so different, as pointed out by Dissing and Hoffman (1990), the likelihood of a common anion flux is low.

Effect of Altering the ADP/ATP Ratio on Anion Efflux

It was known from previous work that increasing the steady state concentration of ADP relative to that of ATP resulted in inhibition of the pump's exchange of Na_i for K_o (see Kennedy et al., 1986). It thus appeared that this effect might offer another approach to test the evident linkage between the effluxes of anions (SO_4 and P_i) and Na in the uncoupled efflux mode, that is, in the absence of Na_o and K_o. The results

TABLE V

The Effect of External Protons on the Coefflux of Na, SO_4 and P_i from DIDS-treated Intact Red Cells during Uncoupled Na Efflux

Experiment	pH_o	pH_i	$^oM_{Na}^{o\,uab}$	$^oM_{SO4}^{o\,uab}$	$^oM_{Pi}^{o\,uab}$
A	6.2 (6.5)	—	0.387 ± .035	0.129 ± .122*	0.001 ± .002
B	6.2 (6.4)	7.3 (7.0)	0.338 ± .038	0.172 ± .129*	−0.001 ± .002
	7.4 (7.4)	7.3 (7.3)	0.978 ± .038	0.430 ± .086‡	0.072 ± .002
C	7.4 (7.3)	7.3 (7.1)	0.869 ± .033	0.387 ± .043§	0.056 ± .002¶
	8.2 (8.0)	7.3 (7.4)	0.377 ± .050	0.172 ± .043‖	0.026 ± .002¶

The results of three different experiments are presented. In each experiment the cells were separated into three groups, depending on whether they were labeled with ^{22}Na or $^{35}SO_4$ or remained unlabeled, during the 3-h preincubation in which $(CL)_{i,o}$ was replaced by $(SO_4)_{i,o}$ by suspension in a medium that contained 95 mM Na_2SO_4 + 5 mM NaH_2PO_4 as described in Materials and Methods. The efflux was carried out at 37°C, where the hematocrit values for ^{22}Na and $^{35}SO_4$ loaded cells were 1.5–2% and ~10% for the unlabeled cells, in media of different pH that contained 220 mM $MgSO_4$ + 10 μM DIDS (to provide better $pH_{i,o}$ control even though the cells were also DIDS-treated as usual during the preincubation) and either 10 mM $(Tris)_2SO_4$ (pH_o 7.4 or 8.3) or PIPES (pH_o 6.2). All flux media were preequilibrated with N_2 at pH 5.0 to reduce CO_2 contamination before being titrated to their final pH's. The pH_o and pH_i values shown were determined on aliquots removed after the cells had been added to their respective media just before and after the 35 min flux period was started. The pH's given in parentheses represent the latter values and indicate the effectiveness of the pH clamp (see Heinz and Hoffman, 1990). The effluxes were measured in the absence and presence of 100 μM ouabain but only the ouabain-sensitive components are presented. Na_i values for cells used in experiments A, B, and C were 17.6, 18.8, and 16.4 mmol/liter cells, respectively, where $(SO_4)_i$ was ~43 mmol/liter cells in all instances. The flux values presented represent the means ± SEM where n = 3, and P values are: *0.25 to 0.5; ‡0.025 to 0.05; §0.01 to 0.025; ‖<0.005; ¶<0.001. See text for discussion.

presented in Table VI indicate that this is the case since at different preset levels of ADP and ATP, there is a concurrent inhibition of all three fluxes as the ADP/ATP ratios are increased. (Direct comparison of the different kinds of effluxes is not possible because each column presented in Table VI represents the results of a separate experiment.) Kennedy et al. (1986) found that the onset of inhibition of ouabain-sensitive Na_i/K_o exchange was already apparent when the ratio of the levels of ADP/ATP were equal to or greater than one. This also applies to the uncoupled efflux mode, as seen in Table VI, when partial inhibition of $^oM_{Na}^{ouab}$, $^oM_{SO_4}^{o\,uab}$, and $^ok_{32p}^{ou\,ab}$ has occurred when the concentration of ADP and ATP were the same. Increasing the ADP/ATP ratio further markedly increases the level of inhibition of

TABLE VI

The Effects of Altering the ATP/ADP Ratio on the Coefflux of Na, SO_4 and ^{32}P from DIDS-treated Resealed Ghosts during Uncoupled Na Efflux

ADP μmol/liter ghosts	$^oM^{o\,uab}_{Na}$ mmol/liter ghosts × h ATP μmol/liter ghosts		$^oM^{ouab}_{SO_4}$ mmol/liter ghosts × h ATP μmol/liter ghosts		$^ok^{ouab}_{32P}$ h^{-1} ATP μmol/liter ghosts	
	24 ± 2*	49 ± 2‡	10 ± 1§	201 ± 11‖	10 ± 1¶	201 ± 11**
0.20 ± .02	0.825 ± .060	0.925 ± .044	0.482 ± .007	0.464 ± .009	0.257 ± 004	0.209 ± .004
5.7 ± 0.2	0.775 ± .044	—	0.460 ± .006	—	0.254 ± .002	—
10.9 ± 0.5	0.775 ± .060	0.875 ± .070	0.314 ± .006	—	0.154 ± .003	—
25 ± 2	0.400 ± .044	0.925 ± .044	0.073 ± .007	—	0.036 ± .004	—
52	—	0.500 ± .044	—	—	—	—
105 (103, 107)	—	—	—	0.412 ± .009	—	0.183 ± .004
214 (212, 216)	—	—	—	0.344 ± .009	—	0.125 ± .004
502 ± 21	—	—	—	0.228 ± .009	—	0.022 ± .004

