# Sodium Pump-mediated ATP:ADP Exchange

## *The Sided Effects of Sodium and Potassium Ions*

JACK H. KAPLAN

From the Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

ABSTRACT Resealed human red cell ghosts containing caged ATP (Kaplan et al., 1978) and [$^3$H]ADP were irradiated at 340 nm. The photochemical release of free ATP initiated a rapid transphosphorylation reaction (ATP:ADP exchange), a component of which is inhibited by ouabain. The reaction rate was measured by following the rate of appearance of [$^3$H]ATP. The sodium pump-mediated ATP:ADP exchange reaction showed high-affinity stimulation by Mg ions (<10 $\mu$M) and was inhibited at higher levels. At optimal [Mg], extracellular Na ($Na_o$) had a biphasic effect. $Na_o$ progressively inhibited the reaction rate between 0 and 10 mM and stimulated at higher levels. Intracellular Na ($Na_i$) activated the reaction; the rate was maximal when $Na_i$ was 1 mM and remained unaltered up to 115 mM $Na_i$ at constant $Na_o$. Extracellular K ions ($K_o$) inhibited the reaction; at high $Na_o$, half-maximal inhibition was observed with 0.9 mM $K_o$. $Li_o$ inhibited the exchange rate with a lower affinity than $K_o$; half-maximal inhibition was produced by ~50 mM $Li_o$. Intracellular K ions were without dramatic effect on the reaction rate in the concentration range where $K_o$ inhibited completely. The relationship between these observations and previous studies on porous preparations is discussed, as well as the extent to which these observations support the hypothesis that the sodium pump-mediated ATP:ADP exchange reaction accompanies the Na:Na exchange transport mode of the sodium pump.

### INTRODUCTION

When human red blood cells are incubated in K-free media rich in Na ions, a ouabain-sensitive exchange of intracellular and extracellular Na ions is observed (Garrahan and Glynn, 1967*a*). Na:Na exchange has been most widely studied in red blood cells (Garay and Garrahan, 1973; Glynn and Hoffman, 1971; Sachs, 1970) and has also been measured in giant axons of squid (Caldwell et al., 1960; De Weer, 1970) and in skeletal muscle (Keynes

Address reprint requests to Dr. Jack H. Kaplan, Dept. of Physiology, G4, 37th and Hamilton Walk, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/82/12/0915/23 $1.00 915
Volume 80 December 1982 915–937

and Steinhardt, 1968; Kennedy and De Weer, 1976). The studies in red blood cells and resealed ghosts have shown that the Na:Na exchange rate is increased by elevated levels of ADP, is independent of ATP in the concentration range 300–1,500 $\mu$M (Glynn and Hoffman, 1971), and will not take place in ghosts prepared to contain ADP but not ATP (Cavieres and Glynn, 1979). Although ATP is required for the pump to operate in this mode, there is apparently no associated hydrolysis of ATP (Garrahan and Glynn, 1967*c*).

This set of observations supports the suggestion that Na:Na exchange transport is accompanied by the ATP:ADP exchange reaction (Glynn and Hoffman, 1971), first observed in partially purified Na,K-ATPase preparations by Fahn et al. (1966). Subsequent work has shown that the rate of ATP:ADP exchange shows a complex dependence upon Na ion concentration with enzyme isolated from a variety of sources (Wildes et al., 1973; Beauge and Glynn, 1979; Kaplan and Hollis, 1980; Kaplan et al., 1981).

The asymmetric effects of Na and K ions on Na:Na exchange transport have been previously characterized in human red cells (Garrahan and Glynn, 1967*a*, *b*; Garay and Garrahan, 1973), where the effects of internal and external cations can be separately studied. To investigate whether the ATP:ADP exchange reaction accompanies the Na:Na exchange transport, it is necessary to similarly characterize the sided effects of cations on the ATP:ADP exchange rate.

Until recently it has not been possible to measure sodium pump-mediated ATP:ADP exchange rates in resealed ghosts. The intracellular compartment contained high levels of adenylate kinase activity, which would obscure the sodium pump-mediated component of ATP:ADP exchange. Furthermore, endogenous phosphatase (including ATPase) activities caused substantial breakdown of ATP during the incubation at 37°C, which is necessary to restore the low cation permeability to the resealed ghosts. It was also necessary to devise a method of initiating the ATP:ADP exchange reaction in the intracellular compartment after completion of the ghost preparation, because the isotopic equilibration would be completed during the resealing procedure if it was allowed to proceed during the preparation of the resealed ghosts.

This paper describes the application of caged ATP in a resealed human red cell ghost system that enabled us to circumvent these problems and to investigate the sidedness of the cation-activating effects of ATP:ADP exchange. Caged ATP is an ester of ATP bearing a 2-nitrobenzyl group on the terminal phosphate. It has previously been shown (Kaplan et al., 1978) that caged ATP is resistant to ATPase activity and it does not bind to or inhibit the Na, K-ATPase. After brief photolytic irradiation of caged ATP with light of 340–350 nm wavelength, the nitrobenzyl ester is cleaved and free ATP is released (Kaplan et al., 1978). The stability of the compound avoids problems of endogenous ATPase activity in the ghosts, and the photolability enabled us to initiate the exchange reaction in the intracellular compartment and measure the initial rates of the exchange reaction. Brief reports of the effects of external Na on the ATP:ADP exchange rate using caged ATP have been published previously (Kaplan, 1980; Kaplan and Hollis, 1980).

## METHODS

### *Preparation of Resealed Ghosts*

Freshly drawn blood was centrifuged at 10,000 *g* for 10 min and the plasma and buffy coat were removed by aspiration. The cells were then washed three times by centrifugation and resuspension in 140 mM choline chloride, 10 mM HEPES-Tris, pH 7.6 (henceforth buffer A). Between the second and third washes the cell suspensions were incubated at room temperature for 15 min to allow completion of pH equilibration. After the final centrifugation the packed cells were suspended at ~10% hematocrit (2 ml in 20 ml buffer A) and cooled in ice. The cell suspension was then applied to the top of the gel filtration column (K 50/60; Pharmacia Fine Chemicals, Piscataway, NJ), which was surrounded by a cooling jacket maintained at −1°C. The column had previously been filled with Bio-Gel A-50m beads (Bio-Rad Laboratories, Richmond, CA) and equilibrated with the lower four-fifths in 10 mM PIPES, 0.1 mM EDTA, pH 6.0 (with Tris), and the upper one-fifth with buffer A. The nylon screen supplied with the column was replaced with a larger mesh screen to prevent clogging (30-μm mesh, Small Parts, Inc., Miami, FL). All solutions were degassed by aspiration at the water pump before use in the column. The cells were subsequently eluted from the column at a rate of ~2–3 ml $min^{-1}$. The major band of hemoglobin penetrated to about one-third of the depth of the column when the eluate became turbid. Fractions of the turbid eluate were then collected in tubes in an ice bath. The most turbid fractions were contained in ~30 ml. These fractions were pooled and the membranes were collected by centrifugation at 20,000 *g* for 5 min at 0°C; the supernatant was removed and the isotonic mixture of salts and nucleotides was added to the membranes. In most experiments these solutions contained NaCl plus choline Cl (total 140 mM), 10 mM HEPES, pH 7.4, 10 μM $MgCl_2$, 75 μM caged ATP, 25–50 μM [$^3$H] ADP, and 100 μM Ap5A. After incubation of the membrane suspensions at 37°C for 45 min with gentle shaking, the resealed ghosts were collected and washed by centrifugation and resuspension in buffer A at 0°C. The ghosts were then packed in plastic tubes at 20,000 *g* for 5 min and held on ice until use.

This procedure is a modification of the gel filtration technique previously described (Kaplan and Hollis, 1980). The major modification is the elimination of 15 mM HEPES, pH 7.6, between buffer A and the PIPES solution on the column. If the HEPES solution is used, the resulting ghosts are composed of at least two fractions. One fraction is very leaky to Na ions and these ghosts lose their cation content on resuspension after packing. The major fraction is relatively impermeable to Na ions. The fraction of leaky ghosts can comprise 20–40% of the total population. The size of the leaky fraction is dependent upon column flow rate and the volume of the HEPES pH 7.6 solution used in the band between buffer A and the PIPES solution on the column. Apparently, the more time the ghosts at hemolysis spend in the high-pH buffer (pH 7.6), the greater is the size of the leaky fraction of ghosts. These observations are similar to those previously made by Lepke and Passow (1972), who showed that the optimal pH at hemolysis for maximizing the yield of resealed ghosts that were least permeable to K ions was pH 6.0–6.4, and that higher pH values increased the proportion of ghosts that did not reseal to K ions. The elimination of the HEPES solution yields an essentially homogeneous (>90%) population of ghosts when the kinetics of Na efflux are examined. Since the completion of this work, a preliminary report has appeared in agreement with the effects reported here on the properties of resealed ghosts prepared using gel filtration procedures (Wood, 1982). Whatever distribution of ghosts is obtained in terms of Na content, all of the ghosts are impermeable to nucleotides.

