Inhibition of the Na,K Pump by Vanadate in High-Na Solutions

Modification of the Reaction Mechanism by External Na Acting at a High-Affinity Site

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ABSTRACT We have examined vanadate inhibition of the Na,K pump in the presence of external Na (Na_o). Na_o protects against inhibition of the Na,K pump by vanadate, but not against inhibition by phosphate or arsenate. Protection by Na_o is reversed by external K (K_o). Although the site at which Na exerts its protective effect has properties similar to the two transport sites for K at the outside of the pump, it is not one of the transport sites. The data can be qualitatively accounted for if it is postulated that there is a protective site, separate from the transport sites, at which Na_o and K_o compete. When the site is empty or bound to K, vanadate combines with high affinity with pumps that have two K ions bound to the transport sites, but not with pumps that have Na bound to the protective site, even if K is bound to the transport sites. The protective site has a high affinity for both Na and K; the apparent $K_{1/2}$ for external Na is <2 mM, which is similar to that of a previously described site at which Na_o inhibits a number of the partial reactions of the pump. Na_o protects against vanadate inhibition of the K-K exchange in the absence of cell Na, and against vanadate inhibition of *p*-nitrophenylphosphatase activity of the pump in the absence of ATP. The protective site is a manifestation of an E_2 conformation of the pump. The protective effect of Na_o is not changed by altering the intracellular Mg^{2+} concentration.

INTRODUCTION

There is a great deal of evidence (De Weer, 1983; Glynn, 1985) that the reaction mechanism of the Na,K pump is adequately described by the Albers-Post model; for convenience in following the discussion, a version of the model is shown in Fig. 1. The model is ping-pong (Cleland, 1963) with respect to intracellular Na (Na_c) and external K (K_o) in that Na is released to the outside before K adds at that side and K is released to the inside before Na_c adds. Because the Na pump

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can carry out an efflux of Na into Na- and K-free solutions that is not coupled to the influx of a cation (uncoupled Na efflux), it is not possible to use the standard methods of steady state kinetics to distinguish between the ping-pong model and alternative models in which Na and K are bound to the pump simultaneously at some point in the transport cycle (Sachs, 1979, 1986*a*). However, by examining the effects of external and internal Na and K on the characteristics of pump inhibition by oligomycin (Sachs, 1980) and by vanadate (Sachs, 1986*b*), it is possible to conclude that, at least in Na-free solutions, Na is released to the outside before K adds and, at least in K-free cells, K is released to the inside before Na adds. Moreover, the presence of two cation exchanges

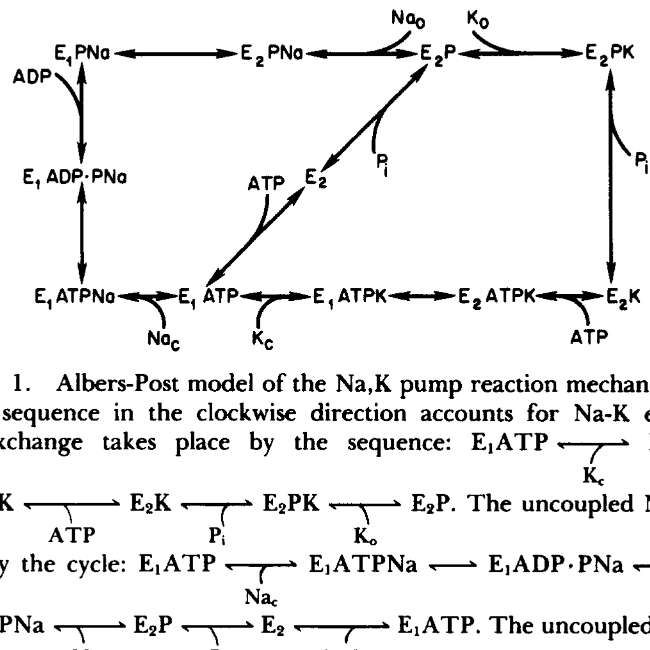

FIGURE 1. Albers-Post model of the Na,K pump reaction mechanism. Operation of the sequence in the clockwise direction accounts for Na-K exchange. The K-K exchange takes place by the sequence: $E_1ATP \underset{K_c}{\longleftrightarrow} E_1ATPK \longleftrightarrow E_2ATPK \underset{ATP}{\longleftrightarrow} E_2K \underset{P_i}{\longleftrightarrow} E_2PK \underset{K_o}{\longleftrightarrow} E_2P$. The uncoupled Na efflux takes place by the cycle: $E_1ATP \underset{Na_c}{\longleftrightarrow} E_1ATPNa \longleftrightarrow E_1ADP \cdot PNa \underset{ADP}{\longleftrightarrow} E_1PNa \longleftrightarrow E_2PNa \underset{Na_o}{\longleftrightarrow} E_2P \underset{P_i}{\longleftrightarrow} E_2 \underset{ATP}{\longleftrightarrow} E_1ATP$. The uncoupled K efflux takes place by the cycle: $E_1ATP \underset{K_c}{\longleftrightarrow} E_1ATPK \longleftrightarrow E_2ATPK \underset{ATP}{\longleftrightarrow} E_2K \underset{P_i}{\longleftrightarrow} E_2PK \underset{K_o}{\longleftrightarrow} E_2P \underset{P_i}{\longleftrightarrow} E_2 \underset{ATP}{\longleftrightarrow} E_1ATP$.

carried out by the pump, an exchange of K_o for K_c (Simons, 1974) and an exchange of Na_c for Na_o (Garrahan and Glynn, 1967*b*), provide strong support for the ping-pong model (Sachs, 1986*a*).

However, Na_o modifies pump behavior in several ways that are not readily accommodated by the simple ping-pong mechanism. When, in K-free solutions, Na_o is increased from 0 to ~5 mM, a number of effects are produced: the uncoupled Na efflux (Garrahan and Glynn, 1967*a*, *b*), a small saturable Na influx (Sachs, 1970), a K efflux that is not coupled to the influx of another cation (Sachs, 1986*a*) (uncoupled K efflux), ATPase activity activated by Na_c (Glynn and Karlish, 1976), and the exchange of phosphate between ATP and ADP catalyzed by the pump (Kaplan, 1982) are all inhibited. Using a purified Na,K-ATPase preparation, in which the side at which cation effects are exerted is not certain, Beaugé and Glynn (1979) reported that similar low concentrations of Na, presumably acting at external sites, slow the dephosphorylation of enzyme phosphorylated by ATP. When Na_o is increased beyond 5 mM, still in K-free solution, ATP-ADP exchange increased (Kaplan, 1982) and an ADP-dependent Na-Na exchange appears (Garrahan and Glynn, 1967*b*), Na_c-dependent ATPase activity increases (Glynn and Karlish, 1976), and an Na-Na exchange that requires ATP but not ADP can be demonstrated (Lee and Blostein, 1980; Forgac and Chin, 1982; Blostein, 1983).

Even when the concentration of K_o is high enough to saturate the external pump sites, Na_o still modifies pump behavior. Kennedy et al. (1986) showed that, at high K_o, Na_o slows the rate of the Na-K exchange if the intracellular ADP/ATP ratio is high, and Hobbs and Dunham (1978) showed that Na_o increases the rate at which ouabain binds to the pump whether or not K_o is present at saturating concentrations.

Finally, the way in which K_o modifies pump inhibition by vanadate depends on whether or not the external solution is Na free. In Na-free solutions, pump inhibition is strictly uncompetitive with respect to K_o; a plot of pump rate as a function of the concentration of K_o is nearly hyperbolic, although the apparent V_M (the pump rate at saturating K_o) and the apparent $K_{½}$ for K_o (the concentration of K_o at which the pump rate is half-maximal) are both decreased by vanadate (Sachs, 1986*b*). When the same measurement is made in solutions containing Na, the curve is biphasic; the velocity first increases with increasing K_o, passes through a maximum, and then decreases (Beaugé and Glynn, 1977; Beaugé, 1979; Bond and Hudgins, 1979; Beaugé et al., 1980; Beaugé and Berberian, 1983). This complex interaction between Na_o, K_o, and vanadate cannot be readily accounted for by the Albers-Post model.

This article presents some observations on the characteristics of vanadate inhibition of the Na,K pump when Na_o is present, and discusses their implications for the Albers-Post model.

METHODS

Venous blood was collected from normal volunteers into citrate-phosphate-dextrose solution, or it was anticoagulated with heparin. For experiments in which intact red cells or resealed ghosts were used, the cells were either used immediately or stored for as long as 3 d in citrate-phosphate-dextrose solution. For the preparation of broken membranes, cells stored for as long as 35 d were used.

The intracellular cation concentrations of intact cells were altered by a modification of the *p*-chloromercuribenzenesulfonate (PCMBS) method first described by Garrahan and Rega (1967). Cells were washed three times (by centrifugation, aspiration of the supernatant, and resuspension in the washing solution) in an isosmotic (107 mM), unbuffered $MgCl_2$ solution, and then incubated for 36 h at 4°C in buffered solutions of suitable composition containing PCMBS. During the incubation, the cells became permeable to cations and the intracellular cation concentrations approached the extracellular concentrations. The cells were then separated from the PCMBS solution and incubated for 1 h at 37°C in a solution containing dithiothreitol instead of PCMBS. The method has been described in detail (Sachs, 1986*b*). Cells prepared by this method have a final ATP concentration of 1–1.5 mmol/liter cells.

Resealed ghosts were prepared by a gel filtration method similar to that described by Kaplan (1982); the method has been described in detail (Sachs, 1986*b*). Briefly, cells were washed with an isosmotic choline chloride solution buffered to pH 5.5 until the pH of the cell suspension was ~6.0. The cell suspension was then run into a column filled with Biogel A-50 beads (Bio-Rad Laboratories, Richmond, CA), equilibrated with a hypotonic choline chloride solution adjusted to pH 6.0, and maintained at −1 °C. The cells hemolyzed on the column and intracellular contents were retained by the beads. The ghosts were eluted, collected, and resealed by incubation for 1 h at 37 °C in a solution of appropriate composition and adjusted to pH 7.4.

PCMBS-treated cells loaded with ^{42}K or ^{22}Na were used for the measurement of Na and K efflux. If the cells were to be used for the measurement of K efflux, they were incubated after resealing in an isosmotic Tris phosphate solution for 30 min at 37 °C in order to increase the intracellular phosphate concentration (phosphate is a required substrate for the K-K exchange). The cells were washed three times in isosmotic $MgCl_2$ solution, suspended at ~2% hematocrit in a solution appropriate for the efflux measurement, and incubated at 37 °C. Samples were taken at appropriate intervals, the cells were separated from the suspension, and the supernatants were saved and counted. A sample of the suspension was also counted. In order to correct for hemolysis, the absorbance of the supernatants and of the hemolyzed suspension was measured at 540 nm after they were counted. Efflux rate constants were calculated as previously described (Sachs and Welt, 1967). The compositions of the solutions used are given in the figure legends.