Each column represents the results of a separate experiment in which an arginine kinase/creatine kinase based nucleotide buffering system[3] was incorporated into the ghosts in order to regulate the indicated ATP and ADP concentrations during the time course of the efflux measurements. The ATP and ADP concentrations (in units of μmol/liter ghosts) listed in the table were determined on resealed ghosts sampled just before the efflux assay and the values presented represent the means ± SEM, where n = 4–6. The value of n here represents the average of duplicate measurements in four to six separate experiments. Single values listed represent the average of duplicate determinations on ghosts in a single experiment. The cells to be used in the Na efflux measurements were loaded in a NaCl/choline Cl/Tris Cl medium while the cells to be used for the SO_4 and ^{32}P efflux measurements were loaded in a Na_2SO_4/$(Tris)_2SO_4$ medium as described in Materials and Methods. The preparation of resealed ghosts containing either ^{22}Na, $^{35}SO_4$ or $[\gamma\text{-}^{32}P]ATP$ were prepared as described in the legend to Table I except for the use of Cl rather than SO_4 solutions for the ^{22}Na loaded ghosts. Na efflux $^oM^{o\,uab}_{Na}$ in mmol/liter ghosts was measured from ghosts suspended in a medium that contained 160 mM choline Cl + 20 mM Tris Cl at pH 7.4; for SO_4 ($^oM^{o\,uab}_{SO_4}$ in mmol/liter ghosts) and ^{32}P ($^ok_{32P}$, in h^{-1}) efflux, the medium contained 10 mM $(Tris)_2SO_4$ together with sufficient $(NMG)_2SO_4$ to bring the final osmolality to 300 mosmol. The effluxes were all carried out at 37°C at hematocrits between 1.5 and 2% in the absence and presence of 100 μM ouabain. Only the ouabain-sensitive difference in the effluxes are shown where the values presented represent the mean ± SEM where n = 3. Na_i and $(SO_4)_i$ in all cases was ~25 and 43 mmol/liter ghosts, respectively.

both the SO_4 and ^{32}P effluxes. The presumed mechanism by which ADP exerts its effect is as a product inhibitor, restraining the release of ADP from an $E_1P\cdot ADP$ form if not promoting its back reaction to an E_1ATP form (see Kennedy et al., 1986 for further discussion).

[3] The nucleotide regeneration system incorporated into the ghosts that were used in connection with the results presented in Table VI are given in the following where for each experiment the values listed refer to each column top to bottom (four flux conditions each) for the constituents in their respective order for Arg, ArgP, Cr, CrP, ATP, and ADP are (in micromolar): (*)8, 1, 100, 100, 50, and 0; 8, 1, 100, 100, 50, and 10; 8, 40, 100, 100, 50, and 25; 8, 20, 100, 100, 50, and 50; (‡) 8, 1, 100, 100, 25, and 0; 8, 1, 100, 100, 25, and 5; 8, 1, 100, 100, 25, and 10; 8, 1, 100, 40, 25, and 25; (§) 10, 1, 20, 10, 10, and 0; 10, 1, 20, 10, 10, and 2; 40, 1, 20, 10, 10, and 8; 100, 5, 50, 10, 10, and 23; (‖) 1,000, 1, 10, 500, 200, and 0; 1,000, 1, 10, 500, 250, and 50; 1,000, 1, 10, 250, 300, and 100; 5,000, 100, 200, 100, 400, and 300. (¶) same as §; (**) same as ‖.

Influx of Na and SO₄

Since the question of the stoichiometry of Na to SO_4 effluxes is considered in the next section, it is important to know whether there are significant influxes of these ions, relative to their effluxes, in order to quantitate their net movements across the cell membrane. The results presented in Table VII show that there is no significant ouabain-sensitive influx of either Na or SO_4 when Na_o is raised from zero to 5 mM. Note that this is the same circumstance in which 5 mM Na_o markedly inhibits both the Na and SO_4 efflux (see Table I). In addition it is evident that the SO_4 influx at 140 mM Na_o is also negligible. Therefore these results indicate that measurements of the effluxes of these ions provide a direct measure of their net movements in the

TABLE VII

The Effect of Na_o on the Influx of Na and SO_4 into DIDS-treated Resealed Red Cell Ghosts