By using this ghosting procedure we have also been able to eliminate the need for the inclusion of nucleoside diphosphokinase inhibitors that were used in our earlier work in order to observe the ouabain-sensitive ATP:ADP exchange reaction (Kaplan and Hollis, 1980). We have continued to include Ap5A, the adenylate kinase inhibitor, although adenylate kinase activity is also greatly diminished. If ghosts are prepared without Ap5A there is a small increase before irradiation in the amount of radioactivity that migrates with AMP. This amount is higher than that appearing as a contaminant in the [$^3$H]ADP (often 7–8% compared with 4–5% contamination) and is presumably due to some residual adenylate kinase activity. However, this amount does not change during the course of the ATP:ADP exchange reaction after irradiation of the ghosts. Since Ap5A can be expected to chelate Mg ions, we have continued to include it in the ghosts in the present work, so that the present data are comparable to our results in the earlier studies. It seemed likely that at the nucleotide levels used in the present studies, even if Ap5A were not included, the residual adenylate kinase activity in the ghosts is sufficiently low so as not to introduce large errors in measured rates. The results of two experiments illustrating this point are shown in Table I. It is

TABLE I

ATP:ADP EXCHANGE RATES IN RESEALED GHOSTS WITH AND WITHOUT Ap5A

| | | ATP:ADP exchange rate | | |
|---|---|---|---|---|
| Experiment | [Ap5A] | 140 mM $Na_o$ | 140 mM $Na_o$ + ouabain | Na pump component |
| | *μM* | *μmol Pi/liter ghost/h* | | |
| 1-18 | 100 | 113.8 | 39.3 | 74.5 |
| 1-18 | 0 | 107.1 | 42.4 | 64.7 |
| 2-27 | 100 | 108.3 | 70.5 | 37.8 |
| 2-27 | 0 | 83.1 | 37.5 | 45.6 |

ATP:ADP exchange rates in resealed ghosts with and without Ap5A. Ghost preparation and the initiation and measurement of ATP:ADP exchange were carried out as described in Methods. The data shown are from two representative experiments of this type. The rates of ATP:ADP exchange are the means of duplicate determinations the value of which agreed to within <10%.

clear that ouabain-sensitive ATP:ADP exchange rates are measurable in the absence of added adenylate kinase inhibitor and are probably not influenced by the presence of Ap5A.

### *Measurement of Ghost Contents*

Intracellular Na + K concentrations were measured after dilution of the packed ghosts using a Beckman flame photometer (Beckman Instruments, Inc., Fullerton, CA) with Li as an internal standard. Intracellular ADP concentrations and ATP (after photolysis) were measured on ghosts suspended at 10% suspension (vol/vol). The suspensions were diluted 10-fold in distilled water, placed in a boiling water bath for 2 min, and then held on ice until assay. The nucleotides were assayed using a modification of the procedure of Kimmich et al. (1975), which uses the luciferin bioluminescence procedure after the enzymatic conversion of ADP and AMP to ATP. In one experiment, samples of ghosts were irradiated under identical conditions and the nucleotide contents of the ghosts were analyzed. The photolytic release of ATP

was highly reproducible. After irradiation of the ghosts, the ATP concentration was 32.8 ∓ 1.7 μM (SD, $n = 8$) and the ADP concentration was 34.8 ∓ 2.4 μM (SD, $n = 8$). 50 μM caged ATP and 30 μM ADP had been present in the resealing solution. Intracellular ion and nucleotide concentrations were corrected for the extracellular space obtained in the pellet after packing the ghosts which was between 25 and 30% when either [$^{14}$C]ADP or $^{86}$Rb were used as markers of the extracellular space.

### *Photolytic Initiation and Measurement of the Exchange Reaction*

The ATP:ADP exchange reaction was initiated by irradiation of the ghosts suspended in appropriate buffered salts at 10% cell suspension (vol/vol) in glass cuvettes with a 1-mm path length. The optical arrangement for photolysis has been described previously (Kaplan et al., 1978). In the present studies the ghost suspensions (125 μl) were irradiated using a defocused light spot (3 cm diam) for 15 s. Aliquots (20 μl) were then taken at suitable time intervals (usually 30, 60, 90, and 120 s after the initiation of photolysis) and added to ice-cold 8% trichloroacetic acid (20 μl). The samples were rapidly mixed and held on ice. Samples for assay of nucleotide content were also taken and processed as described above.

The samples in trichloroacetic acid were then frozen and thawed, precipitated protein was pelleted by centrifugation for 1 min (Eppendorf [Hamburg, Federal Republic of Germany] microfuge, 13,000 *g*), and samples of the supernatant (4–6 μl) were spotted onto polyethyleneimine thin-layer chromatography plates (Brinkmann Instruments Inc., Westbury, NY). The spots were dried and overlaid with a spot of solution containing 4 mM each of ATP, ADP, and AMP. The plates were developed in 0.4 M K phosphate, pH 3.5 (with HCl), and visualized with a hand-held ultraviolet lamp. The nucleotides migrated with $R_f$ values of 0.12, 0.35, and 0.55 for ATP, ADP, and AMP, respectively. The areas containing the nucleotides were cut out, placed in scintillation vials in 1 M HCl (1 ml), heated to 120°C for 20 min, and cooled, and scintillation fluid was added. The amount of radioactivity in each nucleotide was then measured in a liquid scintillation counter (7500; Beckman Instruments, Inc.). The recovery of radioactivity was always >96% and the distribution of radioactivity between the nucleotides was shown in control experiments to be unaffected by the acid-stop recovery procedure. In a typical experiment the decline in ATP concentration during the course of the exchange assay was <5%. The exchange rate was measured during the initial 20% progress towards equilibrium.

### *Calculation of Exchange Rate*

The calculation of the exchange rate (expressed in micromoles of Pi transferred to ADP liter ghosts$^{-1}$ h$^{-1}$) was performed using the equation derived by McKay (1943). The rate constant for the reaction is obtained from a plot of $-\ln(1 - a_t^*/a_\infty^*)$ vs. time, where $a_t^*$ is the fraction of total counts per minute in ATP at time $t$ and $a_\infty^*$ is the fraction of the total counts per minute in ATP at equilibrium. The value of $a_\infty^*$ is obtained for each experiment from the measured concentrations of ATP and ADP in the resealed ghosts. The slopes of the straight lines obtained yield the rate constant, which is converted to a rate by multiplying by the factor [ATP][ADP]/[ATP] + [ADP]. The conversion of typical data to rates is shown in Figs. 1*a* and *b*. The difference between the two rates obtained from these figures, in the presence and absence of $10^{-4}$ M ouabain, is the component mediated by the sodium pump. Some comment is appropriate on the nonzero intercept at zero time in the semilog plots of Fig. 1*b*. We have previously shown that when the ghosts are not irradiated, i.e., when ATP is not photoreleased, the radioactivity migrating with ATP in the experimental samples is <0.04% of the total radioactivity (Kaplan and Hollis, 1980). If the system

were in steady state throughout its time course, then the lines in Fig. 1*b* should be straight and pass through the origin. In our experimental system an apparent burst of [$^3$H]ATP occurs at early times. Such a burst has been observed previously when the ATP:ADP exchange reaction was initiated by the rapid addition of [$^3$H]ADP and ATP to the purified enzyme (Yamaguchi and Tonomura, 1977). The explanation of this burst can be found in the relative values of the rate constants involved in the reaction. It is observed that the rate at which $E_1P$ (ADP-sensitive phosphoenzyme) is converted to $E_2P$ (ADP-insensitive phosphoenzyme) is much slower than the rate at which $E_1P$ reacts with ADP to release ATP (J. Froehlich, personal communication). This means that (*a*) $[E_1P]$ is higher during the early stages (pre-steady state) of the

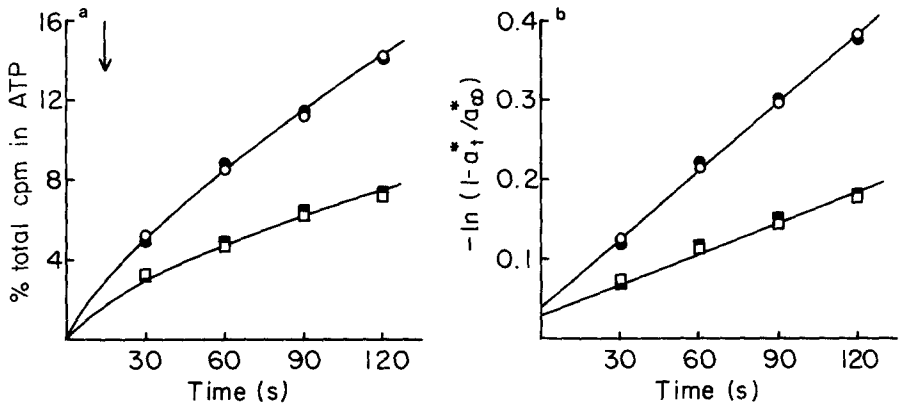



FIGURE 1. Initial time course of appearance of [$^3$H]ATP after illumination of the ghost suspensions. (*a*) Ghosts were prepared to contain [$^3$H]ADP, caged ATP, Ap5A, and salts as described in Methods. They were then suspended in 140 mM NaCl, 10 mM HEPES, pH 7.5, and irradiated with 340-nm light. The vertical arrow indicates when photolysis ended. Samples were taken at the indicated times after the initiation of photolysis and mixed with trichloroacetic acid. The data plotted are the percent total counts per minute migrating with ATP on thin-layer chromatography plates. Circles indicate duplicate photolysis and assays in 150 mM NaCl, 10 HEPES, pH 7.5. Squares indicate duplicate photolysis and assays in the same media plus $10^{-4}$ M ouabain. (*b*) The data from *a* are plotted as described in the text so that the slopes of these lines yield the rate constants for the ATP:ADP exchange reaction. Symbols are identical to those in *a*.

reaction than when the steady state distribution between $E_1P$ and $E_2P$ is established; (*b*) since the ATP:ADP exchange reaction depends upon $[E_1P]$ and since $[E_1P]$ reacts rapidly with [$^3$H]ADP, there will be a pre-steady state burst of [$^3$H]ATP. The size of the burst is not directly related to the number of pump sites per cell but is a complicated function of several rate constants, relating the number of times $E_1P$ turns over with ADP before its steady state distribution with $E_2P$ is established, i.e., the system cycles through reactions A, B, and C many times faster than it progresses through $E_1P \rightleftharpoons E_2P$ (*D*).

$$E_1 + \text{ATP} \overset{(A)}{\rightleftharpoons} E_1\text{ATP} \overset{(B)}{\rightleftharpoons} E_1 \sim \text{P}\cdot\text{ADP} \overset{(C)}{\rightleftharpoons} E_1P \overset{(D)}{\rightleftharpoons} E_2P$$

*Materials*

[$^3$H]ADP, [$^{14}$C]ADP, $^{86}$Rb, and $^{22}$Na were obtained from Amersham Corp., Arlington Heights, IL. Salts, buffers, AMP, ADP, ATP (grade 1, prepared by phosphorylation of adenosine), phosphoenolpyruvate, luciferin-luciferase (type L-0633), myokinase, and trichloroacetic acid were obtained from Sigma Chemical Co., St. Louis, MO. Pyruvate kinase and diadenosine pentaphosphate were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN.