Measurement of unidirectional influx of Na or K into intact cells was made as previously described (Sachs, 1977). Cells were distributed to tubes containing ice-cold solutions of appropriate composition and ^{42}K or ^{22}Na; the final hematocrit was ~2%. The influx was started by placing the tubes in a 37 °C water bath, and the cells were kept in suspension by periodic mixing. The measurement was terminated by immersing the tubes in an ice-cold water bath. The cells were separated from the suspension and washed three times with isosmotic $MgCl_2$ solution, and the washed cells were hemolyzed in distilled water and counted. Cation uptake was calculated from the amount of ^{42}K or ^{22}Na taken up by the cells and the specific activity of the suspending solution. Uptake was not corrected for back-diffusion since it amounted to a few percent or less of the total uptake.

Broken red cell membranes were prepared by osmotic lysis followed by freezing and thawing (Sachs, 1980). ATPase activity was measured, with and without 2.5×10^{-4} M ouabain, by a coupled enzyme assay in which rephosphorylation of ADP by pyruvate kinase and phosphoenolpyruvate (PEP) is coupled to the oxidation of NADH by lactic dehydrogenase. A complete description of the method has recently been published (Sachs, 1986*b*).

p-Nitrophenolphosphatase (pNPPase) activity was measured by incubating membranes or ghosts in the presence of 5 mM *p*-nitrophenolphosphate (pNPP), 5.5 mM $MgCl_2$, 0.5 mM EDTA, and other substances indicated in the figure legends. After incubation at 37 °C, the reaction was stopped by adding to the reaction mixture an equal volume of a solution containing 4 mM EDTA, 0.2 mM NaOH, and 25 g/liter sodium lauryl sulfate. The samples were mixed and the *p*-nitrophenol concentration was measured at 410 nm.

Intact cells were incubated with the appropriate concentrations of vanadate for 30 min before the flux measurements were started, to allow time for equilibration of the inhibitor across the cell membrane (Cantley et al., 1978). When measurements were made with resealed ghosts, vanadate at appropriate concentrations was present in the resealing solutions, wash solutions, and influx solutions.

Red cell cation concentrations were estimated by flame photometry as previously described (Sachs and Welt, 1967). ATP concentrations were estimated by a method that uses luciferin-luciferase (Kimmich et al., 1973). For the calculation of free Mg concentrations, the dissociation constant of MgATP was taken as 0.050 mM, that of MgEGTA was 5.0 mM, that of MgEDTA was 0.251 μM, and that of MgPEP was 0.025 M.

Ouabain-sensitive values are the difference between measurements made in the presence and absence of 10^{-4} M ouabain unless otherwise indicated. Determinations were made in quadruplicate, and when the curves described a rate equation, they were fitted to the data by a nonlinear least-squares method; the points were weighted from the variances. In the figures, each point is the mean of four determinations, and the SEM is indicated, unless it is smaller than the symbol.

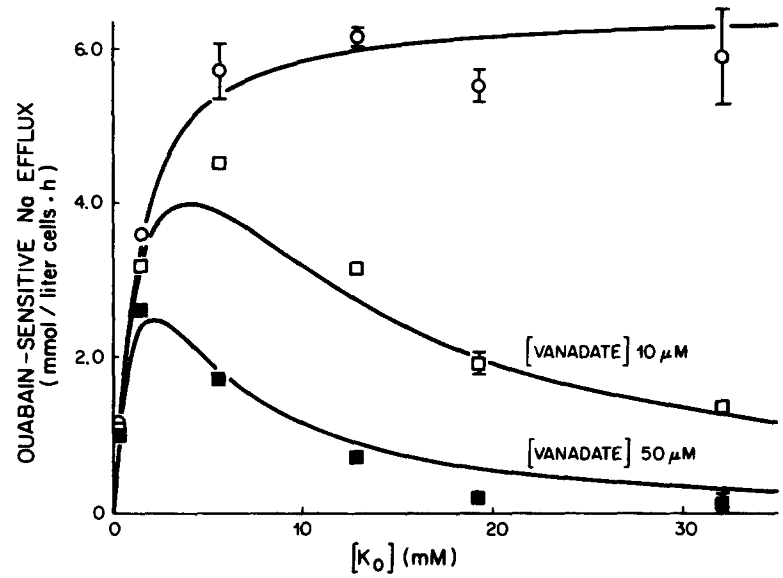

FIGURE 2. Ouabain-sensitive Na efflux vs. K_o concentration. Intracellular cation content was altered by the PCMBS procedure so that the cells contained 17.06 mmol/liter cells Na and 1.15 mmol/liter cells K; the remainder of the cation was choline. The extracellular solution contained 112 mM Na and the indicated concentrations of K; KCl was replaced by choline chloride. The solution contained 10% by volume of an isosmotic (295 mosmol/kg H_2O) $MgCO_3$-glycylglycine solution adjusted to pH 7.4, and 10 mM glucose. The solution also contained 0 (○), 10 μM (□), or 50 μM (■) vanadate. The curves are drawn to Eq. 1; for each curve, V_M = 6.49 mmol/liter cells·h, K_K 0.548 mM; K'_K = 4.40 mM, K_N = 0.888 mM, and K_I = 0.834 μM.

RESULTS

The phenomenon with which this article is concerned is illustrated in Fig. 2; similar results have been published previously (Bond and Hudgins, 1975, 1979; Beaugé and Glynn, 1977; Beaugé, 1979; Beaugé et al., 1980; Beaugé and Berberian, 1983). In the presence of vanadate, the relationship between pump activity (in this case, ouabain-sensitive Na efflux) and K_o is biphasic; activity first increases with the K_o concentration, passes through a maximum, and then decreases as the concentration of K_o increases. The biphasic relation between the pump rate and the K_o concentration occurred only when the external solution contained Na. When the pump rate was measured as a function of the K_o concentration in Na-free solutions, vanadate reduced both the apparent V_M and the apparent $K_{1/2}$ for K_o, but the curve increased monotonically; i.e., vanadate was a noncompetitive inhibitor with respect to K_o (Beaugé and Berberian, 1983; Sachs, 1986*b*). Fig. 3 shows the results of an experiment in which ouabain-sensitive Na efflux was measured in high-Na and Na-free solutions as a function of the concentration of K_o; all solutions contained 20 μM vanadate. The results show that the biphasic curve occurs because Na_o, at low K_o, protects against vanadate inhibition.

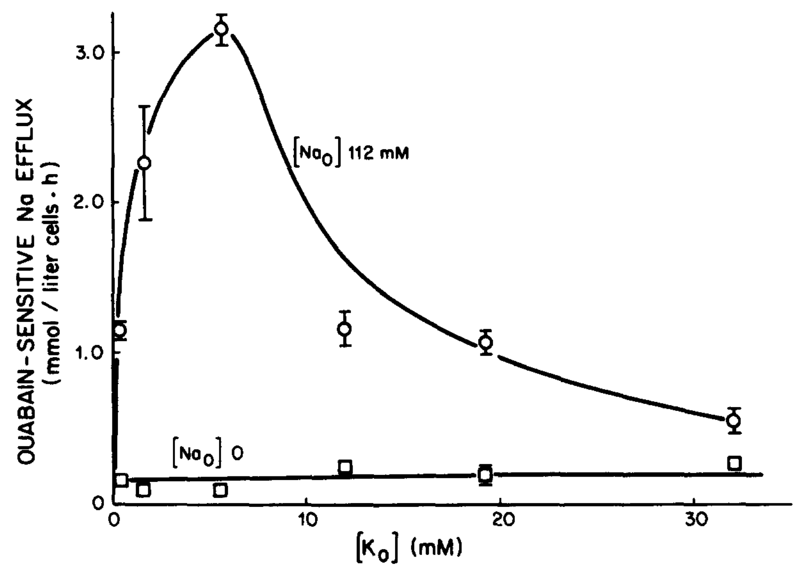

FIGURE 3. Ouabain-sensitive Na efflux vs. K_o concentration. Intracellular cation content was altered by the PCMBS method so that the cells contained 22.8 mmol/liter cells Na and 1.79 mmol/liter cells K; the remainder of the cell cation was choline. The extracellular solution contained 122 (○) or 0 (□) mM Na; NaCl was replaced by choline chloride. The composition of the extracellular solution was otherwise the same as that described in the legend to Fig. 2. 20 μM vanadate was present in all solutions. The curves were drawn by eye.

The biphasic curve occurred when ouabain-sensitive K influx was measured as a function of the K_o concentration in solutions containing Na and vanadate, when the ouabain-sensitive Na,K-ATPase activity of broken cell membranes was measured as a function of the K concentration in high-Na solutions containing vanadate, or when an Na efflux experiment similar to that shown in Fig. 2 was performed in high-Na, vanadate-free solutions with cells that had been preloaded with vanadate (results not shown). The biphasic curve does not, therefore, result from the measurement of a particular pump function, nor does it result from an effect of Na_o on the permeability of the cell to vanadate. We measured ouabain-sensitive ATPase activity in high-Na solutions as a function of K concentration up to 48 mM in the presence of 40 mM arsenate and in the presence of 25 mM phosphate; both significantly inhibited Na,K-ATPase activity, but a plot of activity against K concentration rose monotonically and showed no evidence of being biphasic (results not shown). Na_o reversed vanadate inhibition, but it did not reverse arsenate or phosphate inhibition, although it is believed that the three anions inhibit by combining with the same enzyme form, E_2K (Fig. 1). Both Tris (Nørby et al., 1983) and imidazole (Schuurmans Stekhoven et al., 1986) ions, presumably acting at external pump sites, inhibit dephosphorylation of enzyme phosphorylated by ATP, as does Na_o at low concentrations; only Na in the external solution prevents vanadate inhibition of the Na efflux (not shown).

Relationship of the Site at Which Na_o Modifies Vanadate Inhibition to the K_o Activation Sites

The site at which Na_o modifies vanadate inhibition is similar in several ways to the sites at which K_o activates Na-K exchange. The K congeners Tl, Rb, Cs, and NH_4 reverse the effect of Na_o on vanadate inhibition, and the relative affinity of the modifying site for the ions is in about the same order as the relative apparent affinity of the activation sites (Bond and Hudgins, 1979). The apparent affinity of the modifying site for K_o increases as the concentration of Na_o decreases; the apparent affinity of the modifying site for K_o increases while the ability of any given concentration of Na_o at constant K_o to reverse vanadate inhibition decreases as the vanadate concentration increases (Beaugé, 1979; Bond and Hudgins, 1979). These results indicate that competition between Na_o and K_o occurs at the modifying sites just as it does at the activation sites. Since the modifying site has properties similar to those of the activation sites, it seemed possible that the two sites might be the same, and that Na_o might reverse vanadate inhibition by competing with K_o at sites at which K both activates transport and increases the steady state concentration of an enzyme form that binds vanadate. However, such competition between Na and K at the activation site (or at two sites, both of which must be filled by K before a transport cycle occurs) does not result in a biphasic activation curve in the presence of vanadate, such as that shown in Fig. 2, but rather in inhibition that is uncompetitive with K_o. Fractional inhibition increases as the pump is activated, but activation curves in the presence of inhibitor rise monotonically with the concentration of K_o rather than being biphasic.