	Influx					
	Na			SO₄		
	mmol/liter ghosts × h			mmol/liter ghosts × h		
Na₀	Control	+ Ouabain	ΔOuabain	Control	+ Ouabain	ΔOuabain
mM						
0	—	—	—	3.265 ± .002	3.261 ± .003	0.004 ± .004
0.5	0.128 ± .003	0.126 ± .003	0.002 ± .004	—	—	—
1.0	0.203 ± .004	0.200 ± .004	0.002 ± .006	—	—	—
5.0	0.390 ± .005	0.382 ± .005	0.008 ± .007	3.213 ± .003	3.211 ± .002	0.002 ± .004
140	—	—	—	3.258 ± .002	3.258 ± .003	0.000 ± .004

The influx of Na and SO_4 were carried out in separate experiments on ghosts prepared in the same way. The ghosts contained (see Materials and Methods) an arginine kinase/creatine kinase regenerating system that consisted of (in µM): 100 ATP, 800 Arg, 1 ArgP, 10 Cr and 500 CrP. The concentrations of ATP and ADP, determined on ghosts sampled at the time of the influx measurement were, respectively, 107 and 0.24 µmol/liter ghosts. The SO_4-loaded cells were suspended in a medium that contained 10 mM $(Tris)_2SO_4$ (pH 7.4) + sufficient $(NMG)_2SO_4$ to bring the solution to 300 mosmol. The indicated concentrations of Na_o were added to the medium as Na_2SO_4 in substitution for isosmolar $(NMG)_2SO_4$. The influxes were performed at 37°C, at an hematocrit of 1.5 to 2 percent, as described in Materials and Methods in the absence and presence of 100 µM ouabain. Na_i and $(SO_4)_i$ in both experiments were found to be ∼25 and 40 mmol/liter ghosts, respectively. The influx values presented represent the means ± SEM where $n = 3$.

uncoupled Na efflux mode. The results also indicate that the Na_i/Na_o exchange does not occur to any appreciable extent at 5 mM Na_o, in support of the view that type IB uncoupled efflux is Na_o-insensitive. It is reasonable to assume that this also applies in the case of P_i influx although no ouabain-sensitive uptake measurements of this ion were made.

Stoichiometry of Na to SO_4 and P_i Effluxes

Table VIII summarizes results concerned with the stoichiometry of the ouabain-sensitive effluxes of Na to SO_4 and Na to P_i that occur in the uncoupled efflux mode. The results of experiments 1 and 2, taken from Table I (where the unidirectional effluxes of Na and SO_4 were measured on different batches of resealed ghosts

prepared at the same time) indicate that the ratio of the Na_o-sensitive component of Na efflux to the ouabain-sensitive component of SO_4 efflux is 2.15 and 1.76, respectively. These are similar to the four estimates of this ratio as reported by Dissing and Hoffman (1990). The mean ± SEM for these six sets of values is 1.95 ± .09, which is not significantly different from 2 ($P \leq 0.77$). The stoichiometric ratio of the ouabain-sensitive and Na_o-insensitive effluxes of Na to P_i, as measured on intact cells (experiment 3) in the presence of 5 mM Na_o was found to be 2.25. (The presumed reason that the ouabain and Na_o-insensitive value of Na efflux in this experiment was somewhat smaller than usual is that Na_i was lower than usual, i.e., 12 compared to 25 mmol Na_i/liter cells.) In addition, in the two other experimental results presented in Table V (B and C) that were carried out on intact cells at pH_o 7.4,

TABLE VIII

Stoichiometry of the Efflux of Na with SO_4 and P_i during Uncoupled Na Efflux in DIDS-treated Resealed Ghosts and Intact Red Cells

Experiment	Flux component	Efflux			Stoichiometry		
		Na	SO_4	P_i	Na/SO_4	Na/P_i	$Na/(SO_4 + P_i)$
1	Na_o-sensitive	.775	.360	—	2.15	—	—
2	Na_o-sensitive	.775	.440	—	1.76	—	—
3	Ouabain-sensitive	.108 ± .030	—	.048 ± .004	—	2.25	—
4	Ouabain-sensitive (B)	.978	.430	.072	—	—	1.95
	Ouabain-sensitive (C)	.869	.387	.056	—	—	1.96

The results of four experiments are shown. The effluxes of Na and SO_4 in experiments 1 and 2 are taken, respectively, from experiments 1 and 2 in Table I where the results on resealed ghosts are expressed, in mmol/liter ghosts × h, as the Na_o-sensitive efflux (i.e., the difference in efflux obtained in 0 and 5 mM Na_o). The effluxes of Na and P_i (experiment 3) and Na, SO_4 and P_i (experiment 4) were determined on intact cells where the efflux units are mmol/liter cells × h. The results of experiment 4 are taken from those presented in Table V (B and C at pH_o 7.4) but experiment 3 represents a separate measurement. Experiment 3 was carried out following the same protocol as that described in Table V, where pH_o was 7.4, except that the Na_o concentration was 5 mM by using 2.5 mM Na_2SO_4 to replace an osmotic equivalent concentration of $MgSO_4$ in order to eliminate the Na_o-sensitive uncoupled Na efflux component (i.e., type IA). Because the effluxes were measured in the absence and presence of 100 μM ouabain, the residual ouabain-sensitive Na efflux could thus be compared to the ouabain-sensitive efflux of P_i (i.e., type IB uncoupled Na efflux). The efflux values presented for experiment 3 represent the means ± SEM where $n = 4$ (Na_i was 12 mmol/liter cells on cells sampled just before the efflux measurement). See text for discussion.