## RESULTS

*Effects of Internal Mg*

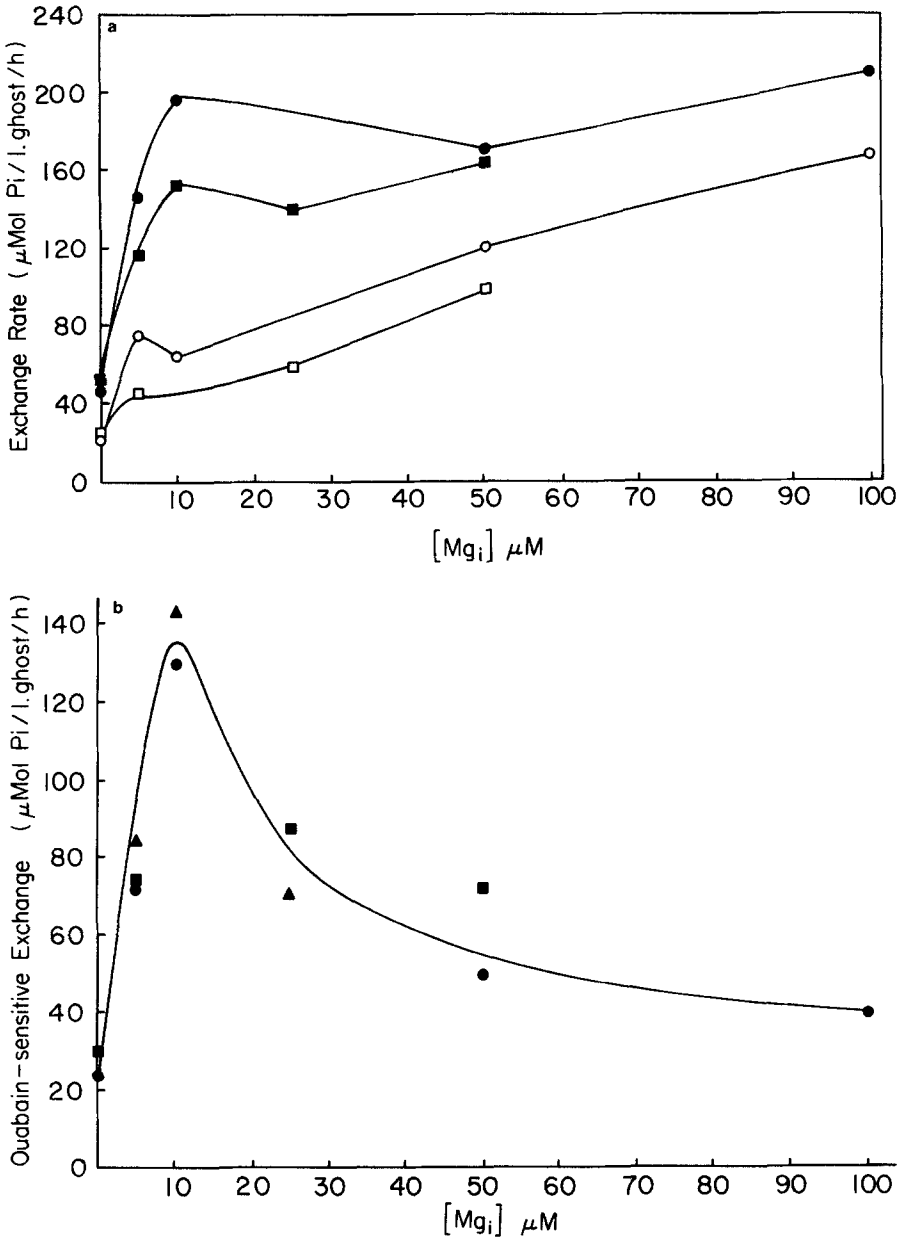
We have previously described the initial observations of ouabain-sensitive ATP:ADP exchange in resealed red cell ghosts (Kaplan and Hollis, 1980). These measurements were performed using a fixed intracellular total Mg concentration of 10 $\mu$M.

In preliminary experiments we had observed that high Mg levels (>100 $\mu$M) made the detection of ouabain-sensitive exchange difficult since the ouabain-insensitive background rate was elevated as the Mg concentration was raised. In Fig. 2*a* are shown the results of experiments where the intracellular Mg has been varied between nominally zero and 100 $\mu$M. Although the absolute values of the total rates vary in the different batches of ghosts, the overall pattern is the same. There is a rapid increase of the rate in the low concentration range (up to 10 $\mu$M), followed by a slower increase in rate. In spite of the variation in total rates, the ouabain-sensitive component of the exchange rate showed a reasonable reproducibility in these experiments, as shown in Fig. 2*b*. The Na pump-mediated exchange reaction in the presence of 140 mM Na in the external medium is activated by a site with very high affinity for Mg (Fig. 2*b*). At higher Mg concentrations, the Na pump-mediated exchange is inhibited by increasing levels of Mg. The Mg concentrations indicated in Figs. 2*a* and *b* represent the total Mg sealed into the ghosts at reversal. Calculation of the free Mg concentration under these conditions is difficult because of the complexity of the mixture of components capable of binding Mg in the intracellular compartment. Although in several experiments no Mg was added to the ghosts at reversal, a measurable rate of ATP:ADP exchange with a significant ouabain-sensitive component was observed. If we assume that Mg is absolutely required for ATP:ADP exchange, inspection of the curves shown in Figs. 2*a* and *b* enables an estimate of the residual Mg in the ghosts of ~2–3 $\mu$M to be made. Inclusion of 100 $\mu$M EDTA in the resealing medium did not completely eliminate the exchange reaction. 1 mM EDTA was required to reduce the rates in the absence of added Mg to a level that was not reliably different from zero, i.e., <2 $\mu$mol Pi transferred per liter ghosts$^{-1}$ h$^{-1}$.

*Activation by $Na_o$*

When the ATP:ADP exchange is initiated in ghosts suspended in various concentrations of Na, a characteristic activation curve is seen (Fig. 3). This is

similar to the data previously reported using ghosts containing nucleoside diphosphokinase inhibitors (Kaplan and Hollis, 1980). At concentrations of $Na_o$ between 0 and 10 mM, an inhibition is observed, whereas at concentrations from 20 to 140 mM, a monotonic increase in exchange rate is seen (with a rather shallow rate of increase with increasing Na concentration). Variations



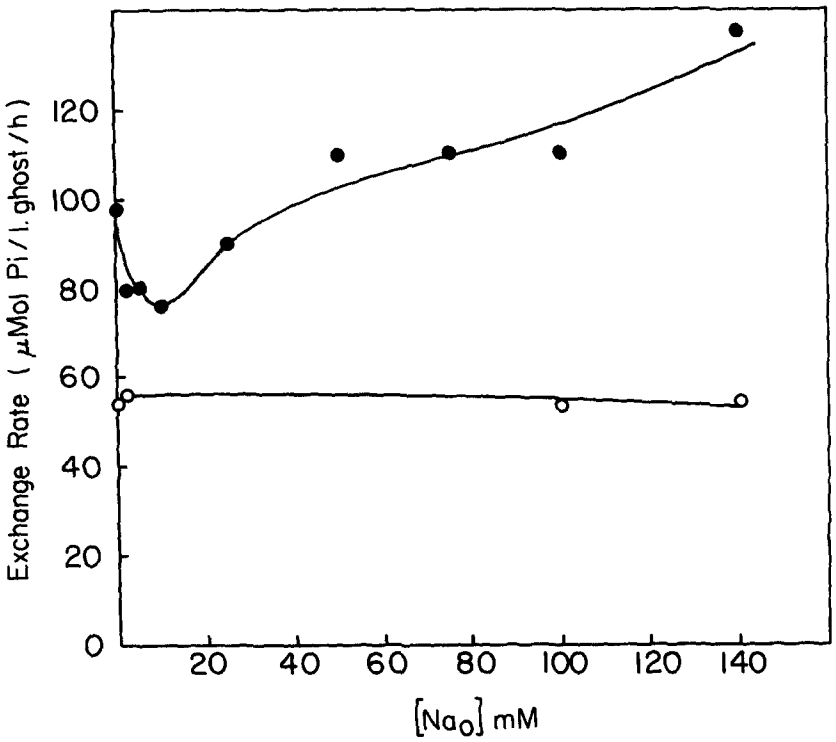



FIGURE 3. Effect of $Na_o$ on the rate of ATP:ADP exchange. The ATP:ADP exchange rate was determined in a preparation of resealed ghosts suspended in buffered NaCl solution in the absence (●) and presence (○) of $10^{-4}$ M ouabain. The sum of NaCl plus choline Cl was 150 mM. The results shown are illustrative of six different experiments that yielded essentially similar data. The points shown are larger than the range of duplicate determinations at each value of $Na_o$.

