Interaction of External Alkali Metal Ions with the Na-K Pump of Human Erythrocytes

A Comparison of Their Effects on Activation of the Pump and on the Rate of Ouabain Binding

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ABSTRACT   The effects of external alkali metal ions on the rate of ouabain binding and on the rate of the Na-K pump were examined in human red blood cells. In Na-containing solutions, K, Cs, and Li decreased the rate of ouabain binding. For K and Cs, the kinetics of this effect were similar to those for their activation of the pump. In Na-free (choline-substituted) solutions the rate of ouabain binding was decreased by K whereas it was promoted by Cs and Li. External Na increased the rate of ouabain binding whether or not external K was present, and the kinetics of this effect were not the same as those for inhibition of the pump by Na. These findings are interpreted to mean that not only do the cations affect ouabain binding at the external loading sites on the pump from which ions are translocated inward, but that there are additional sites on the external aspect of the pump at which cations can promote ouabain binding, and that these sites can be occupied by Li, Na, and Cs. It is postulated that these latter sites are those from which Na is discharged after outward translocation by the pump.

The inhibitory action of cardioactive steroids on the Na-K pump in human erythrocytes has been a subject of study by numerous laboratories in the hopes of acquiring a better understanding of the mechanism of the pump's action (Glynn, 1957; Hoffman, 1966; Beaugé and Adragna, 1971; Gardner and Conlon, 1972, Sachs, 1974; Gardner and Frantz, 1974; Bodemann and Hoffman, 1976 *a*, *b* and *c*; Hobbs and Dunham, 1976). It is known that these steroids act on the outside of the membrane (Hoffman, 1966; Perrone and Blostein, 1973), and that the ionic composition of the external medium affects the rates at which the binding of the steroid and the inhibition of the pump occur (Hoffman, 1966;

Beaugé and Adragna, 1971; Gardner and Conlon, 1972; Sachs, 1974; Gardner and Frantz, 1974). One experimental approach to this problem has been to study the effects of monovalent cations on the steroid binding in comparison with the effects of these same ions on the pump rate. For example, Hoffman (1966), noting that external K and Cs both activate the Na-K pump, but that only K will antagonize digoxin binding to the pump, concluded that the monovalent cation sites activating the pump and those influencing cardioactive steroid binding must be different.

Studies of this kind, however, have led to contradictory results. Gardner and Frantz (1974) studied the binding of tritiated ouabain to human red cells and proposed a model in which the external aspect of the pump had separate binding sites for monovalent cations, divalent cations, and steroids. In a somewhat different model, Sachs (1974) proposed that the external loading sites are identical to the sites at which monovalent cations modify binding of strophanthidin to the pump. If it can be assumed that ouabain and strophanthidin bind to the same site and by a similar mechanism (Sachs, 1974), then at least two disparities arise: (a) in the number of monovalent cation sites involved in the regulation of steroid binding; and (b) in their relationship to the loading sites from which inward translocation by the pump takes place. For this reason a study of the effects of various alkali metal ions on both activation of the pump and binding of ouabain to the pump was undertaken. A preliminary report of some of these results has been presented (Hobbs and Dunham, 1975).

MATERIALS AND METHODS

Solutions and Chemicals

Standard incubation media for both influx and efflux experiments contained the following: 150 mM NaCl; 5 mM glucose; and 10 mM Tris·HCl, pH 7.45 (adjusted at room temperature). Where specified, KCl, Choline chloride, or LiCl were substituted in equimolar amounts for Na. Osmolarity of solutions was checked with an Advanced osmometer (Advanced Instruments, Inc., Needham, Mass.) and adjusted to 300 ideal mosmol/kg H_2O. (In isotonic cesium solutions this necessitated a CsCl concentration of 158 mM rather than 150 mM.) Ouabain was added to the experimental solutions to a concentration of 10^{-4} M or less as desired.

Choline chloride was obtained from Hoffman Taff, Inc. (now Chemical Division of Syntex Agri-Business Inc., Springfield, Mo.) and was not further purified. CsCl was Specpure (Johnson Matthey Chemicals, Ltd., London, England) or certified grade (Fisher Scientific Co., Pittsburgh, Pa.). Nystatin was obtained as Mycostatin (E. R. Squibb & Sons, Inc., Princeton, N.J.). [137]Cs, [42]K, and [24]Na were obtained as chlorides (International Chemical and Nuclear Corp., Isotope and Nuclear Div., Irvine, Calif.). All other chemicals used were standard analytical grade reagents.