the stoichiometric ratio of the ouabain-sensitive effluxes of Na to $[SO_4 + P_i]$ was found to be 1.95 and 1.96, respectively. Had the Na_o-sensitive component of Na efflux been evaluated in these latter two experiments it would have been possible to estimate separately the stoichiometric ratios of Na to SO_4 and Na to P_i as done in experiments 1 and 2 (Table VIII). Nevertheless, the results presented in Table VIII are consistent with the idea that the efflux of Na in the uncoupled mode is electroneutral (see Dissing and Hoffman, 1990) involving a balanced coefflux of anions which in the instance shown, are comprised of SO_4 and P_i. If this is so then these results would also imply that the valence of the cotransported P_i was two, but this would need to be confirmed by other approaches (e.g. Gunn, Milanick, and Froehlich, 1980).

Stoichiometry of Uncoupled Na Efflux and ATP Utilization

Table IX presents results of experiments where the ouabain-sensitive Na efflux and ATPase activity (see Materials and Methods) were measured on the separately labeled batches of the same preparation of ghosts that were prepared to maintain different set concentrations of ATP and ADP and incubated under conditions where the concentration of Na_o was either 0, 5, or 140 mM (e.g., Fig. 1 has plotted the data of experiment 2 of Table IX). The results are of interest for several reasons. First, it is evident that, regardless of the relative concentrations of ATP and ADP, the ratio of the number of Na_i ions effluxed per ATP hydrolyzed is close to 3 (mean value is 2.78 ± .06). These results are similar to the values reported previously by Glynn and Karlish (1976) and Blostein (1983) even though the experimental conditions are different and the range of variations in both the ATP and ADP concentrations is greatly extended in the present work. Thus, it is clear that for uncoupled Na efflux, measured at 0 mM Na_o, the absolute values of the ouabain-sensitive Na efflux and ATP utilization are approximately the same whether or not ADP is present (experiments 1, 2, and 4). In addition, the stoichiometric ratio of Na ions pumped per ATP used is ~3 in the presence and absence of ADP.

Second, it is also clear, as found by Glynn and Karlish (1976), that both the ouabain-sensitive Na effluxes as well as the ATPase activities are concordantly reduced in the presence of 5 mM Na_o and that ADP is essentially without effect on the resultant stoichiometry. These results in the presence and absence of 5 mM Na_o are important because of the questions they raise regarding the mechanism(s) responsible for the evident electroneutral transport stoichiometry of Na to anions for both of the two types (IA and B) of uncoupled Na efflux (Dissing and Hoffman, 1990). Thus, as discussed before (Table VIII), the transport stoichiometry of the Na_o-sensitive component (type IA) of uncoupled Na efflux is 2 Na/SO_4 while that for the Na_o-insensitive component (type IB) is 2 Na/PO_4. How these results can be fit to or rationalized with an ATPase activity where the number of Na ions transported per ATP utilized is ~3 is not as yet clear.

Third, increasing Na_o to 140 mM in the absence of ADP does not appreciably alter the Na efflux (or its associated ATPase activity) above its value at zero Na_o, as also reported by Glynn and Karlish (1976), in any of the experiments presented in Table IX, even though, as discussed below, an influx of Na_o is initiated by this maneuver (see Lee and Blostein, 1980; and Blostein, 1983). The extent to which this increase in the Na efflux that occurs in the presence of 140 mM Na_o represents a relief of the inhibition seen at 5 mM Na_o or an activation of a new process (i.e., an exchange of Na_o for Na_i or both will be considered in the next section. On the other hand, Na efflux at high Na_o is markedly stimulated by the addition of ADP and also promotes an exchange of Na_o for Na_i (see Garrahan and Glynn, 1967a,d; and Glynn and Hoffman, 1971). The main difference between the two kinds of exchange of Na_i for Na_o referred to above is that net hydrolysis of ATP occurs in the former but not in the latter instance (Blostein, 1983; and Garrahan and Glynn, 1967d).

Influx of Na at 140 mM Na_o and the Effect of ADP

Table X presents the results of Na influx measurements that were carried as part of the same experiments under which Na efflux was determined (experiments 3 and 4,

TABLE IX

The Relationship between the Ouabain-sensitive Efflux of Na to ATP utilization in DIDS-treated Resealed Red Cell Ghosts as a Function of Na_o and Various ATP/ADP Ratios