---

FIGURE 2. (*a*) Dependence of ATP:ADP exchange rate on total intracellular Mg ion concentration. The data are shown from two experiments. Closed symbols are ATP:ADP exchange rates measured in ghosts suspended in 100 mM NaCl, 10 mM HEPES, pH 7.5, 40 mM choline chloride; open symbols indicate the presence of $10^{-4}$ M ouabain. In each experiment the membranes from the column were divided into separate tubes and tonicity restored as described in Methods so that the ghosts contained the indicated amounts of $MgCl_2$. All determinations of exchange rate were determined in duplicate, the range of values at each point is smaller than the symbol in the figure. (*b*) Dependence of Na pump-mediated ATP:ADP exchange on total intracellular Mg ion concentration. The difference in rates in the absence and presence of $10^{-4}$ M ouabain shown in *a* have been plotted vs. total intracellular Mg ion concentration. The data from a third experiment not shown in *a* for reasons of clarity are included here (△).

in rates are observed in different preparations of ghosts, but the same overall pattern of Na dependence is seen. Table II summarizes results of several similar experiments illustrating this point.

TABLE II

THE BIPHASIC EFFECTS OF $Na_o$ ON SODIUM PUMP-MEDIATED ATP:ADP EXCHANGE IN RESEALED GHOSTS

| | | | | ATP:ADP exchange rate | | |
|---|---|---|---|---|---|---|
| Experiment | [ATP] | [ADP] | [$Na_o$] | | Plus ouabain | Na pump component |
| | *μM* | *μM* | *mM* | | *μmol Pi/liter ghost/h* | |
| III-11 | 20.0 | 13.0 | 0 | 34.3 | 21.5 | 12.8 |
| | | | 5 | 27.7 | 19.4 | 8.3 |
| | | | 140 | 39.0 | 18.9 | 20.1 |
| III-20 | 20.0 | 24.6 | 0 | 92.7 | 39.1 | 53.6 |
| | | | 5 | 68.2 | 39.1 | 29.1 |
| | | | 140 | 108.6 | 32.5 | 76.1 |
| II-4 | 23.3 | 17.6 | 0 | 68.2 | 45.4 | 22.8 |
| | | | 5 | 56.8 | 37.5 | 19.3 |
| | | | 140 | 96.1 | 37.5 | 58.6 |
| II-9 | 3.2 | 5.6 | 0 | 12.3 | 6.7 | 5.6 |
| | | | 3 | 10.7 | 5.5 | 5.2 |
| | | | 140 | 24.2 | 6.1 | 18.1 |
| II-12 | 16.9 | 11.2 | 0 | 53.6 | 34.8 | 18.8 |
| | | | 3 | 42.5 | 37.4 | 5.1 |
| | | | 140 | 60.3 | 32.4 | 27.9 |
| I-16 | 22.5 | 20.2 | 0 | 81.6 | 43.2 | 38.4 |
| | | | 5 | 58.5 | 43.2 | 15.3 |
| | | | 140 | 117.7 | 52.8 | 54.9 |
| X-16 | 24.1 | 19.8 | 0 | 70.1 | 34.2 | 35.9 |
| | | | 3 | 56.4 | 32.0 | 24.4 |
| | | | 100 | 109.2 | 34.2 | 75.0 |

The biphasic effects of $Na_o$ on sodium pump-mediated ATP:ADP exchange in resealed ghosts. Ghost preparation and the initiation and measurement of ATP:ADP exchange rates were performed as described in Methods. In all of the experiments shown in Table II, [$Mg_i$] was 10 μM and [$Na_i$] varied between 5 and 15 mM.

### *Effects of $Na_i$*

The dependence of the rate of ATP:ADP exchange on $Na_i$ was measured in a series of experiments in which $Na_i$ was varied while $Na_o$ was held constant (100 mM). The rate of the ATP:ADP exchange reaction remained constant above 1.5 mM $Na_i$ (Fig. 4), and we saw no evidence of substantial inhibition at elevated $Na_i$ levels (see also Fig. 7).

*Effects of $K_o$*

If K ions are added to the external medium containing high levels of Na, inhibition of the ouabain-sensitive exchange rate is observed (Fig. 5*b*). The concentration of K ions giving half-maximal inhibition is ~1 mM. When experiments are performed at elevated K levels, there is an apparent partial inhibition of ouabain-sensitive ATP:ADP exchange (compare columns 7 and 6, Table III). The reason for this is shown in Table III, where it can be seen

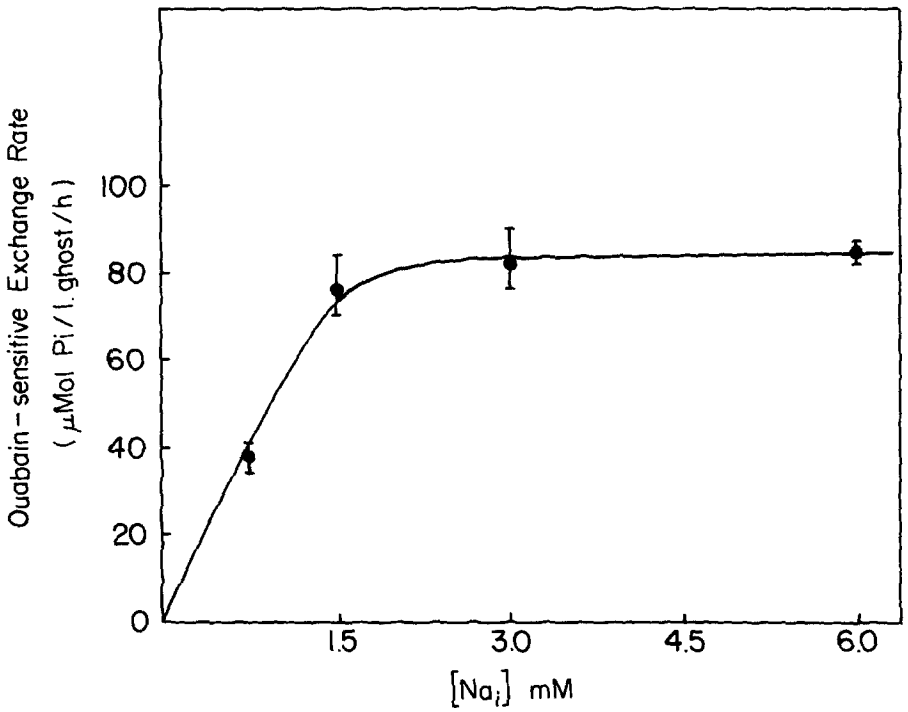



FIGURE 4. Dependence of the rate of ouabain-sensitive ATP:ADP exchange on $Na_i$. Resealed ghosts containing the indicated concentration of Na ions were suspended in 140 mM NaCl, 10 HEPES, pH 7.6, in the absence and presence of $10^{-4}$ M ouabain. The data shown are the difference in rates caused by the presence of ouabain in the external medium. The experimental points show the mean and range of duplicate determinations. In the experiment illustrated, the ghosts contained 5 mM KCl in addition to the NaCl indicated.

that the exchange rate in the presence of ouabain has been stimulated by high $K_o$. Evidently, as $K_o$ is increased, the ouabain-insensitive exchange rate is stimulated. The dependence on $[K]_o$ of these two effects can be seen in Fig. 5*a*. Whereas $K_o$ inhibits the ouabain-sensitive component of ATP:ADP exchange, the ouabain-insensitive component is stimulated. The stimulation by K of the ATP:ADP exchange reaction in the presence of ouabain and high Na concentrations can also be observed in experiments on porous red cell membranes,

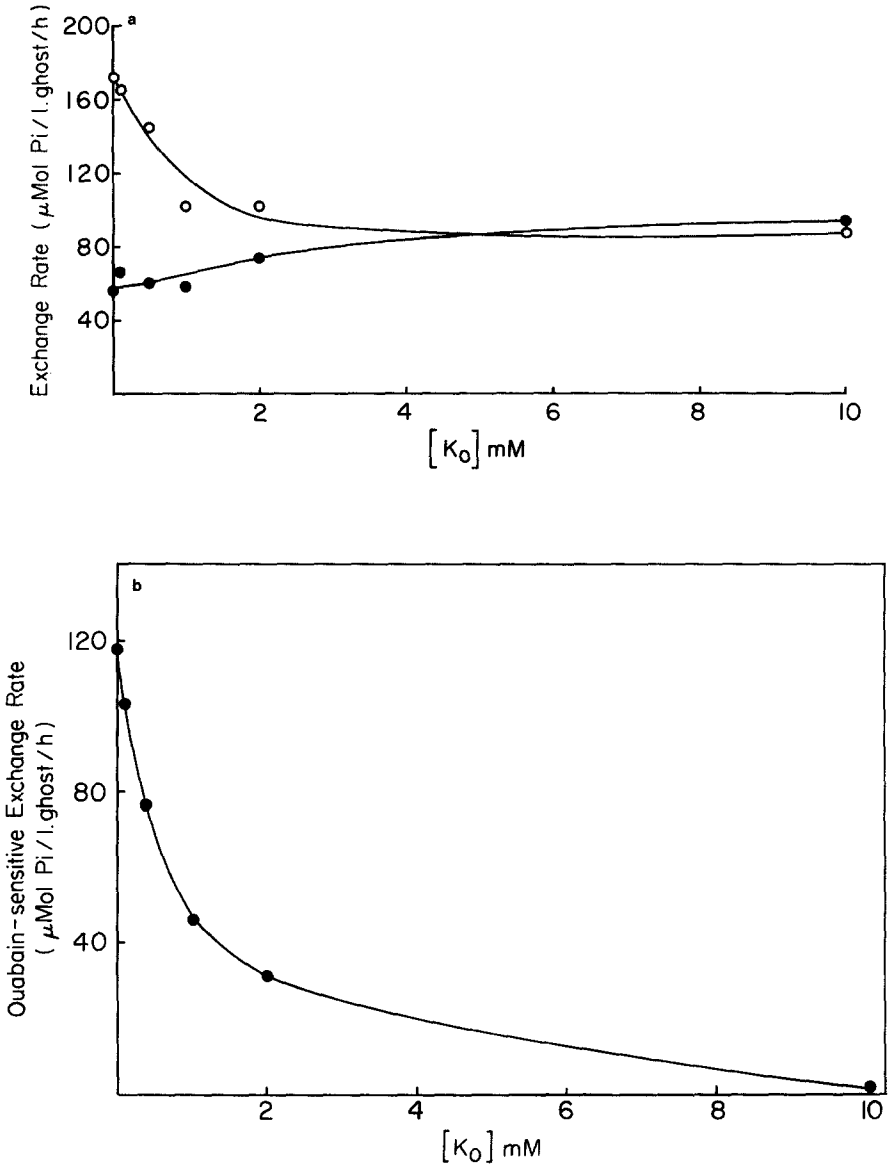



FIGURE 5. (*a*) Dependence of ATP:ADP exchange rate on $K_o$ in the presence and absence of ouabain. The ATP:ADP exchange rate was determined in a batch of ghosts suspended in 100 mM NaCl, 10 mM HEPES in the presence (●) and absence (○) of $10^{-4}$ M ouabain in the presence of the indicated [$K_o$]. (*b*) Inhibition of ouabain-sensitive ATP:ADP exchange by $K_o$. The rate of ATP:ADP exchange was determined in ghosts suspended in 100 mM NaCl, 10 mM HEPES containing the indicated [$K_o$] where [$K_o$Cl] + choline Cl = 40 mM. The differences in rates in the presence and absence of $10^{-4}$ M ouabain at each [$K_o$] are shown.