There is, however, reason to believe that there are two sites for K at the outside of the pump, and that a transport cycle can occur when only one of the sites is combined with K (Sachs, 1977; Kropp and Sachs, 1977; Livengood, 1983). If such cycles are possible, one can propose a model in which pumps with both activating sites combined with K, pumps with one site bound to K and the other site empty, and pumps with K bound at one site and Na at the other are all capable of transport, but only the two Na-free species can combine with vanadate. Such a model predicts a biphasic activation curve in the presence of inhibitor, such as that shown in Fig. 2; however, not all the characteristics of the experimental curve are reproduced by the model. In appraising this model, two fates must be considered for the Na that is bound to an activation site: (*a*) the Na might remain outside so that only the bound K is moved into the cell, or (*b*) the bound Na and bound K might be transported in together.

If, as in the first case, Na is bound to one of the outside transport sites and K to the other in cycles in which Na_o protects against vanadate inhibition, but only the K ion is transported into the cell, the protected cycles should have a coupling ratio of 3 Na out to 1 K in:

$$\begin{array}{l} 3\ Na_i \rightarrow 3\ Na_o \\ \quad\quad\quad\quad\quad\quad\quad \text{(protected cycles)} \\ 1\ K_i \leftarrow 1\ K_o \end{array}$$

At concentrations of Na_o and K_o at which pumps are protected against vanadate inhibition, the measured pump rate will be the sum of the rate for such protected cycles and the rate for normal cycles in which the coupling ratio is 3 Na out to 2 K in:

$$\begin{array}{l} 3\ Na_i \rightarrow 3\ Na_o \\ 2\ K_i \leftarrow 2\ K_o \end{array}$$

At concentrations of Na_o and K_o at which a significant fraction of the pump cycles are those in which Na_o protects against vanadate inhibition, the measured coupling ratio should be >1.5; if half the cycles are protected cycles, the coupling ratio will be 2.25. We measured net ouabain-sensitive Na efflux and K influx in high-Na solutions with and without vanadate at three concentrations of K_o; the results of two such experiments are shown in Table I. In the presence of vanadate, the pump rate at 32 mM K_o is much less than the rate of 2.4 mM K_o; in the second experiment, it is less than half as great. At 2.4 mM K_o, therefore, Na_o protects against vanadate inhibition in a significant fraction of the transport cycles (see also Figs. 2 and 3), but the coupling ratio is not much different from 1.5 under any circumstance. The result is not consistent with the proposal that protected cycles are cycles in which Na is bound to one of the external transport sites and K is bound to the other, but only K is transported into the cell.

In the second case, as in the first, Na binds to one of the external transport sites and K to the other in cycles in which Na_o protects against vanadate inhibition, but both ions are transported into the cell. A net coupling ratio of 2 Na out to 1 K in would be expected in protected cycles:

$$\begin{array}{l} 3\ Na_i \rightarrow 3\ Na_o \\ 1\ K_i \leftarrow 1\ K_o\ \text{(protected cycles)} \\ 1\ Na_i \leftarrow 1\ Na_o \end{array}$$

A ratio of 2:1 rather than 1.5:1 in 50% of the cycles would be difficult to detect in an experiment such as that shown in Table I. However, if this is the way in which Na_o protects against vanadate inhibition, protected cycles should have a ouabain-sensitive Na influx of the same magnitude as the ouabain-sensitive K influx. Fig. 4 shows the results of an experiment in which we attempted to demonstrate such an influx. We simultaneously measured ouabain-sensitive Na efflux and ouabain-sensitive Na influx in high-Na solutions with and without 10 μM vanadate at several concentrations of K_o. In the presence of vanadate, the difference between the ouabain-sensitive Na efflux at 3.2 mM K_o, at which Na_o protects against vanadate inhibition, and the efflux at 9.6 mM K_o, at which K_o counteracts the protective effect of Na_o (see Fig. 3), provides an estimate of the magnitude of the efflux protected against vanadate inhibition by Na_o; the

TABLE I

Coupling Ratio (Na Efflux/K Influx) of Na-K Exchange at Several Values of $[K_o]$ in the Presence and Absence of Vanadate

	Net Na efflux ± SEM	Net K influx ± SEM	Ratio ± SEM
mM	*mmol/liter cells·h*		
Experiment 1			
0 Vanadate			
K_o 2.4	2.55±0.20	1.57±0.03	1.63±0.13
16.0	3.15±0.19	2.26±0.05	1.39±0.09
32.0	3.57±0.14	2.06±0.16	1.73±0.12
5 μM Vanadate			
K_o 2.4	2.30±0.11	1.48±0.04	1.56±0.08
16.0	2.19±0.20	1.61±0.06	1.36±0.13
32.0	1.35±0.13	1.03±0.04	1.31±0.14
Experiment 2			
0 Vanadate			
K_o 2.4	—	2.11±0.03	—
16.0	4.27±0.08	3.12±0.03	1.38±0.03
32.0	4.51±0.12	3.18±0.08	1.42±0.05
5 μM Vanadate			
K_o 2.4	3.13±0.27	2.15±0.07	1.45±0.13
16.0	2.62±0.18	1.58±0.06	1.66±0.13
32.0	1.20±0.12	0.89±0.09	1.34±0.18

The intracellular cation content was altered by the PCMBS method; the cells used for experiment 1 contained 13.0 mmol/liter cells Na and 1.99 mmol/liter cells K and the cells used for experiment 2 contained 13.8 mmol/liter cells Na and 2.09 mmol/liter cells K. The cells were incubated at 37°C in a solution that contained 112 mM Na and the indicated concentrations of K and vanadate; K was replaced by choline. The solution contained 10% by volume of an isosmotic (295 mosmol/kg H_2O) $MgCO_3$-glycylglycine solution adjusted to pH 7.4 (the approximate composition of the buffer is 575 mM glycylglycine and 51 mM $MgCO_3$), and 10 mM glucose. Samples were taken at 15 and 135 min, the cells were washed three times with isosmotic $MgCl_2$ solution, and the intracellular Na and K concentrations were determined. Net fluxes are the differences between fluxes in the presence and absence of 10^{-4} M ouabain.

difference is ~1 mmol/liter cells·h. At $K_o > 9.6$ mM, further inhibition of Na efflux in the presence of vanadate would be expected (see Figs. 2 and 3), so that 1 mmol/liter cells·h is a minimum value for the protected Na efflux. If one Na ion is transported inward during each vanadate-resistant cycle, it should contribute 0.33 mmol/liter cell·h to the ouabain-sensitive Na influx. The measured ouabain-sensitive Na influx at 3.2 mM K_o was 0.170 mmol/liter cells·h in the absence of vanadate and 0.185 in its presence, and this influx must have included Na-Na exchange that is not completely suppressed by K_o until the K activation sites are saturated (Garrahan and Glynn, 1967*c*; Sachs, 1970). Therefore, there is not enough ouabain-sensitive Na influx for every cycle that is protected from vanadate inhibition at 3.2 mM K_o to be accompanied by the influx of an Na ion, although the possibility that some of the cycles occur by this mechanism cannot be completely excluded by the results in Fig. 4.

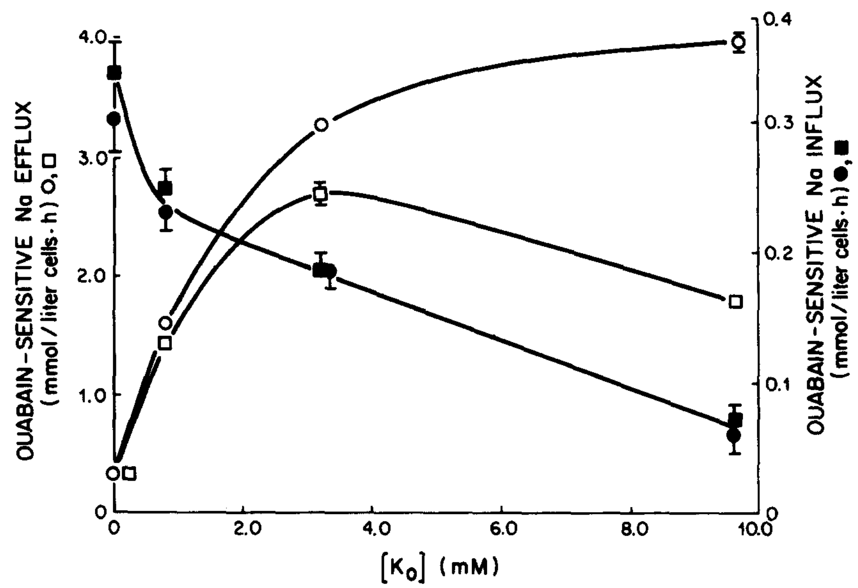

FIGURE 4. Ouabain-sensitive Na efflux and Na influx vs. extracellular K concentration. The measurements were made with fresh cells; half the cells were incubated with ^{22}Na and used for the measurement of Na efflux; the other half were incubated under similar circumstances but without ^{22}Na and used for the measurement of Na influx. Intracellular Na was 15.1 and intracellular K was 90.3 mmol/liter cells. The measurements were made in the presence (squares) and absence (circles) of 10 μM vanadate. The solutions in which the measurements were made contained 112 mM Na and the indicated concentrations of K; K was replaced by choline. The solution contained 10% by volume of an isosmotic (295 mosmol/kg H_2O) $MgCO_3$-glycylglycine solution adjusted to pH 7.4, and 10 mM glucose. The curves were drawn by eye.

Since the interpretation of the results of this experiment is complicated by the existence of an Na-Na exchange, we prepared Na-free cells (in which Na-Na exchange is not possible) containing phosphate and a high concentration of K, and simultaneously measured K influx and Na influx at two Na_o concentrations and several K_o concentrations; the results are shown in Fig. 5. The concentrations of Na_o and K_o were within the range in which Na_o prevents vanadate inhibition (see below). In this experiment, the ouabain-sensitive influx of K was in exchange for cell K; evidence is presented later to show that Na_o prevents vanadate inhibition of the K-K exchange just as it prevents inhibition of the Na-K exchange. If vanadate-insensitive cycles are characterized by the paired influx of one Na and one K ion, the Na influx in this experiment should have increased as the K influx increased with increasing K_o; no such increase was apparent. The results of these experiments make it unlikely that Na_o prevents vanadate inhibition by interacting with the K activation sites.