Expt.	ATP	ADP	Na	Ouabain-sensitive component		Ratio A/B
				Na efflux (A)	ATPase activity (B)	Na flux/ATP used
	μmol/liter ghosts		*mM*	*mmol/liter ghosts* × *h*		
1	207	0.11	0	0.800 ± .100	0.317 ± .008	2.52
(*n* = 3)			5	0.325 ± .050	0.117 ± .008	2.78
			140	0.825 ± .100	0.339 ± .009	2.43
		43	0	0.850 ± .075	0.302 ± .006	2.81
			5	0.350 ± .050	0.138 ± .006	2.54
			140	1.975 ± .100	0.508 ± .011	3.89
2	121	0.33	0	0.950 ± .100	0.336 ± .012	2.83
(*n* = 3)			5	0.275 ± .050	0.103 ± .007	2.67
			140	1.025 ± .100	0.378 ± 010	2.71
		21	0	0.925 ± .080	0.321 ± .004	2.88
			5	0.250 ± .050	0.093 ± .010	2.69
			140	1.375 ± .100	0.518 ± .012	2.65
		53	0	0.850 ± .100	0.310 ± .006	2.74
			5	0.175 ± .050	0.064 ± .007	2.73
			140	1.925 ± .100	0.760 ± .015	2.53
3	118	0.22	0	0.575 ± .100	0.214 ± .007	2.69
(*n* = 3)			140	0.475 ± .130	0.166 ± .006	2.86
		53	0	0.375 ± .100	0.135 ± .007	2.77
			140	1.400 ± .100	0.534 ± .006	2.62
4	24	0.15	0	0.245 ± .015	0.081 ± .009	3.02
(*n* = 3)			140	0.260 ± .005	0.096 ± .008	2.71
		12	0	0.240 ± .010	0.088 ± .009	2.73
			140	0.670 ± .015	0.255 ± .015	2.63

The results of four separate experiments are shown. In each experiment, the ghosts before resealing were separated into two (or three, see Table X, legend) batches that were otherwise treated in the same way. One batch was labeled with [22]Na and the other with [γ-^{32}P]ATP in order to measure, respectively, Na efflux and ATP utilization (by measuring the rate of liberation of ^{32}P from [γ-^{32}P]ATP) as described in Materials and Methods. To control the indicated ATP and ADP concentrations during the time course of the efflux and ATPase assays, an arginine kinase/creatine kinase nucleotide regeneration system was incorporated[4] into the ghosts as previously described. The ATP and ADP values given in the table were measured on samples of ghosts removed at the time of the efflux and ATPase assays. The ghosts were prepared to contain 25 mM Na_i as Na_2SO_4 + 10 mM $(Tris)_2SO_4$ + sufficient $(NMG)_2SO_4$ to bring the final osmolality to 300 mosmol, except that in experiment 4, Na_i was 5 mmol/liter ghosts and in experiment 1, the ghosts contained a NaCl/choline Cl/Tris Cl medium as described in Materials and Methods. The efflux and ATPase assays were carried out at 37°C at an hematocrit of 1.5 to 2% on ghosts suspended either in 300 mosmol $(NMG)_2SO_4$ containing 10 mM $(Tris)_2SO_4$ with Na_2SO_4 being substituted for $(NMG)_2SO_4$ at the indicated concentrations of Na_o or in 160 mM choline Cl + 20 mM Tris Cl with NaCl being substituted for choline Cl at the indicated concentrations of Na_o. The effluxes of Na and the ATPase assays were carried out at the same time in the absence and presence of 100 μM ouabain. Only the ouabain-sensitive difference in the effluxes of Na and ATPase activity are shown (except for experiment 2 where the Na efflux results are graphed in Fig.1) with the values presented representing the means ± SEM, where *n* = 3. See text for discussion.

Table IX). Values of Na efflux (Table IX) for the indicated conditions are repeated in Table X for ease of comparison. Although the stoichiometry of ADP-independent Na_i for Na_o exchange may be 1 to 1 or 3 to 2 (see Blostein, 1983; Yoda and Yoda, 1987), the expected stoichiometry for ADP-dependent Na_i for Na_o exchange is 1:1 (Garrahan and Glynn, 1967a). For the results presented in Table X it is evident that in both of the above kinds of Na_i/Na_o exchange efflux exceeds influx. The reasons these results differ from those of others are not known but may be related to the different red cell preparations studied (Blostein, 1983, used inside-out vesicles; Garrahan and Glynn, 1967a, used intact cells) and/or the various conditions and/or constituents (e.g., the use of SO_4 instead of Cl) that obtained during the flux measurements. (Glynn, Lew, and Luthi, 1970, report that the stoichiometry for ADP-dependent Na_i/Na_o exchange may be related to the cellular level of orthophosphate but whether or not this is a factor here would need to be evaluated.)

TABLE X

The Effect of ADP, at High Values of Na_o, on the Influx and Efflux of Na in DIDS-treated Resealed Red Cell Ghosts

Experiment		ATP	ADP	Na_o	Na influx			Na efflux (Δ ouabain)
					mmol/liter ghosts × h			
					Control (A)	+ ouabain (B)	Δouabain (A—B)	
		μmol/liter ghosts		*mM*				*mmol/liter ghosts × h*
3	A	121	0.22	140	4.286 ± .014	4.109 ± .004	0.177 ± .015	.475 ± .130
	B	118	53	140	4.506 ± .017	4.056 ± .021	0.450 ± .021	1.400 ± .100
4	A	27	0.15	140	3.963 ± .009	3.885 ± .006	0.078 ± .011	.260 ± .005
	B	22	12	140	4.094 ± .003	3.889 ± .006	0.206 ± .007	.670 ± .015

The ghosts used in the experiments shown in this Table were prepared as the third batch in conjunction with experiments 3 and 4 as described in the legend of Table IX, in which they were not labeled with any radioactive isotope but otherwise treated the same as the other two batches of each experiment. The influx was carried out under the same circumstances as that described for efflux in Table IX but following the protocols described in Materials and Methods (see also Table VII, legend). For ease of comparison with influx the respective ouabain-sensitive efflux values from Table IX are repeated here. All influx values presented represent the means ± SEM where n = 4. See text for discussion.