but not in experiments with partially purified Na,K-ATPase from canine renal medulla (J. H. Kaplan, M. D. Mone, and R. J. Hollis, unpublished observations). A possible source of the K-activated ouabain-insensitive exchange activity in red cell membranes is discussed below.

We have observed that unlike $K_o$, $Li_o$ does not stimulate the ouabain-insensitive ATP:ADP exchange component in resealed ghosts. In the presence of high $Na_o$, $Li_o$ inhibits the Na pump-mediated ATP:ADP exchange reaction but with a significantly lower affinity than $K_o$ (Fig. 6). The biphasic nature of the effect of $Li_o$ has not been further investigated. More detailed investigation would be required to establish the significance of the small amount of inhibition seen at low levels of $Li_o$, apparently acting at sites of higher affinity.

### *Effects of $K_i$*

In several experiments the effects of $K_i$ on the ATP:ADP exchange were examined. In the concentration range where $K_o$ inhibits the ouabain-sensitive

TABLE III

THE EFFECTS OF HIGH [$K_o$] ON ATP:ADP EXCHANGE IN RESEALED GHOSTS

| | | | | ATP:ADP exchange rate | | | |
|---|---|---|---|---|---|---|---|
| Experiment | [ATP] | [ADP] | [$Na_i$] | 100 mM $Na_o$ | 100 mM $Na_o$ + ouabain | 100 mM $Na_o$ + 20 mM $K_o$ | 100 mM Na + 20 mM $K_o$ + ouabain |
| | *μM* | *μM* | *mM* | *mol Pi/liter ghosts/h* | | | |
| X-16 | 24.1 | 19.8 | 3.6 | 109.2 | 34.2 | 66.9 | 66.9 |
| X-21 | 32.8 | 27.0 | 2.5 | 102.2 | 29.3 | 72.0 | 72.0 |
| X-29 | 33.5 | 25.4 | 3.6 | 72.0 | 32.5 | 53.3 | 48.6 |

The effects of high [$K_o$] on ATP:ADP exchange in resealed ghosts. Ghost preparation, ATP:ADP exchange, and other procedures in these experiments were performed as described in Methods. The data (from three representative experiments) show the rates of ATP:ADP exchange in resealed ghosts suspended in 100 mM $Na_o$ in the presence or absence of ouabain and 20 mM [$K_o$].

ATP:ADP exchange rate completely (see Fig. 5*b*), $K_i$ has only a small inhibitory effect, which is reduced or absent at high $Na_i$ concentrations (Fig. 7). The data presented in Fig. 7 also show that at high $Na_i$ concentrations there is no inhibition of the rate of ATP:ADP exchange. It is also evident in Fig. 7 that $K_i$ has no effect on the rate of ATP:ADP exchange in the presence of ouabain.

## DISCUSSION

The objective of the present studies was to characterize the sodium pump-mediated ATP:ADP exchange in resealed red cell ghosts so that the effects of Na and K ions could be unambiguously ascribed to inward- or outward-facing cation sites on the pump protein.

Two aspects of our experimental approach to the problem have potentially wider applications for other physiological studies, and in particular, sodium pump studies in intact-sided systems. The gel filtration procedure used in the

present work produces resealed red cell ghosts with minimal retention of cytoplasmic enzyme activities. The very high levels of adenylate kinase activity (~5 mol/liter cells/h) present in human red blood cells have been greatly reduced so that even in the absence of added inhibitor, the Na pump component of the ATP:ADP exchange reaction was measurable (see Table I). It is interesting that even though the gel procedure involves a substantial dilution of cytoplasm and an efficient washing procedure in situ, there are still significant amounts of Mg associated with the membranes. The presence of residual Mg associated with washed red cell membranes has been noted previously (see Flatman and Lew, 1980), and the levels involved (~10 μmol/

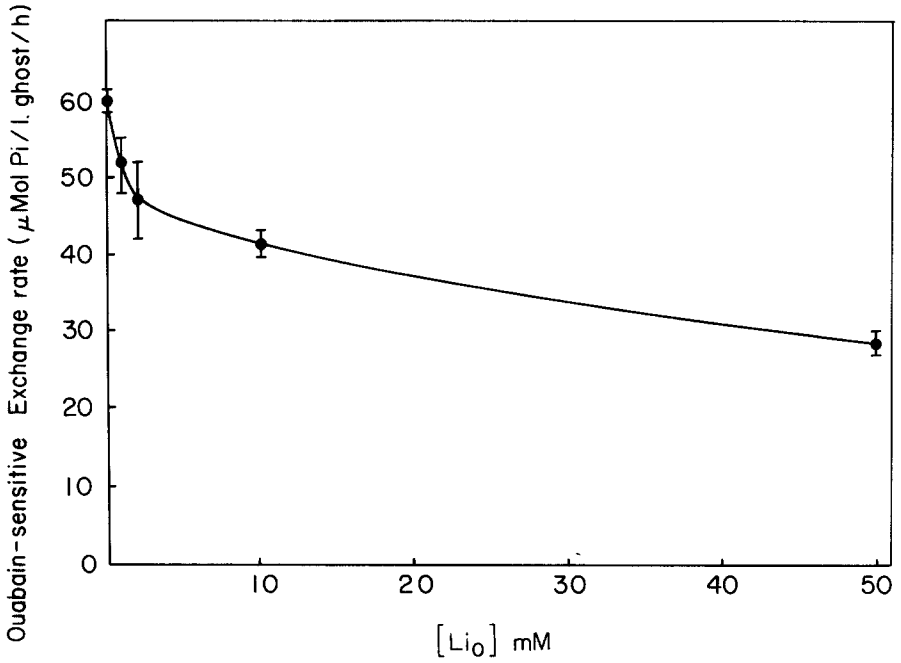



FIGURE 6. Inhibition of ouabain-sensitive ATP:ADP exchange by $Li_o$. The experimental procedure was the same as in Fig. 5 but here [$Li_oCl$] + choline Cl = 50 mM.

liter cells; Welt, 1964) are similar to those inferred to be present in the ghosts prepared by gel filtration. The gel filtration procedure makes possible the independent manipulation of ATP and ADP concentrations uncomplicated by the presence of kinase activities converting ADP to ATP. The use of caged ATP to initiate photochemically the intracellular exchange reaction enabled us to make initial rate measurements in a controlled manner, which would have been extremely difficult without this approach. Since ATP is used by a variety of endogenous enzyme activities, the ability to retain an intracellular supply of ATP in a protected form during the ghost preparation makes possible several types of experiment that otherwise would be less straightforward. The use of ATP-regenerating systems was not appropriate in the present

experiments, and in other types of studies on the sodium pump the associated accumulation of phosphate ions from an ATP-regenerating system might complicate the experiments. Essentially, the properties of caged ATP recommend its use when either (*a*) a stable source of ATP is required in ghosts or vesicles which themselves contain endogenous ATP-consuming enzymes, or (*b*) the rapid release of ATP and initiation of ATP-dependent processes in a closed compartment is desired.