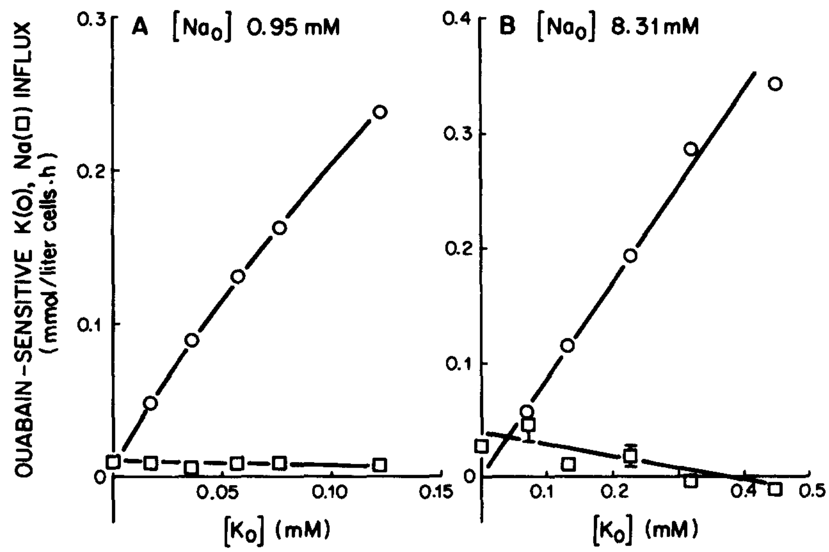

FIGURE 5. Ouabain-sensitive K influx and the simultaneously determined Na influx vs. K_o concentration. Intracellular cation content was altered by the PCMBS method; all solutions including those used during cation alteration and during the flux measurements contained 27.2 mM phosphate. Intracellular Na was 0.51 and intracellular K was 93.9 mmol/liter cells. The solutions in which the influx measurements were made contained the indicated concentrations of Na and K; the remainder of the cation was choline, except that all solutions contained 10% by volume of an isosmotic (295 mosmol/kg H_2O) Tris phosphate solution adjusted to pH 7.4. The solutions also contained 10 mM glucose.

Affinity of the Modifying Sites for Na_o and K_o

On the basis of previously reported observations (Beaugé, 1979; Bond and Hudgins, 1979), we developed a descriptive relation between the pump rate and the concentration of Na_o, K_o, and vanadate. The model is based on the following assumptions: there are two external activation sites, both of which must be occupied by K for a transport cycle to occur; there is a separate modifying site at the outside to which Na binds; the binding of K_o to a site (or sites) other than the activation sites completely prevents Na binding to its modifying site (these K sites may or may not be the same as the modifying site to which Na binds); and combination of Na or K with the modifying sites does not affect the interaction of K_o with the activation sites, nor does it alter the maximal pump rate. Vanadate combines only with those pumps that have both activation sites filled with K (Sachs, 1986*b*), and whose modifying sites are either empty or bound to one or two K ions, but vanadate does not bind to pumps whose modifying sites are combined with Na. Assuming that binding of Na_o and K_o to the activation sites is rapid and not rate-limiting for the overall transport cycle, and that the other substrates of the exchange (Na_c, ATP) are present at constant concentrations, one can derive a rate equation:

$$v = \frac{V_M}{(1 + K_K/K_o)^2 + (I/K_I)\,(1 + K_o/K'_K)^2/[Na_o/K_N + (1 + K_o/K'_K)^2]}, \quad (1)$$

where V_M is the maximal Na-K exchange at saturating K_o in the absence of vanadate, K_K is the apparent dissociation constant for K_o of the activation sites, K_N is the apparent dissociation constant for Na_o of the modifying site, K'_K is the apparent dissociation constant for K_o of the modifying sites, K_I is the apparent dissociation constant for vanadate, and I is the vanadate concentration.

Eq. 1 with I set at zero was fitted to the data in Fig. 2 obtained in the absence of vanadate, and V_M and K_K were estimated; using these values, we then fitted the equation to the data obtained at 10 and 50 μM vandate, and K'_K, K_N, and K_I were estimated. The curves fitted the data fairly well. The assumption of a single modifying site for K_o (the exponent for the expression $[1 + K_o/K'_K]$ in Eq. 1 set at 1 rather than 2) resulted in a poorer fit, but a plot of fractional inhibition against the concentration of K_o was sigmoid, so that the sigmoid relation found experimentally (see Fig. 8) does not require the existence of two modifying sites for K_o. It should be emphasized that the model is very sketchy; many known interactions, such as the complicated effects of Na_o on the K activation sites (Sachs, 1977), have been ignored, and the assumptions are greatly simplified. Nevertheless, the model demonstrates that the assumptions listed above account for the complicated interactions of Na_o, K_o, and vanadate at least to a first approximation; we were unable to find another set of assumptions that predict biphasic K activation curves in the presence of an inhibitor such as the curves shown in Fig. 2.

We were surprised at the low calculated value of K_N (0.9 mM) obtained from the fit; previous reports have proposed that the site at which Na_o modifies vanadate inhibition is a low-affinity site (Bond and Hudgins, 1979, 1982; Smith et al., 1980). With the assumption of two modifying sites for K_o, as in Eq. 1, the apparent dissociation constant of each site was relatively high (4.4 mM); if the equation was altered so that only a single modifying site for K_o was assumed, the apparent dissociation constant was lower (0.9 mM), but the fit of the curve to the data was poorer. We performed some experiments to obtain independent estimates of the apparent affinity of the modifying sites for Na_o and K_o.

Since the apparent affinity of the modifying sites for Na_o decreases with increasing concentration of K_o and vanadate, we estimated the apparent $K_{1/2}$ for the effect of Na_o at a fixed vanadate concentration and several concentrations of vanadate. Fig. 6 shows the results of an experiment in which we measured the ouabain-sensitive K influx at 20 μM vanadate, three fixed concentrations of K_o, and varying concentrations of Na_o; the procedure used for estimating the $K_{1/2}$ for

Na_o, the concentration of Na_o at which the reversal of vanadate inhibition was half-maximal, is described in the figure legend. Extrapolation of a plot of the resulting values of $K_{1/2}$ against the concentration of K_o to zero K_o resulted in a value for $K_{1/2}$ of 0.4 mM. Fig. 7 shows the results of the complementary experi-

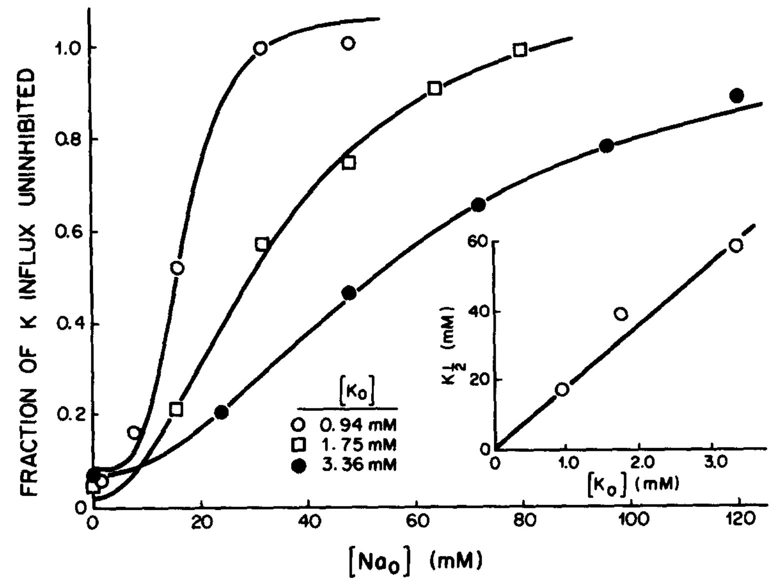

FIGURE 6. Fraction of the ouabain-sensitive K influx resistant to vanadate inhibition vs. Na_o concentration. Intracellular cation content was altered by the PCMBS method; intracellular Na was 33.5 and intracellular K was 4.3 mmol/liter cells, and the remainder of the cation was choline. Influx measurements were made at the three indicated concentrations of K and the indicated concentrations of Na, with and without 20 μM vanadate; the remainder of the cation was choline. The solution contained, in addition, 10% of an isosmotic (295 mosmol/kg H_2O) $MgCO_3$-glycylglycine solution adjusted to pH 7.4. The fraction uninhibited was calculated by dividing the value of the ouabain-sensitive influx at each Na and K concentration in the presence of vanadate by the corresponding value in the absence of the inhibitor. The values were fitted to the equation: fraction of influx inhibited = $a + b/[1 + (K_{1/2}/Na_o)^n]$. The relation is completely empirical; a is the fraction of the influx uninhibited in Na-free solutions, b is the maximum fraction of the influx protected against vanadate inhibition by Na_o, $K_{1/2}$ is the concentration of Na_o at which Na_o half-maximally protects against inhibition by vanadate, and n is an integer whose value is selected to obtain the best fit to the data. For the curve at 0.94 mM K_o, a = 8.50, b = 98.27, $K_{1/2}$ = 16.9 mM, and n = 4; at 1.75 mM K_o, a = 2.15, b = 114.50, $K_{1/2}$ = 34.8, and n = 2; at 3.36 mM K_o, a = 6.62, b = 97.62, $K_{1/2}$ = 58.3, and n = 2. The inset is a plot of $K_{1/2}$ vs. the K concentration; the line is $K_{1/2}$ = 0.434 + 17.53 [K_o].

ment in which we measured the value of the ouabain-sensitive K influx at a fixed concentration of K_o, several fixed concentrations of vanadate, and various concentrations of Na_o. The values of the $K_{1/2}$ for Na_o were calculated as described in the figure legend, plotted against the vanadate concentration, and extrapolated to zero vanadate. If the value for $K_{1/2}$ for Na_o obtained in Fig. 6 at zero K_o but with 20 μM vanadate (0.4 mM) is corrected to zero vanadate using the data of Fig. 7, the combined estimate for the value at zero K_o and zero vanadate is 0.18 mM; if the value for $K_{1/2}$ for Na_o obtained in Fig. 7 with zero vanadate but with 1.96 mM K_o is corrected to zero K_o using the data of Fig. 6, the combined estimate at zero K_o and zero vanadate is 0.17 mM. All estimates for $K_{1/2}$ for Na_o are low, and the true value is probably not much more than 1 mM.

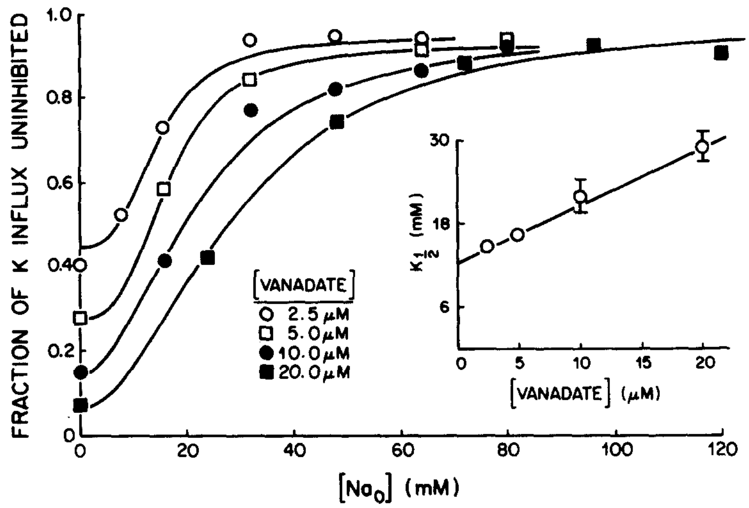

FIGURE 7. Fraction of the ouabain-sensitive K influx resistant to vanadate inhibition vs. Na_o concentration. Intracellular cation content was altered by the PCMBS method; intracellular Na was 30.7 and intracellular K was 3.4 mmol/liter cells, and the remainder of the cation was choline. Influx measurements were made at 1.96 mM K_o, the indicated concentrations of Na_o, and the indicated vanadate concentrations; the remainder of the cation was choline. The solution also contained 10% by volume of an isosmotic (295 mosmol/kg H_2O) $MgCO_3$-glycylglycine solution adjusted to pH 7.4. The fraction uninhibited was calculated by dividing the value of the ouabain-sensitive influx at each Na and K concentration in the presence of vanadate by the corresponding value in the absence of inhibitor. The values were fit to the equation given in the legend to Fig. 6. At 2.5 μM vanadate, $a = 44.67$, $b = 49.34$, $K_{1/2} = 14.6$, and $n = 3$; at 5.0 μM vanadate, $a = 27.40$, $b = 64.72$, $K_{1/2} = 21.91$, and $n = 2$; at 20.0 μM vanadate, $a = 6.46$, $b = 91.71$, $K_{1/2} = 29.0$, and $n = 2$. The inset is a plot of $K_{1/2}$ vs. the vanadate concentration; the line is $K_{1/2} = 12.37 \pm 0.823$ [vanadate] (micromolar).