Nevertheless, it is clear from what has already been discussed that when Na_o and ADP are nominally zero, uncoupled Na efflux proceeds with a stoichiometry of ~3 Na/ATP (Table IX). It should also be recalled that the influx of Na_o is negligible for

[4] The nucleotide regenerating systems incorporated into the ghosts that were used in connection with the results presented in Table IX are given in the following where for each experiment (1–4), the values listed refer to each condition tested, i.e., different combinations of ATP and ADP concentrations. Note that the ATP and ADP concentrations incorporated are different from the measured estimates given in the table. Thus, the constituents incorporated in their respective order for Arg, ArgP, Cr, CrP, ATP, and ADP are (in μM): (*1*) 1,000, 1, 10, 500, 200, 0; 1,000, 2, 10, 500, 250, and 20; (*2*) 5, 1, 20, 100, 125, and 0; 100, 1, 50, 5, 125, and 25; 100, 10, 50, 5, 125, and 50; (*3*) 500, 2, 10, 250, 125, and 0; 1,000, 1, 10, 500, 125, and 50; (*4*) 8, 1, 100, 100, 25, and 0; 8, 1, 100, 50, 25, and 10.

values of Na_o between 0 and 5 mM (Table VII). Because the efflux of Na at zero ADP is essentially the same at zero and 140 mM Na_o, the implication is that the magnitude of uncoupled Na efflux is the same in these two situations. But this cannot be so because there is a significant influx of Na_o at 140 mM Na_o (Table X, experiments 3A and 4A), an indication that uncoupled Na efflux must have been inhibited. The extent of this inhibition of (or the level of the residual) uncoupled Na efflux can be estimated by subtracting the amount of the Na_i/Na_o exchange efflux from the total Na efflux. The amount of the exchange efflux is estimated by using the Na_o influx values and assuming a Na_o for Na_i exchange stoichiometry of either 1 for 1 or 2 Na_o for 3 Na_i (Lee and Blostein, 1980; Blostein, 1983; Yoda and Yoda, 1987). If the Na_o/Na_i exchange stoichiometry is 1 for 1 then for experiment 3A (Table X) the corrected efflux is 0.475–0.177 = 0.298 mmol Na/liter ghosts × h; if the stoichiometry is 3 Na_o/2 Na_i then the corrected efflux is 0.475–0.266 = 0.209 mmol Na/liter ghosts × h. Since in this experiment, uncoupled Na efflux at zero Na_o (see Table IX) was 0.575 mmol Na/liter ghosts × h, the inhibition was 48 and 64%, respectively, for the above stoichiometries. (For experiment 4A, the comparable percent inhibition was 26 and 42.) Because the usual level of inhibition by 5 mM Na_o of uncoupled Na efflux is between 60 and 80% (Dissing and Hoffman, 1990 and the present work) of its value at zero mM Na_o, it would appear that uncoupled Na efflux (i.e., type IA) remains at least partially inhibited in the presence of 140 mM Na_o. A separate question not addressed by these experiments is whether or not in the absence of ADP the resultant efflux of Na (and its associated ATPase activity) that takes place in the presence of high Na_o represents two separate processes (i.e., an uncoupled Na efflux [that is partially inhibited] and a Na_i/Na_o exchange component) or two aspects of the same process. In addition the results presented in Tables IX and X do not provide any clear way to analogously dissect the various components of Na efflux that occur in the presence of ADP and high Na_o (Table X, experiments 3B and 4B) because the proportional contribution of the net ATP utilizing components may change in the presence of ADP.

Effect of Na_o on $^{32}P_i$ Efflux in the Absence of ADP

The idea that Na_o could act as a surrogate K_o was suggested by Lee and Blostein (1980) and Blostein (1983) as an explanation for their observation that high Na_o not only stimulated Na_o for Na_i exchange but was accompanied by ATP hydrolysis. This was based in part on the fact that this kind of Na_o for Na_i exchange took place in the absence of ADP and was therefore distinct from the kind of ADP-dependent Na_o for Na_i exchange that did not result in any net utilization of ATP (Garrahan and Glynn, 1967d; and Glynn and Hoffman, 1971). While it was reported above (Fig. 4) that 140 mM Na_o was without effect on the ^{32}P efflux that takes place during type IB uncoupled Na efflux, these previous measurements were made on ghosts that contained ADP. The question now to consider is whether in the nominal absence or very low concentration of ADP, high Na_o would act like K_o to inhibit ^{32}P efflux. Therefore, resealed ghosts were prepared (protocol analogous to that described in the legend of Table VI) to contain (per liter ghosts): 204 μmol [$\gamma^{32}P$]ATP, 1.6 μmol ADP and 23 mmol Na_i. The ^{32}P efflux rate constants (in h^{-1} ± SEM, where n = 4) at

zero and 140 Na_o, respectively, were 0.189 ± .006 and 0.197 ± .005. Thus, it is unlikely that Na_o in this situation acts as a congener of K_o.