*Effects of Na Ions*

Previous studies in partially purified microsomal preparations of the Na,K-ATPase have revealed a complex dependence of the rate of ATP:ADP

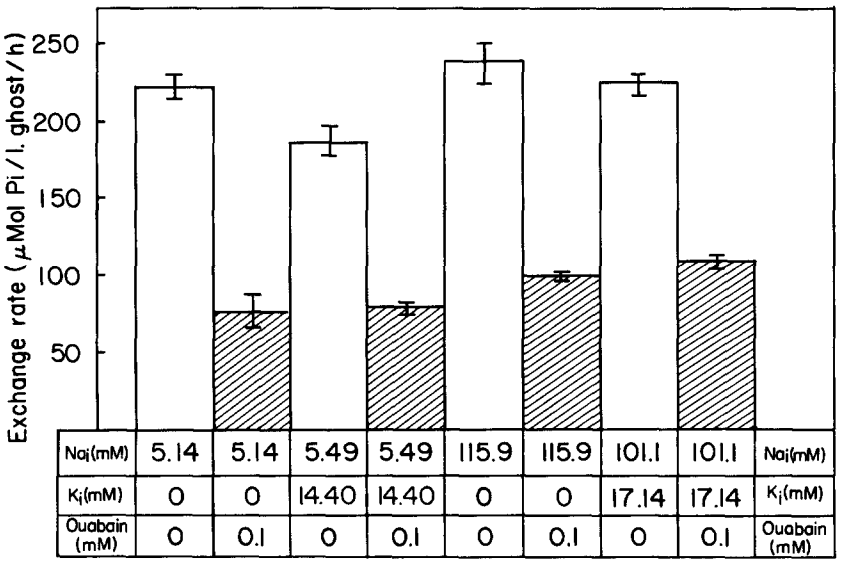



FIGURE 7. The effect of $K_i$ on the rate of ATP:ADP exchange. Resealed ghosts were prepared containing the indicated concentrations of Na or Na + K ions. The rate of ATP:ADP exchange was subsequently measured, as described in Methods, when the ghosts were suspended in 150 mM NaCl, 10 mM HEPES, pH 7.6, in the presence or absence of $10^{-4}$ M ouabain. The range of duplicate measurements of rate is indicated by the bars.

exchange on Na ion concentration (Wildes et al., 1973; Beauge and Glynn, 1979; Kaplan et al., 1981). The same complex pattern is also seen in studies using porous human red cell membranes (Kaplan and Hollis, 1980). In the range 0–5 mM Na, the ATP:ADP exchange rate is stimulated; between 5 and 15 mM Na, an inhibition is observed, followed by an activating effect of Na ions that saturates beyond the physiological range. In the present work we have shown that the sites for $Na_i$ have a high affinity for and are saturated by ~1.5 mM Na. This is in keeping with the high affinity for Na (<1 mM) in supporting phosphorylation of the enzyme (from various sources) by ATP (e.g., Post et al., 1965; Foster and Ahmed, 1976; Flashner and Robinson,

1979; but cf. Mardh and Post [1977], who obtained a value of 5–8 mM at 21°C). This suggests that the first activating limb of the curve relating Na ion concentration and ATP:ADP exchange rate in porous preparations is due to intracellular sites for Na. We had previously inferred this sidedness on the basis of studies on the effects of $Na_o$ on ATP:ADP exchange rates in resealed red cell ghosts (Kaplan and Hollis, 1980). Although the internal sites for Na ions in the present work are saturated at suitably low levels, it would be unwise to directly compare these values with those obtained from phosphorylation studies. In the present work a fairly complex multistep reaction is being considered; in phosphorylation studies, although the reaction is simpler, Na ions potentially have simultaneous access to sites on both sides of the membrane. Recent work has shown that $Na_o$ as well as $Na_i$, at 37°C, may affect phosphoenzyme levels (Blostein, 1979). Also, in one of the recent studies of phosphorylation, using a rapid mixing apparatus, an effect of Na at low concentrations (1–8 mM) has been observed on the level of phosphoenzyme as well as on phosphorylation rate (see Fig. 5 of Mardh and Post, 1977). It would be expected that increased [Na] should increase the initial rate of phosphorylation without affecting the maximum level of phosphoenzyme. The high affinity of intracellular sites for Na has previously been demonstrated by Karlish and Glynn (1974) in their studies of Na-ATPase activity in resealed red cell ghosts, and by Blostein (1979) using inside-out red cell membrane vesicles.

The present studies confirm our earlier observations indicating the existence of at least two classes of sites for Na ions at the extracellular surface of the Na pump; an inhibitory site of relatively high affinity (inhibition being observed in the range 0–5 mM $Na_o$) and a site that stimulates the reaction with a much lower affinity for Na ions. The mechanism by which increasing Na ions stimulate the exchange reaction can be interpreted on the basis of earlier work, which has shown that the proportion of total phosphoenzyme that is sensitive to ADP ($E_1P$) is increased by high concentrations of Na ions (Kuriki and Racker, 1976; Taniguchi and Post, 1975; Hara and Nakao, 1981). The way in which lower concentrations of extracellular Na ions inhibit the exchange reaction is less clear. In the absence of K ions the rate of decomposition of the phosphoenzyme is probably rate-limiting for the overall ATPase activity and the major portion of the phosphoenzyme (in the presence of low Na ions) is ADP insensitive ($E_2P$). This means that the ADP-insensitive phosphoenzyme is out of commission for the ATP:ADP exchange reaction until it has been dephosphorylated and returned to the ADP-sensitive form ($E_1P$) after rephosphorylation. Na ions at low concentration have been observed to decrease the rate of dephosphorylation of the phosphoenzyme (Beauge and Glynn, 1979), and this effect might account for the inhibition of ATP:ADP exchange by $Na_o$ in the range 0–5 mM seen in the present studies. If this interpretation is correct, we might expect to observe a relief by low concentrations of $K_o$ of the inhibition caused by low $Na_o$, since $K_o$ stimulates the breakdown rate of phosphoenzyme and low concentrations of $K_o$ have been reported to relieve the inhibition of Na-ATPase caused by low $Na_o$ concentrations (Beauge et al., 1979). We have attempted in a series of

preliminary experiments to test this prediction but were unable to detect a reproducible stimulation by $K_o$ of the rate of ATP:ADP exchange in the presence of 5 mM $Na_o$.

It should be borne in mind that the interpretations of the effects of Na ions on ATP:ADP exchange rates in resealed ghosts rely heavily on extrapolations from data obtained in studies of enzyme isolated from a variety of sources. Although it is clear that Na pump enzymes from different sources behave in a similar fashion, it is also clear that differences do exist. In particular, the proportion of phosphoenzyme that is ADP-sensitive under comparable experimental conditions seems to vary when enzymes from different sources are examined (Kuriki and Racker, 1976; Fukushima and Nakao, 1980; Hara and Nakao, 1981). The major difference seems to be in the effects of Na ions on the distribution of phosphoenzyme between ADP-sensitive and ADP-insensitive forms. Although increasing Na ions increase the former, the "affinity" for Na in influencing this distribution varies with the source of enzyme. The interpretation of the effects of Na ions on ATP:ADP exchange in resealed ghosts offered in the present work will be placed on a firmer basis when the Na dependence of the distribution of phosphoenzyme between the $E_1P$ and $E_2P$ forms in red blood cell Na pump protein has been obtained. It seems likely that the red cell enzyme in this regard more closely resembles the kidney enzyme than the brain enzyme. This can be inferred by the continual shallow increase in ATP:ADP exchange rate observed in the present work with $Na_o$ (up to 140 mM $Na_o$), which more closely resembles the Na dependence of $E_1P$ levels in kidney (Hara and Nakao, 1981) than brain (Fukushima and Nakao, 1980).

*Effects of K and Li Ions*

At high (100–140 mM) $Na_o$, K ions in the external medium clearly inhibit the exchange reaction. The $K_{1/2}$ for the effect of $K_o$ is ~1 mM in the presence of 100 mM $Na_o$. This estimate agrees with the affinity of the enzyme for K sites in activating Na,K-ATPase in red blood cell membranes (Post et al., 1960). Since $Li_o$ did not stimulate the ouabain-insensitive component, the effect of $Li_o$ was studied over a wider range of concentrations. It is apparent that $Li_o$, like $K_o$, inhibits the ATP:ADP exchange rate. We have not systematically investigated the basis of the apparent biphasic inhibitory action of $Li_o$ on the ATP:ADP exchange reaction (see Fig. 6). The rate of ATP:ADP exchange is reduced to one-half of its value in the absence of $Li_o$ by 50 mM $Li_o$. Similar effects of $K_o$ and $Li_o$ in red cells have been observed before, and the poorer affinity for $Li_o$ is well documented (Sachs and Welt, 1967; McConaghey and Maizels, 1962).

Internal K ions were without significant inhibitory effect on the ATP:ADP exchange reaction in the range of concentrations tested.

*Effects of Mg Ions*

The affinity for Mg in supporting ATP:ADP exchange activity in resealed ghosts is high. Because of the low concentrations of nucelotides and Mg and the complex composition of the intracellular compartment, we have not been

able to obtain a precise value for the $K_{1/2}$ for free Mg. However, it is apparent that only low concentrations (micromolar) of Mg ions are sufficient to support the exchange activity and that increased Mg levels inhibit the exchange rate.

The inhibition could be due to Mg ions or Mg-nucleotide complexes. In studies on the purified Na,K-ATPase enzyme where the effects of a range of Mg concentrations on the ATP:ADP exchange were investigated, both inhibitory and activating effects were observed that could not be unambiguously assigned to free Mg ions or Mg-nucleotide complexes (Robinson, 1976; Beauge and Glynn, 1979). In recent work we have found that the inhibition of Na,ATPase activity caused by the addition of K, in the presence of micromolar levels of ATP, is not observed if Mg is also present in the micromolar concentration range. The inhibition by K ions is dependent upon the presence of elevated Mg concentrations. Our interpretation of these observations is that Mg is involved in stabilizing $E_2$ or $E_2K$ forms of the enzyme (M. D. Mone, L. J. Kenney, and J. H. Kaplan, manuscript in preparation). The stabilization of $E_2$ forms of the enzyme by Mg ions might account for the inhibition of ATP:ADP exchange activity observed in the present work and noted previously by others in studies using partially purified enzyme preparations (Robinson, 1976, 1977; Banerjee and Wong, 1972).

*Ouabain-insensitive ATP:ADP Exchange*

The activity observed in the presence of $10^{-4}$ M ouabain can reasonably be attributed to activities other than the Na pump. The inhibitory effects of K on the ouabain-sensitive ATP:ADP exchange reaction were unaffected by preincubation of the ghosts in the presence of ouabain (with or without $K_o$), so we are confident that the effects we observe are not caused by any interaction between K and ouabain that would cause a slow (and limiting) rate of ouabain binding. The total ouabain-insensitive activity is probably composed of several components, but several phenomena lead us to speculate that a contributing activity may be the Ca,Mg-ATPase or Ca pump of the red cell membrane. In summary, the activity is stimulated by $K_o$ but not by $K_i$, is present in red cell membranes, but not in purified Na,K-ATPase from canine renal medulla, and is not stimulated by $Li_o$.