The apparent affinity of the modifying sites for K_o decreases as the concentration of Na_o increases, and increases with increasing vanadate concentration. Fig. 8 gives the results of an experiment in which we measured the fractional inhibition of the ouabain-sensitive K influx (defined in the legend to Fig. 8) at three Na_o concentrations, several concentrations of K_o, with and without 40 μM vanadate. The values were fitted to the equation given in the figure legend. K'_K is a measure of the affinity of the modifying sites for K_o; if it is assumed that there are two K sites, the concentration of K_o that half-maximally promotes vanadate inhibition ($K_{½}$ for K_o) equals 2.4 K'_K. K'_K was plotted against Na_o, but the best-fitting straight line, and in fact a straight line connecting any two of the three points, intersected the *y*-axis well below the origin; the curve that is shown is the parabola that passes through the three points and intersects the ordinate at K'_K = 0.067 mM, or $K_{½}$ for K_o = 0.16 mM. It should be pointed out that

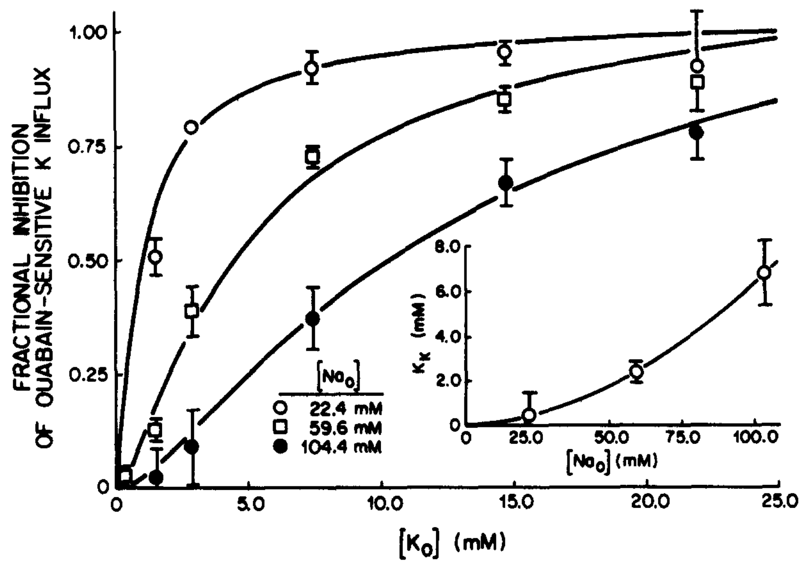

FIGURE 8. Fractional inhibition (FI) of the ouabain-sensitive K influx by vanadate vs. K_o concentration. Intracellular cation content was altered by the PCMBS method. The cells contained 35.0 mmol/liter cells Na and 4.1 mmol/liter cells K. Ouabain-sensitive K influx was measured at the indicated concentrations of Na and K with and without 40 μM vanadate; the remainder of the cation was choline. The solutions also contained 10% by volume of an isosmotic (295 mosmol/kg H_2O) $MgCO_3$-glycylglycine solution adjusted to pH 7.4. Fractional inhibition by vanadate was calculated by dividing the difference between the ouabain-sensitive K influx at a given concentration of Na and K in the absence and presence of vanadate by the value in the absence of inhibitor. The data were fitted to the equation $FI = FI_{max}/(1 + K'_K/K_o)^2$, where FI_{max} is the fractional inhibition at saturating K_o. At 22.4 mM Na_o, FI_{max} = 1.04 and K'_K = 0.451 mM; at 59.6 mM Na_o, FI_{max} = 1.18 and K'_K = 2.357 mM; and at 104.4 mM Na_o, FI_{max} = 1.37 and K'_K = 6.75 mM. In the inset, the values of K'_K are plotted against the concentration of Na_o; the curve is $K'_K = 0.067 + 0.0043\,[Na_o] + 0.00057\,[Na_o]^2$.

Beaugé (1979) has published similar studies, and a plot of his data, similar to the inset in Fig. 8, also suggests that the relation between K'_K and Na_o is nonlinear. From the data given in Fig. 2, it is possible to make similar calculations of the effect of the vanadate concentration on K'_K (not shown); when the values so obtained are plotted against the vanadate concentration and extrapolated to the ordinate, it is found that K'_K at zero vanadate is 2.9 times its value at 40 μM vanadate. The calculated value of $K_{½}$ for K_o at zero Na_o and zero vanadate is therefore 0.5 mM. Although these estimates of the apparent affinity of the modifying sites for Na_o and K_o are not rigorous, it seems clear that the affinity of the sites for both ions is relatively high, less than a few millimolar.

Reversal of Vanadate Inhibition of the K-K Exchange by Na_o

It is known that vanadate, at low concentrations, inhibits the K-K exchange carried out by the Na,K pump just as it inhibits the Na-K exchange (Beaugé et

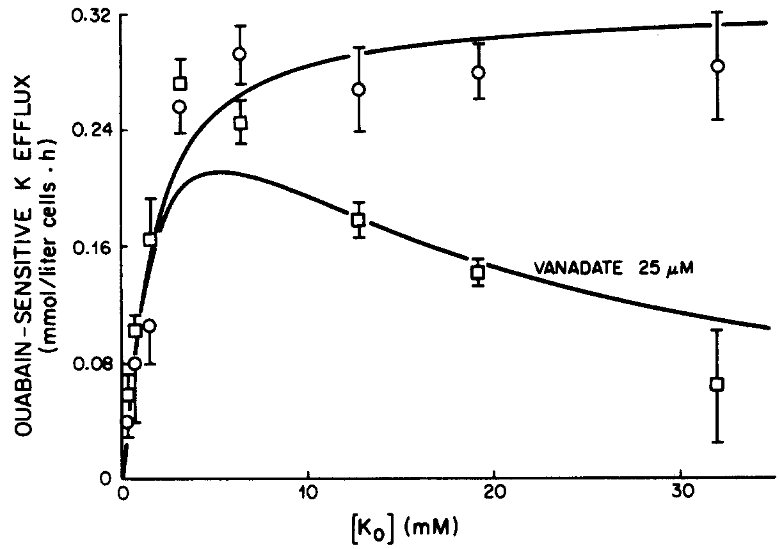

FIGURE 9. Ouabain-sensitive K efflux vs. K_o concentration. Intracellular cation content was altered by the PCMBS method; the PCMBS solutions and the resealing solutions contained 27.2 mM phosphate. After the cells were resealed, they were incubated for 0.5 h at 37°C in isosmotic Tris phosphate solution (pH 7.4). Intracellular Na was 0.12 and K was 10.1 mmol/liter cells. The solutions in which K efflux was measured contained 128 mM Na and the indicated concentrations of K; the remainder of the cation was choline. Vanadate, when present, was 25 μM. For convenience in this and in the following figure, the curves in the absence of vanadate were fitted to the equation $v = V_M/[1 + (K_s/S)]^2$ and the curves in the presence of vanadate were fitted to the equation $v = V_M/[1 + (K_s/S)]^2 \cdot (1 - \{1/[1 + (K_s'/S)]^2\})$, which can be obtained from Eq. 1. The values of V_M and K_s obtained from the curve in the absence of inhibitor were used in fitting the curves in the presence of inhibitor.

al., 1980; Sachs, 1986*b*). We performed some experiments to determine the effect of Na_o on vanadate inhibition of the K-K exchange.

Fig. 9 shows the results of an experiment in which we measured ouabain-sensitive K efflux from nominally Na-free cells into solutions containing 128 mM Na and various concentrations of K; the measurements were made with and without 25 μM vanadate. The relation between ouabain-sensitive K efflux and extracellular K concentration in the presence of vanadate is biphasic, and since K efflux was measured into solutions containing K (such an efflux is known to proceed by means of a K-K exchange; see Fig. 1), it is clear that the interaction between Na_o and vanadate alters the behavior of the K-K exchange.

The experiment shown in Fig. 9 was carried out at low internal K and high Na_o, so it is possible that Na leaking into the cells accumulated sufficiently to interact with the Na transport sites. To eliminate the possibility that such an interaction of Na with the transport sites is necessary to observe reversal of vanadate inhibition by Na_o, we performed an experiment in which we measured K influx into cells that were Na free and high in K from a solution that contained only 24 mM Na; ouabain-sensitive K influx into Na-free cells occurs in exchange

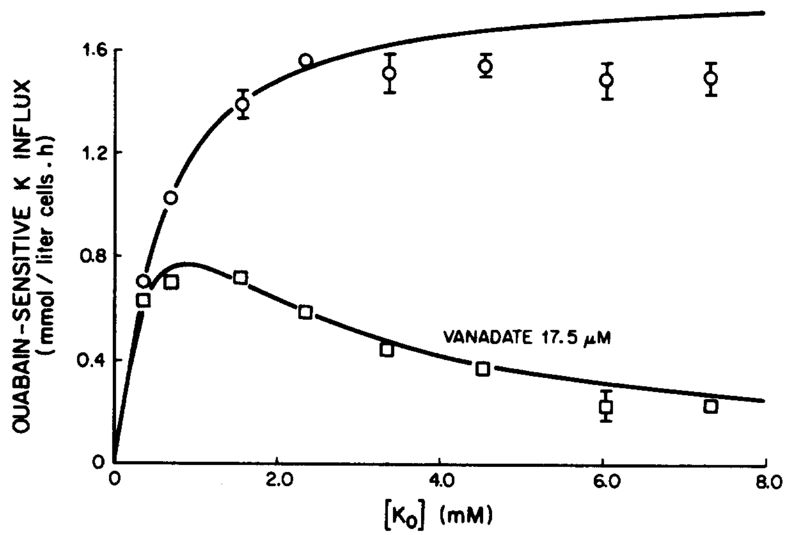

FIGURE 10. Ouabain-sensitive K influx vs. K_o concentration. Intracellular cation concentration was altered by the PCMBS method; the PCMBS solution and the resealing solution contained 27.2 mM phosphate. After resealing, the cells were resuspended for 0.5 h at 37°C in a solution that contained 54.4 mM phosphate as KPO_4, pH 7.4; the remainder of the solution was isosmotic (295 mosmol/kg H_2O) sucrose solution. Intracellular Na was 1.6 and K was 83.3 mmol/liter cells. The solutions in which K influx was measured contained 24 mM Na and the indicated concentrations of K; the remainder of the cation was choline. Vanadate, when present, was 17.5 μM.

for intracellular K (Sachs, 1972). The activation in the presence of vanadate is biphasic, just as it is when Na-K exchange is measured, as shown in Fig. 10.