DISCUSSION

This is the second of three papers concerned with the mechanism by which the human red cell Na/K pump mediates uncoupled Na efflux. The importance of studying uncoupled Na efflux, of course, lies in its potential contribution to understanding how the Na/K pump itself works. The first paper (Dissing and Hoffman, 1990) established that uncoupled Na efflux was in fact linked to the flow of intracellular inorganic anions. It turned out that there were two different kinds as well as two different sources of anions and Dissing and Hoffman (1990) identified the cytoplasm as one source showing that intracellular SO_4 (or Cl) was coeffluxed with Na (i.e. type IA uncoupled Na efflux). The other anion source turned out to be the pump's substrate, ATP, and this is the subject (type IB uncoupled Na efflux) of the present paper.

The third paper (Marín and Hoffman, 1994) concerns a new type of uncoupled Na efflux (type II) that is driven not by ATP but by the combination [ADP + P_i] in a ouabain-sensitive manner and is not discussed further here.

The most important finding presented in this paper is that the Na/K pump can function to transport P_i out of the cell (Table I). This is a unique feature of type IB uncoupled Na efflux for the P_i efflux occurs in a Na_o-insensitive manner from ghosts (Tables I and III) as well as from intact cells (Table IV). The P_i that is transported was shown to originate not only as the gamma phosphate of ATP but to be directly transferred to the pump without mixing with the cytoplasmic pool of orthophosphate (Table II). This result implies that the P_i that is effluxed is identical to the phosphate that forms the phosphointermediate (EP) in the Na/K pump's reaction cycle. If this is so then P_i transport represents a new property of the α-subunit of the Na/K pump, since EP is associated with a specific aspartate residue on this subunit (Post and Kume, 1973). An obvious question (see later) is how the sidedness in the release of this acylphosphate (that is, the dephosphorylation) is directionally defined. Presumably this is controlled by K_o, as suggested before, because when K_o is present P_i efflux is inhibited (Table III, Figs. 2–4). Thus, K_o in binding to the outside of the pump not only converts uncoupled Na efflux into an exchange of Na_i for K_o, it also prevents the release of PO_4 to the outside (see also Marín and Hoffman, 1994). This effect of K_o may also entail K_o's binding to sites other than its transport sites in acting to dissociate P_i efflux from Na efflux (see below). The involvement of regulating sites to account for transport mode conversion has been suggested before in other contexts (Kennedy et al., 1986; Dissing and Hoffman, 1990; Hoffman et al., 1991) but the signaling mechanism remains unknown.

The modified Albers-Post reaction scheme (see Glynn, 1985) shown in Fig. 5 presents possible transphosphorylation steps of the pump (E) that might underlie the translocation of Na_i and ^{32}P (from [γ-^{32}P]ATP) during type IB uncoupled Na efflux. Note that in the scheme presented the terminal ^{32}P of ATP is boxed to make it easier to follow its sequential transfer. Thus, [γ-^{32}P]ATP) and Na_i are seen to bind to the E_1 form of the pump ($E_1ATP^{32}P \cdot Na$) before phosphorylation takes place. After phosphorylation occurs ($E_1^{32}P \cdot ADP \cdot Na$) ADP leaves and the phosphorylated E_1 form with

its bound (occluded) Na undergoes a conformational transition (from inside to outside) to its $E_2{}^{32}P \cdot Na$ state. Na is then deoccluded (thus completing its translocation from Na_i to Na_o) together with the release of $^{32}P_i$ to the outside. (E_2 is seen to spontaneously revert to its E_1 conformation ready for recycling.) It should be emphasized that while Na is depicted as being released before ^{32}P, consistent with the normal operation of the pump (see Glynn, 1985), there is no evidence for this or any order in type IB uncoupled Na efflux. It could be, for instance, that deoccluded Na could not be released to the outside before the accompanying ^{32}P is released. It is also possible that some but not all (that is, the first of two if the stoichiometry is $2Na/PO_4$ as in Table VIII) of the deoccluded Na is released before ^{32}P release (see Yoda and Yoda, 1987) with the remainder Na being released afterwards. On the other hand, if type IB uncoupled Na efflux is electrogenic, for which there is some evidence (see Trace B in Figs. 8 and 9 of Dissing and Hoffman, 1990, where type IB,
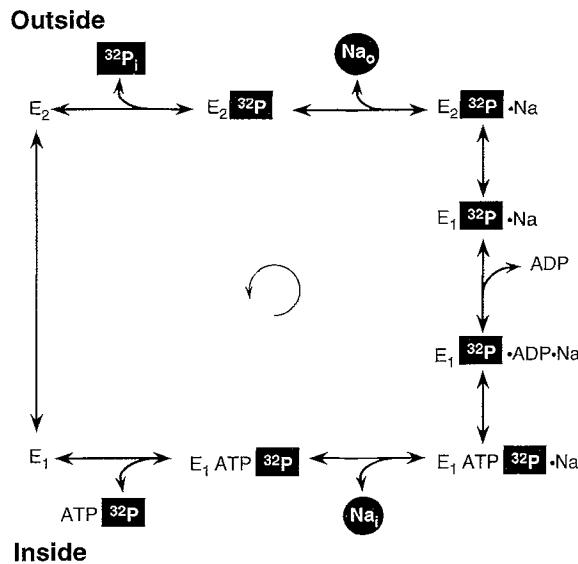