The asymmetric effects of K ions imply that the protein mediating the activity has sided affinities for cations across the membrane and that it penetrates the membrane (since the exchange reaction occurs in the intracellular compartment) with nucleotide sites at the inner surface. These are clearly minimal requirements for a cation ATPase transport system. The stimulation of Ca,Mg-ATPase in red cell membranes by K has been observed and, significantly, Li ions are without effect (Bond and Green, 1971; Larocca et al., 1981). The stimulation of ouabain-insensitive ATP:ADP exchange activity by K ions is observed in experiments using washed porous red cell membranes, but is not observed in experiments using purified Na,K-ATPase enzyme from renal outer medulla. This suggests to us that the stimulation by K is neither an artifact of the procedure used to prepare resealed ghosts nor a property of the sodium pump when ATP:ADP exchange activity is measured. To establish

whether the Ca pump is involved, it will be necessary to repeat the current observations in a situation where nucleotide levels are significantly elevated and Ca and Mg levels can be independently altered. The usual diagnostic tool for Ca pump activity, inhibition by $La^{3+}$ ions, was not observed on K-stimulated ouabain-insensitive ATP:ADP exchange activity in resealed red cell ghosts (J. H. Kaplan, unpublished data). The observations of Schatzmann and Burgin (1978) indicate that although $La^{3+}$ blocks the Ca,Mg-ATPase activity, it might not be expected to inhibit the ATP:ADP exchange reaction mediated by the enzyme (see Fig. 12 of Schatzmann and Burgin, 1978), since it blocks breakdown of phosphoenzyme and enhances its ADP sensitivity. The actions of $La^{3+}$ on the Ca,Mg-ATPase appear to be similar to those of oligomycin or *N*-ethylmaleimide on the Na pump.

*The Relationship of Na,ATPase to ATP:ADP Exchange*

These two partial reactions are both activated by Na ions in the absence of K ions and show similar complex Na-activation curves (Mardh and Post, 1977; Glynn and Karlish, 1976; Beauge and Glynn, 1979; Kaplan et al., 1981; Kaplan and Hollis, 1980; Kaplan, this work). The corresponding transport modes have been observed in red blood cells; they are uncoupled Na efflux for Na,ATPase and Na:Na exchange for ATP:ADP exchange. However, it is not clear that previous observations satisfactorily account for the relationship between these Na-dependent activities. Both enzymatic activities are stimulated by external Na at sites with low affinities (Glynn and Karlish, 1976; Kaplan and Hollis, 1980). However, for Na,ATPase it is difficult to see how the observed change in spontaneous breakdown rate of phosphoenzyme when Na ions are increased from ~20 $\mu$M to 150 mM during dephosphorylation can account for the observed threefold increase in Na,ATPase activity over the same range of Na ion concentrations (Beauge and Glynn, 1979, Fig. 8, cf. Fig. 2) if dephosphorylation is assumed to be the slowest step in the Na,ATPase reaction. By 150 mM Na, the dephosphorlyation rate is only ~40% of its value at 20 $\mu$M Na. It has also been suggested, to account for the effect of high concentrations of Na ions in stimulating ATP:ADP exchange, that the equilibrium $E_1P \rightleftharpoons E_2P$ is driven by high Na ions to the left-hand side, i.e., in the direction of more ADP-sensitive phosphoenzyme (Beauge and Glynn, 1979). If $E_2P$ is involved in the Na,ATPase activity then we must assume that the effects of high Na ions in driving $E_1P \rightleftharpoons E_2P$ in favor of $E_1P$ are compensated for by increasing the rate of $E_2P$ breakdown, or perhaps both $E_1P$ and $E_2P$ breakdown contribute to Na,ATPase activity. Uncertainties in interpretation also exist in accounting for the characteristics of the transport reactions. The effects of high external Na ions on Na influx or Na efflux in ghosts containing low ATP but without ADP have not yet been reported, so that although Na,ATPase activity is stimulated by high $Na_o$ (Glynn and Karlish, 1976), we do not know if uncoupled Na efflux is also stimulated. Previous studies using human red cells or ghosts containing ATP and ADP do not enable us to distinguish between Na:Na exchange and uncoupled Na efflux contributions to the total transport. When only Na efflux is measured in the presence of

$Na_o$, uncoupled Na efflux and Na:Na exchange could contribute to the movements; when only Na influx is measured, uncoupled Na efflux would not be detectable.

The relationship between ATP:ADP exchange and Na,ATPase at moderate to high Na ion concentrations (25–150 mM) points to another area of uncertainty, viz., the number of classes of Na sites at the outside surface of the pump protein. If $Na_o$ stimulates Na,ATPase by enhancing $E_2P$ breakdown and simultaneously stimulates ATP:ADP exchange by increasing the $E_1P/E_2P$ ratio, do two sets of sites exist? If the latter are normally unloading sites for Na, then what are the former? One possibility is that stimulation of Na,ATPase occurs via $Na_o$ acting at what are normally $K_o$ sites. An alternative explanation is that the same sites are involved in both effects and that at these sites Na has a "Na-like effect" (stimulating ATP:ADP exchange) and also a "K-like effect" (stimulating Na ATPase).

Garay and Garrahan (1973) have reported that as $K_i$ is progressively replaced by $Na_i$, the rate of Na:Na exchange falls. They suggested that this was due to a stimulation of Na:Na exchange by $K_i$ (Garay and Garrahan, 1973). Recent studies by Sachs (1981) have shown that intracellular K ions affect the Na pump via predominantly metabolic effects resulting in changes of nucleotide levels. In the present studies, metabolic effects are absent in the ghosts and simple saturation effects of internal Na ions are observed.

### *The Relation between ATP:ADP Exchange and Na:Na Exchange*

To what extent do the results presented here support the idea that ATP:ADP exchange occurs while the pump is carrying out a 1:1 exchange of intracellular and extracellular Na ions? The effects of $Na_o$ clearly show the same biphasic curve as that seen when the effect of $Na_o$ on ouabain-sensitive Na efflux is studied in human red cells (Garrahan and Glynn, 1967*a*; Garay and Garrahan, 1973). It seems reasonable to conclude that the same sites are involved in Na uptake and stimulation of ATP:ADP exchange. The effects of $K_o$ also support this conclusion; $K_o$ inhibits ATP:ADP exchange with a suitable affinity compared with the inhibition by $K_o$ of ouabain-sensitive Na uptake in red cells (Garrahan and Glynn, 1967*b*), so presumably the external K sites that inhibit Na:Na exchange (and stimulate Na:K exchange) also inhibit ATP:ADP exchange. Garay and Garrahan (1973) have shown that in red cells with low $K_i$, $Na_i$ activates Na:K exchange and Na:Na exchange with a rather high affinity, $K_{1/2}$ of ~0.2 mM. In our studies $Na_i$ saturates at low concentrations.

It is apparent that many characteristics of the ATP:ADP exchange reaction also pertain to the Na:Na exchange transport process. However, observations made in several studies suggest that these two processes are not tightly coupled with a constant stoichiometric relationship between their rates. In the presence of oligomycin, the transport process, Na:Na exchange, is inhibited (Garrahan and Glynn, 1967*c*; Sachs, 1980), whereas the ATP:ADP exchange is not inhibited and is perhaps stimulated slightly (Blostein, 1968, 1970). In experiments using resealed red cell ghosts in the absence of $Na_o$, where Na:Na

exchange transport cannot take place, significant rates of ATP:ADP exchange are observed (Kaplan and Hollis, 1980; Kaplan, this work). There exist then situations where ATP:ADP exchange can take place without Na:Na exchange and one form of Na:Na exchange takes place in the absence of ADP (see Lee and Blostein, 1980). In order to define the biochemical events associated with ion movements through the sodium pump apparatus, more detailed studies are required where ion transport and biochemical events are measured simultaneously.

### *Conclusion*

Photolysis of caged ATP inside red cell ghosts has enabled the measurement of ATP:ADP exchange mediated by the Na pump. The asymmetric characteristics of the Na:Na exchange transport are reflected in similar asymmetries in the ATP:ADP exchange reaction. It is likely that the cation binding sites that mediate these processes are identical. If Na entry is coupled to phosphorylation of ADP by the pump phosphoprotein it remains to be seen whether the processes are tightly coupled with fixed stoichiometries.

I am grateful to R. J. Hollis and Linda J. Kenney for excellent technical assistance and to Dr. M. D. Mone for assistance in performing the experiments on purified enzyme. I would like to thank Dr. J. Froehlich, National Institute on Aging, Baltimore, for a helpful discussion of the results of his studies on the reaction rates of phosphoenzyme intermediates of Na,K-ATPase. This work was supported by National Institutes of Health grant HL 28457. J. H. Kaplan is a recipient of Research Career Development Award K04-HL-01092-01.

*Received for publication 4 February 1982 and in revised form 7 June 1982.*

## REFERENCES

BANERJEE, S. P., and S. M. E. WONG. 1972. Effect of potassium on sodium-dependent adenosine triphosphate exchange activity in kidney microsomes. *J. Biol. Chem.* **247**:5409–5413.

BEAUGE, L. A., and I. M. GLYNN. 1979. Sodium ions, acting at high-affinity extracellular sites, inhibit sodium-ATPase activity of the sodium pump by slowing dephosphorylation. *J. Physiol. (Lond.)*. **289**:17–31.

BEAUGE, L. A., I. M. GLYNN, and D. E. RICHARDS. 1979. The biphasic action of external K ions on Na,K-ATPase. *J. Physiol. (Lond.)*. **295**:88p.

BLOSTEIN, R. 1968. Relationships between erythrocyte membrane phosphorylation and adenosine triphosphate hydrolysis. *J. Biol. Chem.* **243**:1957–1965.

BLOSTEIN, R. 1970. Sodium-activated adenosine triphosphatase activity of the erythrocyte membrane. *J. Biol. Chem.* **245**:270–275.