Experimental Procedures

Blood from healthy human donors was drawn into heparinized syringes. The red cells were washed at 4°C three times by centrifugation, aspiration, and resuspension in the NaCl-Tris-Glucose solution (Na-T-G).

Unidirectional Fluxes

For unidirectional influxes, the procedure was similar to that of Sachs et al. (1974). Cells were suspended at 5–10% hematocrit in solutions containing ^{42}K and ^{137}Cs, and incubated at 37°C in a shaker bath for 0.5–1 hr. The reaction was stopped by adding ice-cold choline-Tris-glucose (Ch-T-G) or 115 mM isotonic $MgCl_2$; the cells were then washed three times at 4°C by centrifugation and resuspension in the isotonic $MgCl_2$. Radioactivities of hemolysates and of dilutions of the media were determined with an autogamma counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Fluxes were expressed as millimole per liter of cells per hour after determination of the volumes of cells in each hemolysate from their hemoglobin concentrations.

For unidirectional effluxes, the procedure was that described by Dunham and Hoffman (1971). The cells were preloaded with ^{24}Na at 37°C, then washed and incubated at 2% hematocrit at 37°C. At the times desired, samples of the suspension were taken, the cells were spun down, and the radioactivity of a volume of the supernate was determined.

Determination of Ouabain Binding

To determine rate constants for ouabain binding, k_b, cells were preincubated at 5–10% hematocrit at 37°C with low concentrations (e.g., 10^{-8} M) of ouabain in media with various cation compositions. At desired times, aliquots of the cell suspension were removed, the reaction with ouabain was stopped by sixfold dilution of the suspension with ice-cold Ch-T-G, and the cells were washed free of unbound ouabain by three centrifugations and resuspensions. The remaining, or uninhibited, pump flux was then measured (^{42}K or ^{137}Cs influx, or ^{24}Na efflux). All fluxes were corrected for the ouabain-insensitive flux, measured in 10^{-4} M ouabain. Rate constants for ouabain binding were obtained from the equation:

$$k_b = \ln \left[_tM^P / _aM^P \right] \cdot t^{-1},$$

where $_tM^P$ is the ouabain-sensitive flux remaining after the preincubation in ouabain for time t and $_aM^P$ is the ouabain-sensitive flux before exposure to ouabain. (To clarify the notation: when measuring K influx, $_a^iM_K^P$ and $_t^iM_K^P$ are the ouabain-sensitive fluxes (a) before preincubation with ouabain, time a, and (b) after time t, respectively.)

An effect occasionally seen was an apparent transient stimulation of the Na-K pump by low concentrations of ouabain. After addition of 10^{-8} or 10^{-7} M ouabain to a cell suspension, measurements of $^iM_K^P$ taken during the first several minutes were higher than those of control cells, cells which had not been exposed to ouabain. The maximum early stimulation was ∼ 5%. (In addition, the time-course for ouabain binding consistently extrapolated back to a value at zero time which was slightly higher than that for the control or untreated cells, but this alone could be interpreted as a delay in the onset of binding.) Although it may seem contradictory that low concentrations of ouabain would both stimulate and inhibit the pump, this phenomenon has been observed before. Stimulation of Na,K-ATPase from cardiac muscle cells by low concentrations of a cardiotonic steroid was first reported by Repke (1963). This kind of stimulation has been reported a number of times since for cardiac muscle preparations, not only the ATPase but also intact cells (Peters et al., 1974; Cohen et al., 1976; see Lee and Klaus, 1971, for other references). Similar observations have been made for Na,K-ATPase from rabbit brain and chicken kidney (Palmer et al., 1966) and for active transport in squid axons (Baker and Manil, 1968; Baker and Willis, 1972).

To reduce the importance of stimulation in its effect on the results, ouabain

concentrations and incubation times were chosen which resulted in inhibition between 45% and 90%, at which level of inhibition the rate constants were the most reliable; furthermore several time points were measured for each condition.