From these results, it can be concluded that reversal of vanadate inhibition by Na_o does not require a complete Na-K exchange cycle, nor does it require interaction of cell Na with the Na transport sites.

Effect of Na_o on Inhibition of Ouabain-sensitive, K-dependent pNPPase Activity by Vanadate

Na,K-ATPase preparations are able to carry out a K-dependent ouabain-inhibitable hydrolysis of a variety of phosphate esters, including pNPP; red cell membranes also demonstrate pNPPase activity (Robinson and Flashner, 1979). Since it is possible to measure pNPPase activity in the absence of ATP, it seemed likely that observation of the effect of Na_o on vanadate inhibition of the phosphatase activity might provide information about the substrate requirement for the protective effect of Na_o.

Fig. 11 shows the results of an experiment in which the ouabain-sensitive pNPPase activity of broken red cell membranes was measured at various K concentrations with and without vanadate; the measurements were made in Na-free solutions (Fig. 11*A*) and at 128 mM Na (Fig. 11*B*). In high-Na solutions, a plot of pNPPase activity as a function of K concentration was clearly biphasic when vanadate was present, but in Na-free solutions it was not.

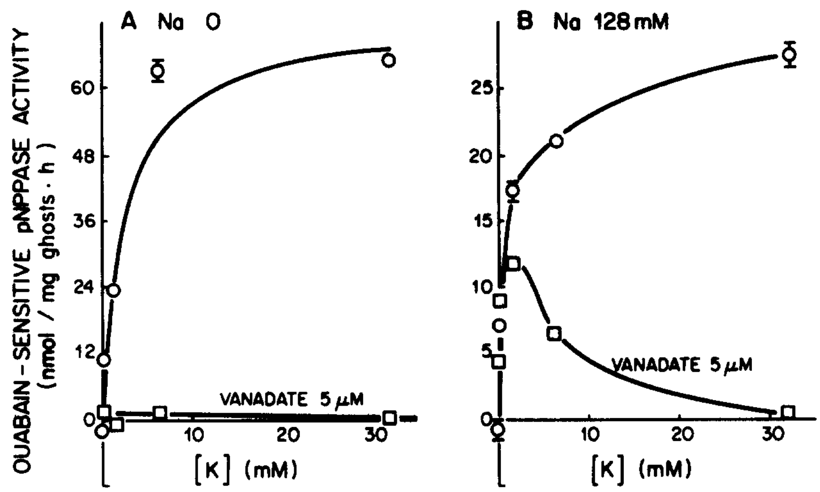

FIGURE 11. Ouabain-sensitive pNPPase activity vs. K concentration. The measurements were made in a solution containing 10 mM Tris HEPES, pH 7.4, 0.5 mM EDTA; 5 mM pNPP, 5 mM $MgCl_2$, and the indicated concentrations of Na and K. Na and K were replaced by choline. The measurements were made with (□) and without (○) 5 μM vanadate. Note that the scale of the ordinate is different for the measurements made in high-Na solutions (*B*) and for the measurements made in Na-free solutions (*A*).

Beaugé and Berberian (1983) performed experiments similar to that shown in Fig. 11 using a purified enzyme preparation from renal medulla; they found a biphasic K activation curve in the presence of vanadate only when Na and ATP were present together while pNPPase activity was measured. Beaugé and Berberian (1983) measured K-activated pNPPase activity, and we measured ouabain-sensitive activity, but in our experiments ouabain-insensitive activity in the presence of Na and vanadate was independent of K concentration, so the different baselines in the two studies would not account for the difference between our observations and those of Beaugé and Berberian (1983). It seemed unlikely that the membrane preparation we used contained enough residual ATP to account for the biphasic curve; nevertheless, we performed two experiments to make certain that residual ATP was not the explanation for the discrepancy in the two studies. In one experiment, ghosts were incubated for 2 h at 37°C in a solution that contained 96 mM Na and 24 mM K. We could detect no ATP in the ghost suspension; however, when the ghosts were diluted 10-fold and pNPPase activity was measured in an experiment similar to that shown in Fig. 11*B*, the activation curve in the presence of vanadate was biphasic (not shown). In a second experiment, ghosts were incubated for 1 h at room temperature in a solution similar to that used to measure pNPPase activity but lacking pNPP and containing 200 mM glucose, 3 mM NADP, 12.8 U/ml hexokinase, and 3.6 U/ml glucose-6-phosphate dehydrogenase. The ghosts were then washed and used for measurement of pNPPase activity in an experiment similar to that shown in Fig. 11*B*; the ATP concentration of the assay solution was 0.39 μM. The activation curve in the presence of vanadate was biphasic, as

TABLE II

Effect of Na_o on K_c-dependent pNPPase Activity of Resealed Ghosts

External solution	K_c-dependent pNPPase activity	5 μM Vanadate	Fractional inhibition
mM	*nmol/mg ghosts·h*		
0 Na, 0 K	58.6±0.07	15.4±0.4	0.74
0 Na, 32 K	43.7±0.2	15.7±0.7	0.64
128 Na, 0 K	43.4±0.2	21.9±0.3	0.50
128 Na, 1.6 K	43.8±0.6	21.2±0.2	0.52
128 Na, 32 K	43.8±0.3	13.2±0.6	0.70

Resealed ghosts were prepared as described in the Methods. The ghosts were prepared to contain 10 mM HEPES, 0.5 mM EGTA, 4.5 mM $MgCl_2$, and 50 mg/100 ml albumin; half the ghosts contained 150 mM K and half contained 150 mM choline and no K; the pH was adjusted to 8.0 with Tris. After the resealed ghosts were washed, pNPPase activity was measured in solutions that contained 10 mM HEPES, 0.5 mM EGTA, 5 mM pNPP, and the indicated concentrations of Na and K; the cation concentration was brought to 160 mM with choline. The solutions were adjusted to pH 7.4 with Tris. K_c-dependent pNPPase activity is the activity measured in the high-K ghosts minus the activity in the K-free ghosts. During the assay, Mg was present only in the resealed ghosts; the external solution contained no Mg. Preliminary experiments showed that, in the absence of Mg, K-dependent and ouabain-sensitive pNPPase activity was negligible, so that the contribution in this experiment of ghosts that failed to reseal to cations to the measured pNPPase activity was also negligible.

in Fig. 11 (results not shown). Finally, we thought it possible that, during the course of the pNPPase assay, the Na,K pump may be phosphorylated by PO_4 and that this phosphorylation may be responsible for the reversal of vanadate inhibition by Na. We therefore measured pNPPase activity in an experiment similar to that shown in Fig. 11*B*, but with 3.24 mg/ml glycogen, 1.0 mM NADP, 0.66 U/ml phosphorylase *a*, and 0.7 U/ml glucose-6-phosphate dehydrogenase in order to minimize accumulation of inorganic phosphate. The activation curve in the presence of vanadate was biphasic (not shown). As a result of these experiments, we concluded that reversal of vanadate inhibition of the red cell Na,K pump by Na does not require ATP, nor does it result from phosphorylation of the pump by inorganic phosphate.

Table II shows the results of an experiment designed to determine the side of the membrane at which Na protects pNPPase activity from vanadate inhibition.

Na-free resealed ghosts were prepared and pNPPase activity was measured in high-Na and Na-free solutions. It can be seen that the inhibition produced by vanadate was less when the solutions contained Na than when they were Na free; at high external K, vanadate inhibition was unaffected by external Na. Na protects pNPPase activity against vanadate inhibition from the outside, just as it protects ATPase activity from the outside.

We performed several experiments designed to determine whether the high-affinity site at which Na_o reverses vanadate inhibition is a property of a conformation of the pump similar to E_1 or of one similar to E_2. Oligomycin inhibits the Na,K pump, and, from the steady state kinetic characteristics of pump inhibition,

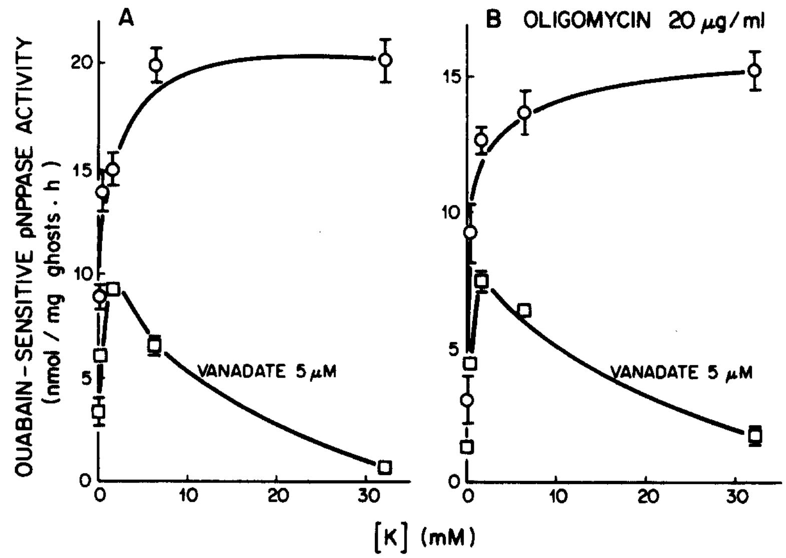

FIGURE 12. Ouabain-sensitive pNPPase activity vs. K concentration. The measurements were made in a solution containing 10 mM Tris HEPES, pH 7.4, 0.5 mM EDTA, 4.5 mM Mg, 5.4 mM pNPP, 122 mM Na, and the indicated concentrations of K; K was replaced by choline. The measurements were made with (□) and without (○) 5 μM vanadate. Note that the scale of the ordinate is different for the measurements made in the presence of 20 μM oligomycin (*B*) and for the measurements made in oligomycin-free solutions (*A*).

it is clear that oligomycin preferentially binds to E_1 conformations (Sachs, 1980). Oligomycin is a poor inhibitor of K-dependent phosphatase activity (Israel and Titus, 1967), but it prevents the stimulation of phosphatase activity that occurs in the presence of Na and ATP (Askari and Koyal, 1971), presumably because E_1 forms occur under these circumstances. Fig. 12 shows the results of an experiment in which we determined the effect of oligomycin on vanadate inhibition of the pNPPase activity measured in a solution high in Na (122 mM) at various concentrations of K; oligomycin, when present, was 20 μM. Oligomycin inhibited the pNPPase activity somewhat, probably because the high concentration of Na converted a significant proportion of the pump to the E_1 form.

However, the K activation curve in the presence of vanadate was biphasic whether or not oligomycin was present, which suggests that E_1 forms, which are removed from the reaction sequence in the presence of oligomycin, are not involved in the reversal of vanadate inhibition by Na_o. We also performed an experiment similar to that shown in Fig. 12, but without Na (not shown). Oligomycin did not inhibit the pNPPase activity, and the K activation curve in the presence of vanadate was not biphasic, so oligomycin did not mimic the effect of Na.