FIGURE 5. Reaction scheme of the red cell Na/K pump that depicts the coupled release of ^{32}P and Na_i from the inside to the outside of the cell during type IB uncoupled Na efflux. It should be noted that this scheme has been simplified since the stoichiometry of Na binding, its occluded forms (cf, Glynn and Karlish, 1990) and the pump's interaction with Mg are not considered here. See text for discussion.

in the absence of type IA, uncoupled Na efflux was studied in tartrate loaded ghosts), Na could precede the release of ^{32}P. This order of release would be consistent with the action of K_o in its conversion of the efflux to a Na_i for K_o exchange together with its obligate reorientation of the dephosphorylation step to the inside, provided that K_o interacted with a transport site(s) independent of whether or not other site(s) were also involved. This not only would preserve the integrity of a ping-pong Type reaction mechanism (see Sachs, 1986) but it would also indicate that some of the deoccluded Na (one?) must leave in order to provide for occupancy of K_o in its sites. Evidence that K_o can bind before all of the Na is released has been discussed by Kennedy et al. (1986) and Yoda and Yoda (1987). But one should be aware that all of the above considerations of ligand interactions relative to order are also dependent upon the actual stoichiometric value of Na to P_i and the average valence of the transported P_i, both of which have yet to be accurately established.

Obviously more work is needed before the detailed relationship between the events of phosphorylation and translocation can be specified in type IB uncoupled Na efflux.

If it is true, as is evidently the case, that the P_i that is released to the outside during type IB uncoupled Na efflux comes directly from E-P, then it is not clear as to how this cotransport, as discussed above, is structurally coordinated. The problem arises because phosphorylation of the pump's α_1-subunit by ATP is known to occur at Asp376 in humans (Kawakami, Ohta, Nojima, and Nagano, 1986). This residue is located on the large cytoplasmic loop between the putative H4 and H5 membrane spanning regions, positioned more than 40 amino acid residues from the H4/cytoplasmic interface. Thus, it is not clear how the phosphorylated Asp376 may be situated/inserted into the hydrophobic region of the membrane, the relationship of its location to the bound/occluded Na, the mechanism of its dephosphorylation such that P_i is vectorially released to the outside along with deoccluded Na and how K_o acts to insure that P_i release is to the inside.

By way of summary, the characteristics of types IA and IB uncoupled Na efflux are directly compared in Table XI. Thus, while the efflux of Na in both types is driven

TABLE XI

A Comparison of the Properties of Two Types (IA and IB) of Ouabain-sensitive,
Anion-coupled Transport that Occur in Uncoupled Na Efflux from
Human Red Blood Cells

Type	Substrate	Anion transported	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_i from ATP)	No	Yes	No	No

by ATP, the kinds of anions that are cotransported (e.g., SO_4 vs P_i) as well as their source (cytoplasm vs ATP) are strikingly different as documented before. In addition, type IA is Na_o-sensitive whereas type IB is not. These differences in properties are distinctive enough that one wonders if types IA and IB represent different kinds of Na pumps (and, if so, are they in the same or different cells?). Clearly, it is only in the uncoupled Na efflux mode that these differences emerge, for the pumps in all their other modes, including Na_i for K_o exchange, appear to function homogeneously. In addition, while it is thought that the α_1 and β_1 are the only isoforms of the pump that are present in human erythroid (as well as renal) tissue (Inaba and Maede, 1986) there is no direct evidence to support this contention. On the other hand, the properties of uncoupled Na efflux as determined on vesicular preparations of Na,K-ATPase made from mammalian kidneys and shark rectal glands are markedly different from those displayed by the human red cell as discussed by Dissing and Hoffman (1990). So while differences are implicated, no conclusion can be drawn until the primary structure of the pump's α and β subunit forms in erythroid tissue if not red blood cells are known.

Another problem associated with the two Types of uncoupled Na efflux concerns the ratios of their fluxes. As mentioned before (see also Dissing and Hoffman, 1990) type IA appears to comprise some 60–80% of the total uncoupled efflux of Na but the mechanism that controls this relative activity is not known. In addition, the puzzle posed by the transport stoichiometries (Tables VIII and IX), as pointed out before, is also in need of an explanation. It may be significant that the measured stoichiometric ratio of Na efflux/ATP used is less than 3, as also observed by Garrahan and Glynn (1967a) and Blostein (1983). In addition more work is needed to verify the type IB stoichiometry of Na to PO_4 coefflux as well as to determine the valency of the transported PO_4 and the extent to which this efflux is electrogenic. This kind of information is needed to justify any speculation about what processes might be involved.

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