Alteration of Intracellular Cation Composition

Internal cation composition of the red cells was varied by using a modification of the nystatin method of Cass and Dalmark (1973). Cells were made with high intracellular Na concentrations, $[Na]_c$, and low intracellular K, $[K]_c$, so that $[Na]_c$ would not be rate-limiting for the activity of the pump and $[K]_c$ would not contribute significantly to $[K]_o$. Cells were incubated for 20–40 min at 0–5°C in Na-T-G containing 20 mM sucrose and 50 $\mu g/ml$ nystatin. Stock solutions of nystatin (5 mg/ml in methanol) were made fresh daily. If a particularly high $[Na]_c/[K]_c$ ratio was desired, the solution was changed several times. To remove the nystatin, cells were then washed three times in Na-T-G plus sucrose (20 mM) and then three times in either Na-T-G or Ch-T-G.

In a few experiments, concentrations of internal cations were altered using p-chloromercuribenzene sulfonate and dithiothreitol (PCMBS and DTT) as described by Sachs (1974), a modification of the method of Garrahan and Rega (1967).

Computations

The fraction of the pump remaining after exposure to ouabain for time t was calculated as $_tM^P/_aM^P$. From the Hill equation, the following terms were calculated: apparent K_m^P (concentration of a particular external ion needed for half-maximal activation of the pump); K_m^G (concentration for half-maximal change in the rate constant for ouabain binding, k_b); V_{max} (maximal pump rate); $\Delta k_{b(max)}$ (maximal increase or decrease in the rate of ouabain binding); and n_P and n_G (the Hill coefficients for the curves for active influx and ouabain binding, respectively). The form of the Hill equation used was:

$$v = \frac{V_{max}}{1 + \left[\dfrac{K_m^P}{[S]}\right]^{n_P}},$$

where v is the rate of active transport at a particular ion concentration, $[S]$. (The same equation, but with K_m^G and n_G, was used for the rate of ouabain binding.) This form of the Hill equation, the same as that used by Albers and Koval (1973), differs from the original (Hill, 1910) and from some currently used forms in which K_m is not raised to the power of n along with $[S]$. The equation above has the feature that K_m has the same units as $[S]$; its K_m can be converted to the K_m in the equation in the "conventional" form simply by raising it to the power of n.

The "best fit" solutions for the equation were determined by an iterative least squares routine called MLAB (Knott and Reece, 1972).

RESULTS

Effects of Nystatin

To correlate data obtained on nystatin-treated cells with those obtained on fresh cells, it is important that the nystatin not alter significantly the properties of the cells being observed. Cass and Dalmark (1973) have shown this to be the case for ouabain-sensitive K influx. Fig. 1 shows the effects of nystatin treatment on the rate of ouabain binding to the pump: both fresh and nystatin-treated cells were incubated in 10^{-7} M ouabain and various K concentrations to obtain the time-

course of the onset of inhibition of the pump. The rate of ouabain binding was only slightly greater in nystatin-treated cells than in fresh cells. The ratio $[Na]_c/[K]_c$ was considerably higher in the nystatin-treated cells, and this difference may account for the small effect on rate of ouabain binding. The effect of increasing $[K]_o$ was indistinguishable in the two types of cells.
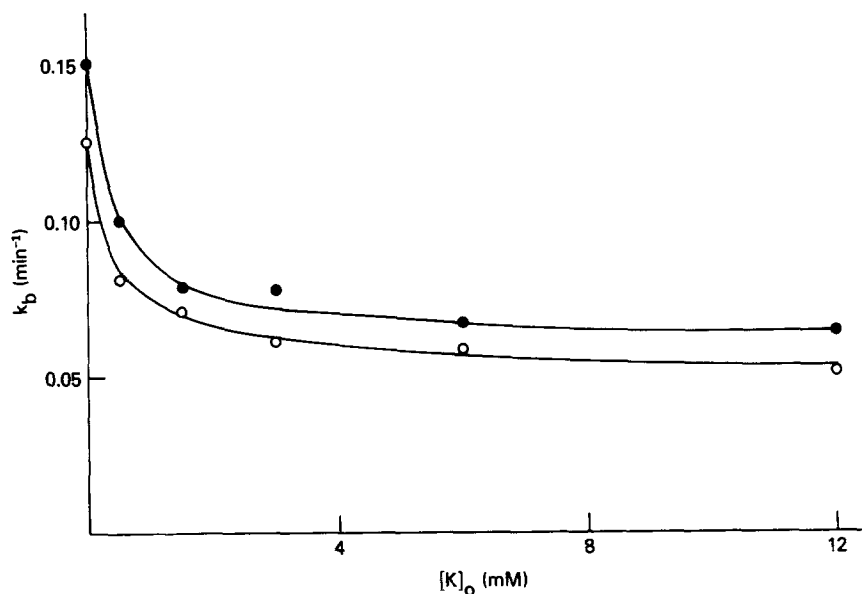
FIGURE 1. Effect of nystatin treatment on rate constant of ouabain binding. Each point represents the rate constant (k_b) determined by incubating aliquots of cells for 15 min at 10^{-7} M ouabain; five determinations of the remaining pump flux were made during this incubation. (O) Fresh cells (not treated with nystatin); $[Na]_c$ = 7 mmol/liter cells; $[K]_c$ = 82 mmol/liter. (●) Nystatin-treated cells; $[Na]_c$ = 43 mmol/liter; $[K]_c$ = 41 mmol/liter. In addition to the indicated external K concentrations, $[K]_o$, the solutions contained NaCl ($[Na]_o + [K]_o$ = 150 mM), 5 mM glucose, and 10 mM Tris-HCl. Curves were fitted by eye.