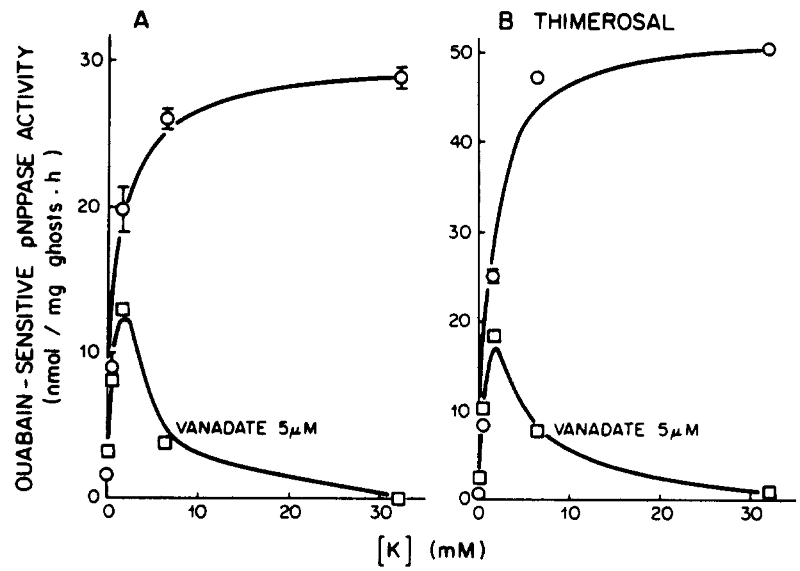

FIGURE 13. Ouabain-sensitive pNPPase activity vs. K concentration. Ghosts were incubated for 15 min at 37°C in solution containing 8.8 mM Tris HEPES, pH 7.4, with and without 1.77 mM thimerosal. The ghosts were then washed four times with an ice-cold 16 mM choline chloride solution. The ouabain-sensitive Na,K-ATPase activity of the ghosts exposed to thimerosal was 42.2 ± 1.5 nmol/mg ghosts·h, and that of the control ghosts was 185.2 ± 4.4 nmol/mg ghosts·h. pNPPase activity was measured in a solution that contained 10 mM Tris HEPES, 0.5 mM EDTA, 4.5 mM Mg, 5.4 mM pNPP, 122 mM Na, and the indicated concentrations of K; K was replaced by choline. The measurements were made with (□) and without (○) 5 μM vanadate. Note that the scale of the ordinate is different for the thimerosal-treated cells (*B*) and for the control cells (*A*).

Ethylmercurithiosalicylate (thimerosal) inhibits Na,K-ATPase, but not its partial reactions, including K-dependent pNPPase activity (Askari et al., 1979). Since, under appropriate circumstances, thimerosal inhibits Na,K-ATPase activity by >90% while stimulating phosphatase activity by 50% (Hansen et al., 1979), it has been suggested that the inhibitor exerts its effect by slowing conformational transitions. Jørgensen and Petersen (1982), by monitoring tryptophan fluorescence, obtained evidence that the inhibitor prevents conformational changes induced by Rb, but not changes induced by Na. Fig. 13 gives the results of an experiment in which the effect of vanadate on the pNPPase activity of control ghosts and ghosts exposed to thimerosal was measured in high-Na solutions at various K concentrations. The ATPase activity of the thimerosal-treated ghosts was 23% that of the control ghosts, and, at high K, the pNPPase activity of the thimerosal-treated ghosts was 1.7 times that of the control ghosts. In thimerosal-treated ghosts, the K activation curve in the presence of vanadate remained biphasic. Table III gives the results of an experiment in which the effect of oligomycin on pNPPase activity was measured using thimerosal-treated ghosts. In this experiment, thimerosal nearly completely inhibited Na,K-ATPase activity; nevertheless, Na reversed vanadate inhibition whether or not oligomycin was present. We concluded that interference with the $E_1 \rightarrow E_2$ conformational transition does not alter Na reversal of vanadate inhibition, nor is the site at which Na exerts this effect a property of the E_1 conformation.

TABLE III

pNPPase Activity of Thimerosal-treated Ghosts

Solution	Ouabain-sensitive pNPPase activity			
	0 Na		128 mM Na	
	0 Vanadate	5 μM Vanadate	0 Vanadate	5 μM Vanadate
	nmol/mg ghosts·h			
0 K	4.7±0.4	0.2±0.2	2.5±0.1	2.1±0.1
1.6 mM K	45.6±0.4	0.7±0.1	31.8±0.2	18.8±0.1
0 K, 20 μg/ml oligomycin	4.7±0.5	0.3±0.1	1.7±0.2	0.9±0.1
1.6 mM K, 20 μg/ml oligomycin	40.6±0.2	0.2±0.1	24.8±0.2	14.7±0.1

Ghosts were incubated for 15 min at 37°C in a solution that contained 8.8 mM Tris HEPES, pH 7.4, and 1.77 mM thimerosal; a portion of the ghosts was incubated in the same solution without thimerosal. The ghosts were washed four times in ice-cold 16 mM choline chloride solution, and ouabain-sensitive Na,K-ATPase activity was measured. The activity of the control ghosts was 220.1 ± 2.3 and that of thimerosal-treated ghosts was 0.5 ± 3.6 nmol/mg ghosts·h. pNPPase activity was measured in a solution that contained 10 mM Tris HEPES, pH 7.4, 0.5 mM EDTA, 4.5 mM Mg, 5.4 mM pNPP, with and without 122 mM Na, and with the indicated concentrations of K. Na and K were replaced with choline.

Effect of Mg^{2+} on Na_o Reversal of Vanadate Inhibition

Vanadate inhibition of the Na,K pump requires Mg (Bond and Hudgins, 1975), and the affinity of the pump for vanadate increases as the Mg concentration increases (Bond and Hudgins, 1979). Since it seemed possible that Na_o antagonizes vanadate inhibition by modifying the interaction of Mg with the pump, we performed two experiments to evaluate the possibility directly. Fig. 14 shows the results of an experiment in which we measured the effect of Na_o on vanadate inhibition of the ouabain-sensitive K influx using cells with low Mg and high Mg concentrations; the intracellular Mg concentration was altered by exposing the cells to solutions with low and high Mg concentrations in the presence of the divalent cation ionophore A23187. The extracellular Na and K concentrations were chosen so that there would be significant protection by Na_o against vanadate inhibition, as demonstrated by the greater concentration of vanadate necessary to produce a degree of inhibition in the presence of Na_o comparable to that in its absence. The effect of Na_o was about the same in the low- and high-Mg cells; the ratios of the vanadate concentrations necessary for half-maximal inhibition in the presence and absence of Na_o was 85.0 in the low-Mg cells and 98.5 in the high-Mg cells.

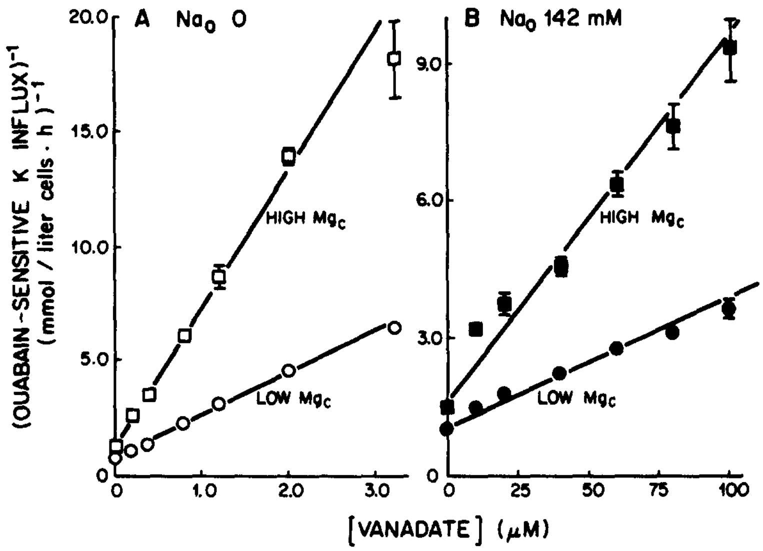

FIGURE 14. The reciprocal of the ouabain-sensitive K influx vs. the concentration of vanadate. Intracellular cation content was altered by exposure of the cells to PCMBS; intracellular Na was 27.3 and K was 7.3 mmol/liter cells. After cation content was altered, the cells were incubated for 30 min at 37°C in solutions that contained 16 mM Tris, 142 mM Na or choline, 1.6 mM K, 0.5 mM EGTA, 1.5 or 9.5 mM Mg, 1 μg/ml A23187, 0.1 vol/vol ethanol, 10 mM glucose, and 20 mg/100 ml albumin; pH was 7.4. After the 30-min incubation, ^{42}K was added and K influx was measured; the final K concentration was 2.52 mM. The lines are: at 0 Na_o, in the high-Mg cells (□) $1/v = 1.21 + 6.14$ [vanadate], and in the low-Mg cells (○), $1/v = 0.79 + 1.87$ [vanadate]; at 142 mM Na_o, in the high-Mg cells (■), $1/v = 1.60 + 0.081$ [vanadate], and in the low-Mg cells (●), $1/v = 1.03 + 0.029$ [vanadate]. From these values, the concentrations of vanadate that half-maximally inhibit the pump are: 0 Na_o, low Mg_c: 0.42 μM; 0 Na_o, high Mg_c: 0.20 μM; 142 Na_o, low Mg_c: 35.7 μM; and 142 Na_o, high Mg_c: 19.7 μM.

Most of the experiments described above were performed with relatively high concentrations of Mg^{2+}. We therefore performed an experiment in which we measured vanadate inhibition of the ouabain-sensitive ATPase activity at various concentrations of K in solutions in which the sum of the EDTA and ATP concentrations was 1.82 mM greater than the Mg concentration, so that the Mg^{2+} concentration was low. The K activation curve in the presence of vanadate was biphasic (not shown); high Mg^{2+} is not necessary to observe the protective effect of Na against vanadate inhibition.

DISCUSSION

The goal of these experiments was to understand how Na_o protects the Na,K pump against vanadate inhibition, and most of the experiments were directed at characterizing the site at which Na_o exerts its protective effect. For convenience in following the discussion, we have given in Fig. 1 a current version of the Albers-Post model for the Na,K pump reaction mechanism; the sequences of the various exchanges referred to below are summarized in the figure legend.