BLOSTEIN, R. 1979. Side-specific effects of sodium on (Na,K)-ATPase. *J. Biol. Chem.* **254**:6673–6677.

BOND, G. H., and J. W. GREEN. 1971. Effects of monovalent cations on the ($Mg^{2+}$ + $Ca^{2+}$)-dependent ATPase of the red cell membranes. *Biochim. Biophys. Acta.* **241**:393–398.

CALDWELL, P. C., A. L. HODGKIN, R. D. KEYNES, and T. I. SHAW. 1960. Partial inhibition of the active transport of cations in the giant axons of *Loligo*. *J. Physiol. (Lond.)*. **152**:591–600.

CAVIERES, J. D., and I. M. GLYNN. 1979. Sodium-sodium exchange through the sodium pump: the roles of ATP and ADP. *J. Physiol. (Lond.)*. **297**:637–645.

DEWEER, P. 1970. Effects of intracellular adenosine $-5^1$-diphosphate and orthophosphate on

the sensitivity of sodium efflux from squid axon to external sodium and potassium. *J. Gen. Physiol.* **56**:583–620.

FAHN, S., G. J. KOVAL, and R. W. ALBERS. Sodium-potassium-activated adenosine triphosphatase of electrophorus electric organ. I. An associated sodium-activated transphosphorylation. *J. Biol. Chem.* **241**:1882–1889.

FLASHNER, M., and J. D. ROBINSON. 1979. Effects of $Mg^{2+}$ on activation of the (Na + K)-dependent ATPase by $Na^+$. *Arch. Biochem. Biophys.* **192**:584–591.

FLATMAN, P. W., and V. L. LEW. 1980. Magnesium buffering in intact red blood cells measured using the ionophore A23187. *J. Physiol. (Lond.)*. **305**:13–30.

FOSTER, D., and K. AHMED. 1976. Na-dependent phosphorylation of the rat brain (Na + K)-ATPase possible non-equivalent activation sites for $Na^+$. *Biochim. Biophys. Acta.* **429**:258–273.

FUKUSHIMA, Y., and M. NAKAO. 1980. Changes in affinity of $Na^+$- and $K^+$-transport ATPase for divalent cations during its reaction sequence. *J. Biol. Chem.* **255**:7813–7819.

GARAY, R. P., and P. J. GARRAHAN. 1973. The interaction of sodium and potassium with the sodium pump in red cells. *J. Physiol. (Lond.)*. **231**:297–325.

GARRAHAN, P. J., and I. M. GLYNN. 1967*a*. The behaviour of the sodium pump in red cells in the absence of external potassium. *J. Physiol. (Lond.)*. **192**:159–174.

GARRAHAN, P. J., and I. M. GLYNN. 1967*b*. Factors affecting the relative magnitudes of the sodium:potassium and sodium:sodium exchanges catalyzed by the sodium pump. *J. Physiol. (Lond.)*. **192**:189–216.

GARRAHAN, P. J., and I. M. GLYNN. 1967*c*. The stoichiometry of the sodium pump. *J. Physiol. (Lond.)*. **192**:217–235.

GLYNN, I. M., and J. F. HOFFMAN. 1971. Nucleotide requirements for sodium:sodium exchange catalyzed by the sodium pump in human red cells. *J. Physiol. (Lond.)*. **218**:239–256.

GLYNN, I. M., and S. J. D. KARLISH. 1976. ATP hydrolysis associated with an uncoupled sodium flux through the sodium pump: evidence for allosteric effects of intracellular ATP and extracellular sodium. *J. Physiol. (Lond.)*. **256**:465–496.

HARA, Y., and M. NAKAO. 1981. Sodium ion discharge from pig kidney Na,K-ATPase Na dependency of the $E_1P \rightleftharpoons E_2P$ equilibrium in absence of KCl. *J. Biochem. (Tokyo)*. **90**:923–931.

KAPLAN, J. H. 1980. The use of caged-ATP to measure ATP:ADP exchange mediated by the Na pump in resealed red cell ghosts. *J. Gen. Physiol.* **76**:31*a*. (Abstr.).

KAPLAN, J. H., B. FORBUSH III, and J. F. HOFFMAN. 1978. Rapid photolytic release of adenosine-5'-triphosphate from a protected analogue: utilization by the Na:K pump of human red blood cell ghosts. *Biochemistry.* **17**:1929–1935.

KAPLAN, J. H., and R. J. HOLLIS. 1980. External Na dependence of ouabain-sensitive ATP:ADP exchange initiated by photolysis of intracellular caged-ATP in human red cell ghosts. *Nature (Lond.)*. **288**:587–589.

KAPLAN, J. H., R. J. HOLLIS, and M. D. MONE. 1981. The regulation of Na Pump-mediated ATP:ADP exchange by extracellular Na ions. *In Advances in Physiological Science.* S. R. Hollan, G. Gardos, B. Sarkadi, editors. Hungarian Academy, Budapest. **6**:293–298.

KARLISH, S. J. D., and I. M. GLYNN. 1974. An uncoupled efflux of sodium ions from human red cells, probably associated with Na-dependent ATPase activity. *Ann. N. Y. Acad. Sci.* **242**:461–470.

KENNEDY, B. G., and P. DE WEER. 1976. Strophanthidin-sensitive sodium fluxes in metabolically poisoned frog skeletal muscle *J. Gen. Physiol.* **68**:405–420.

KEYNES, R. D., and R. A. STEINHARDT. 1968. The components of the sodium efflux in frog muscle. *J. Physiol. (Lond.)*. **198**:581–600.

KIMMICH, G. A., J. RANDLES, and J. S. BRAND. 1975. Assay of picomole amounts of ATP, ADP and AMP using the luciferase enzyme system. *Anal. Biochem.* **69:**87–205.

KURIKI, U., and E. RACKER. 1976. Inhibition of (Na + K)-ATPase and its partial reactions by Quercetin. *Biochemistry.* **15:**4951–4956.

LAROCCA, J. N., A. F. REGA, and P. J. GARRAHAN. 1981. Phosphorylation and dephosphorylation of the $Ca^{2+}$ pump of human red cells in the presence of monovalent cations. *Biochim. Biophys. Acta.* **645:**10–16.

LEE, K. H., and R. BLOSTEIN. 1980. Red cell sodium fluxes catalysed by the sodium pump in the absence of $K^+$ and ADP. *Nature (Lond.).* **285:**338–339.

LEPKE, S., and H. PASSOW. 1972. The effect of pH at hemolysis on the reconstitution of low cation permeability in human erythrocyte ghosts. *Biochim. Biophys. Acta.* **255:**696–702.

MARDH, S., and R. L. POST. 1977. Phosphorylation from adenosine triphosphate of sodium and potassium-activated adenosine triphosphatase. *J. Biol. Chem.* **252:**633–638.

MCCONAGHEY, P. D., and M. MAIZELS. 1962. Cation exchanges of lactose-treated human red cells. *J. Physiol. (Lond.).* **162:**485–509.

MCKAY, H. A. C. 1943. Kinetics of some exchange reactions of the type RI + I* $\rightleftharpoons$ RI* + I in alcoholic solution. *J. Am. Chem. Soc.* **65:**702–706.

POST, R. L., C. R. MERRITT, C. R. KINSOLVING, and C. D. ALBRIGHT. 1960. Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocytes. *J. Biol. Chem.* **235:**1796–1802.

POST, R. L., A. K. SEN, and A. S. ROSENTHAL. 1965. A phosphorylated intermediate in adenosine triphosphate-dependent sodium and potassium transport across kidney membranes. *J. Biol. Chem.* **240:**1437–1445.

ROBINSON, J. D. 1976. The (Na + K)-dependent ATPase. Mode of inhibition of ATP:ADP exchange activity by $MgCl_2$. *Biochim. Biophys. Acta.* **440:**711–722.

ROBINSON, J. D. 1977. $K^+$ stimulation of ATP:ADP exchange catalysed by the (Na + K)-dependent ATPase. *Biochim. Biophys. Acta.* **484:**161–168.

SACHS, J. R. 1970. Sodium movements in the human red blood cell. *J. Gen. Physiol.* **56:**322–341.

SACHS, J. R. 1980. The order of release of sodium and addition of potassium in the sodium-potassium pump reaction mechanism. *J. Physiol. (Lond.).* **302:**219–240.

SACHS, J. R. 1981. Internal potassium stimulates the sodium potassium pump by increasing cell ATP concentration. *J. Physiol. (Lond.).* **319:**515–528.

SACHS, J. R., and L. G. WELT. 1967. The concentration dependence of active potassium transport in the human red blood cell. *J. Clin. Invest.* **46:**65–76.

SCHATZMANN, H. J., and H. BURGIN. 1978. Calcium in human red blood cells. *Ann. N. Y. Acad. Sci.* **302:**125–146.

TANIGUCHI, K., and R. L. POST. 1975. Synthesis of adenosine triphosphate and exchange between inorganic phosphate and adenosine triphosphatase. *J. Biol. Chem.* **250:**3010–3018.

WELT, L. G. 1964. Experimental magnesium depletion. *Yale J. Biol. Med.* **36:**325–349.

WILDES, R. A., J. J. EVANS, and J. CHIU. 1973. Effects of cations on the adenosine diphosphate-adenosine triphosphate exchange reaction catalyzed by rat brain microsomes. *Biochim. Biophys. Acta.* **307:**162–168.

WOOD, P. G. 1982. Does ternary complex formation control the state of the cation barrier in the resealed human red blood cell ghost? *Protides Biol. Fluids Proc. Colloq.* **29:**283–286.

YAMAGUCHI, M., and Y. TONOMURA. 1977. Kinetic studies on the ATP:ADP exchange reaction catalyzed by $Na^+$, $K^+$-dependent ATPase. *J. Biochem. (Tokyo).* **81:**249–260.