From the results presented, it is clear that the protective site(s) has a relatively high apparent affinity for Na_o, with a probable $K_{1/2}$ of ≤ 1 mM. Previous reports suggested that the protective site has a low affinity for Na_o. This conclusion was based on experiments in which the apparent affinity for Na_o was estimated in the presence of K_o and vanadate, both of which reduce it (Bond and Hudgins, 1979), and on equilibrium binding experiments in which Na stabilizes E_1 forms of the enzyme that have a low affinity for vanadate (Smith et al., 1980), a mechanism that is not likely to account for the protective effect of Na_o when the pump carries out Na-K and K-K exchange. K may or may not compete directly at the protective Na sites; K_o competes with a relatively high affinity (moderately high when estimated by fitting Eq. 1 to the curves of Fig. 2, and very high when judged from the experiment shown in Fig. 8). The fit of Eq. 1 to the data of Fig. 2 is better if it is assumed that there are two K_o sites rather than one. Beaugé (1979) and Bond and Hudgins (1979) previously suggested that there must be two sites for K since plots of fractional inhibition by vanadate vs. K concentration, such as those shown in Fig. 8, are sigmoid; however, such sigmoid plots can be generated by an equation similar to Eq. 1, but with the assumption that there is a single site for K_o.

Reversal of vanadate inhibition by Na_o can be demonstrated when Na-K exchange is measured in cells with very low K_c, when K-K exchange is measured in cells nominally free of Na, or when pNPPase activity is measured in the absence of ATP. Beaugé et al. (1980) demonstrated that vanadate does not inhibit the K efflux into high-Na solutions free of K, i.e., during reversed operation of the pump. Reversal of vanadate inhibition does not, therefore, require K_c, Na_c, or ATP; nor does it require turnover of the pump, since it can readily be demonstrated when partial pump reactions are measured, or even when the pump operates in reverse. Beaugé and Berberian (1983) found that the effect of Na on vanadate inhibition of pNPPase activity could not be demonstrated in the absence of ATP; we have no explanation for the difference between their results and ours.

The site at which Na_o exerts its protective effect is a property of an E_2 form of the enzyme. Three kinds of experiments lead to this conclusion. First, when Na-K exchange was measured, the protective effect was observed at Na_o concentrations as low as 8 mM (Fig. 6), and when the K-K exchange was measured in Na-free cells, the effect was present at 20 mM Na_o (Fig. 10). Kaplan and Kenney (1985) showed that at concentrations of Na as low as these, the red cell membrane

Na,K-ATPase exists predominantly as E_2P; there is very little E_1P present. Second, oligomycin, which preferentially combines with E_1 forms bound to Na and therefore should remove them from the reaction sequence, does not alter the protective effect of Na_o. Finally, thimerosal, which inhibits $E_1 \rightarrow E_2$ conformational changes, does not interfere with the protective effect of Na; interference would be expected if the protective effect of Na_o were exerted on E_1 conformations, the transition from E_1Na to E_2 were blocked, and a function, such as pNPPase activity, which is performed by E_2 forms, were measured.

From the results presented above, it is clear that the protective effect of Na_o against vanadate inhibition is not exerted at the two K transport sites. Several other sites at which Na modifies pump behavior seem to exist, however. At high concentrations, Na_o inhibits Na-K exchange when the measurements are made using ghosts with high ADP/ATP ratios (Kennedy et al., 1986), and high external Na concentrations also promote ouabain binding (Hobbs and Dunham, 1978). Both of these effects exhibit a relatively low apparent affinity for Na_o, and both can be demonstrated in the presence of high concentrations of K_o; this site(s) cannot be the site at which Na protects against vanadate inhibition. On the other hand, there is a site at the outside of the pump at which Na inhibits the uncoupled Na efflux (Garrahan and Glynn, 1967*a*, *b*), a small saturable Na influx (Sachs, 1970), the uncoupled K efflux (Sachs, 1986*a*), Na-dependent ATPase activity (Glynn and Karlish, 1976), and the ATP-ADP exchange (Kaplan, 1982), and which has an apparent affinity for Na similar to what we estimate for the site at which Na protects against vanadate inhibition. There is no unequivocal evidence that K_o competes with Na_o at this site. However, Cavieres and Ellory (1975) showed that, at very low K, Dixon plots of the reciprocal of the pump rate against the Na_o concentration are curved, which they interpreted as an allosteric effect of Na at the high-affinity site on the pump rate. At higher K concentrations, the plots are linear (Sachs, 1977), and the results can be explained as straightforward competition between Na and K at one of the K transport sites without the need to invoke allosteric effects; the loss of the allosteric effect might be due to competition between Na and K at the high-affinity site. The high-affinity site at which Na_o modifies the various partial reactions of the pump is a property of an E_2 conformation of the enzyme (Kaplan and Kenney [1985] have observed that inhibition of the ADP-ATP exchange by low Na_o does not occur at 0°C; E_2P is absent at this temperature), and so is the high-affinity site at which Na_o protects against vanadate inhibition. We conclude, then, that the two effects may be exerted at the same site(s).

Although the protective site is not one of the transport sites, it has properties strikingly similar to them, including its relative affinities for the K congeners as competitors with Na (Bond and Hudgins, 1979). If the protective effect is exerted at a single site, it may be the third transport site at the outside of E_2 conformations, which, though not capable of transporting ions, can, by interacting with Na and K, modify the operation of the pump. If this is the case, then the relative affinities of the three transport sites on E_2 at the outside surface decreases as the sites are filled with K; the first site (the Na protective effect) has a high affinity for Na in the absence of K, and, judged by competition experiments (Sachs, 1977), the first transport site has a lower affinity and the second transport site has a very low affinity for Na_o.

On the other hand, the relation between the pump rate and Na_o concentration in Figs. 6 and 7 is sigmoid, the relation between fractional pump inhibition and K_o concentration in Fig. 8 is sigmoid, and a plot of K'_K against Na_o shown in Fig. 8 may be nonlinear. The relation between Na_o, K_o, and vanadate is clearly complex and estimation of the number of sites for either ion involved in the protective effect on the basis of nonlinear plots may be perilous. However, if the nonlinear plots are taken as evidence for multiple ion-binding sites at which the protective effect of Na_o is exerted, it is possible to devise a model that describes the data qualitatively. The model proposes that there are n cation-binding sites at the outside of the pump, separate from the K transport sites and coexistent with them. When all n sites are filled with Na, the pump is protected against inhibition by vanadate. If any of the n sites is occupied by K, inhibition by vanadate occurs, and binding of Na or K to one of the sites does not alter the affinity of the other sites for either ion (i.e., species with some sites occupied by K and the rest by Na can occur). With these assumptions, the fraction of pumps protected from vanadate inhibition is defined by:

$$\text{fraction protected} = \frac{1}{\left[1 + \frac{K_N}{\text{Na}}\left(1 + \frac{K}{K_K}\right)\right]^n},$$

where K_N is the dissociation constant of the sites for Na, K_K the dissociation constant of the sites for K, and n is the number of sites. The model predicts a sigmoid relation between the pump rate and the Na_o concentration in the presence of vanadate (Fig. 6), a linear relation between the apparent $K_{1/2}$ for Na_o at its protective sites and the K_o concentration (Fig. 6), a sigmoid relation between fractional inhibition in the presence of vanadate and the K_o concentration (Fig. 8), and a parabolic relation between the apparent $K_{1/2}$ for K_o promoting vanadate inhibition and the Na_o concentration (Fig. 8). If n is taken as 3, the model is nearly the same as one that describes the interaction of Na and K at the internal surface of the pump at which Na_c activates Na-K exchange, K_c activates K-K exchange, and each ion competes with the other (Sachs, 1986*a*). The n protective sites may be sites on the same α chain as the K transport sites, but in that case it would be necessary to suppose that the same subunit simultaneously contains the two transport sites and the n protective sites. On the other hand, the protective sites may be manifestations of transport sites on the nontransporting monomer of a dimeric enzyme functioning by some variant of a flip-flop mechanism. A somewhat similar proposal was made by Jensen et al. (1984) to account for the effect of Na and K on nucleotide binding to a purified enzyme preparation.

Whatever the physical basis for the protective sites, it is clear that although Na is released to the outside before K adds when Na-K exchange takes place in Na-free solutions (Sachs, 1980), Na and K can bind to the outside of the enzyme at the same time in high-Na, low-K solutions.

The molecular mechanism by which Na_o protects against vanadate inhibition is not clear. Since these measurements were made under steady state conditions

(Sachs, 1986*b*), Na, by binding to a high-affinity external site, must reduce the steady state level of an enzyme form that binds vanadate with high affinity. We have shown that Na does not accomplish this by modifying the interaction of the enzyme with internal Mg^{2+}. It can be shown that, in Na-free solutions, the steady state level of the enzyme form that binds vanadate is increased by both intracellular and extracellular K (Sachs, 1986*b*), and is probably E_2K, or an enzyme form in equilibrium with E_2K, so that it is possible that external Na may reduce the steady state level of this intermediate. However, external Na does not protect against inhibition by phosphate or arsenate, which are also believed to inhibit by combining with E_2K. It may be that phosphate and arsenate combine with an enzyme form different from that which binds vanadate; for instance, high-affinity vanadate binding may require combination of K with both the transport sites and the modifying site, while phosphate and arsenate binding take place when only the transport sites are combined with K. Phosphate (Glynn et al., 1970) and arsenate (Kenney and Kaplan, 1985), at concentrations that inhibit Na-K exchange, support a ouabain-sensitive K-K exchange, but vanadate inhibits rather than supports the exchange, which is a further indication that the way in which the anions interact with the pump is fundamentally different.

There is, however, some reason to believe that the change brought about by Na_o is more extensive, and that, with Na attached to the high-affinity external site, the reaction sequence may be different from what it is when the site is empty. The pathway that operates when Na occupies the protective site eliminates the enzyme form that binds vanadate, but not the enzyme forms that bind phosphate and arsenate and take part in the K-K exchange. Alternative reaction mechanisms for this enzyme have previously been proposed by Shaffer et al. (1978) to account for differences in the characteristics of the phosphatase reaction with acetyl phosphate as substrate from those with pNPP as substrate. Modification of the reaction mechanism by Na_o is consistent with the finding of Beaugé and Glynn (1979) that Na at the high-affinity external site inhibits dephosphorylation of E_2P. Hobbs et al. (1980) reported that phosphoenzyme formed in the presence of Na and K exhibits rapidly and slowly decaying components, and vanadate, at low concentrations, suppresses predominantly the rapidly decaying component. Purified enzyme trypsinized in the presence of Na until about half the ATPase activity is lost has been shown by Jørgensen and Karlish (1980) to be more likely to exist in E_1 conformations under circumstances in which the native enzyme assumes E_2 conformations. Beaugé and Glynn (1978) found that enzyme trypsinized in this way is relatively insensitive to vanadate, and that inhibition does not increase with increasing K. It is possible that, in addition to altering the equilibrium between E_1 and E_2, trypsinization stabilizes the enzyme in the state selected by Na acting at the high-affinity protective site.

Whatever the basis for the modification by Na_o of the affinity of the Na,K pump for vanadate, it is clear that Na_o must profoundly alter the reaction mechanism. A ready explanation for such an effect cannot be deduced from the Albers-Post mechanism (Fig. 1). Although the model has been very successful in accounting for a vast array of observations on the biochemical and physiological characteristics of the pump, it is likely that the mechanism is more complex than the simple model, and modification of the model will be necessary. It is perhaps worth pointing out that at 150 mM Na and 4 mM K, most of the modifying sites will be occupied by Na and therefore most of the Na-K exchange cycles will take place by this alternative pathway.

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