Influxes of K vs. Cs

Inasmuch as both ^{42}K and ^{137}Cs were used to measure "remaining pump," it was important to show that there is only one class of pump sites responsible for transport of K and Cs. Table I shows an experiment in which cesium and potassium are each used, first, during preincubation with ouabain, and second to measure active influx. When either of these ions has been used to modify the ouabain binding rate, "remaining pump" is the same regardless of whether it is measured using Cs or K.

Effects of External Potassium

Fig. 2 shows results of simultaneous measurements of active K influx and rate of ouabain binding with varying K concentrations, measured in solutions

containing Na as the major cation. As external K was raised, the pump rate increased and k_b decreased. The shapes of the curves were similar in that the K concentrations were ~ 2 mM for both half-maximal reduction of the rate of ouabain binding (K_m^G) and half-maximal activation of the pump flux (K_m^P); in addition, the Hill coefficients were between 1.5 and 2 for both curves.

Kinetic constants from the experiment in Fig. 2 and from two additional experiments are shown in Table II A (all carried out in Na-containing solutions). In each experiment K_m^G and K_m^P are similar. These results are consistent with the hypothesis that translocation of ions and modulation of ouabain binding are regulated by the same sites. The results also show that there are more than one of these sites per pump because the Hill coefficients are > 1.0.

Fig. 3 shows a similar experiment performed in the absence of external Na. Again as external K was increased, pump activity increased and the rate of ouabain binding decreased. When fitted to the Hill equation, however, the Hill coefficients are similar, but the apparent K_m^G is substantially lower than the K_m^P (0.16 mM as compared to 0.46 mM). Results from this experiment and from another similar one are shown in Table II B; both sets of results gave values of K_m^G for potassium that were substantially lower than those for K_m^P under the same conditions.

TABLE I

COMPARISON OF ACTIVE INFLUXES OF K AND Cs AFTER PARTIAL
INHIBITION BY OUABAIN

	Fraction of pump remaining		
Pump flux measured in:	Preincubation solution contained ouabain (10^{-7} M) and:		
	2 mM K	2 mM Cs	75 mM Cs
K (10 mM)	0.30	0.08	0.42
Cs (45 mM)	0.34	0.00	0.44

Fresh cells were preincubated for 15 min in media containing potassium or cesium as indicated, plus 10^{-7} M ouabain. The solutions contained, in addition, NaCl ($[Na]_o + [K]_o + [Cs]_o = 150$ mM); 10 mM Tris-HCl, pH 7.45; 5 mM glucose. The extent of the inhibition of the pump by ouabain was then determined as the fraction of the pump flux remaining by measuring the influx of either K or Cs. This fraction was obtained by comparison of these fluxes with those for cells not exposed to ouabain and also cells maximally inhibited by ouabain.

If one assumes that the same sites are involved in modifying both processes (activation of the pump and inhibition of the binding of ouabain) whether Na is present externally or not, then in the absence of Na_o, occupation of one of these sites must be able to affect the rate of ouabain binding far more effectively than it affects the pump translocation process. This follows because a low concentration of external K exerts a much more nearly maximal effect on ouabain binding than on active K influx. An alternative explanation is that K ions, in the absence of Na, inhibit ouabain binding at an additional class of sites to which they, K, do not have access in the presence of Na.

Effects of External Sodium

It had been shown earlier that ouabain binding proceeds more rapidly in the presence of Na than in solutions in which choline replaces Na (Beaugé and
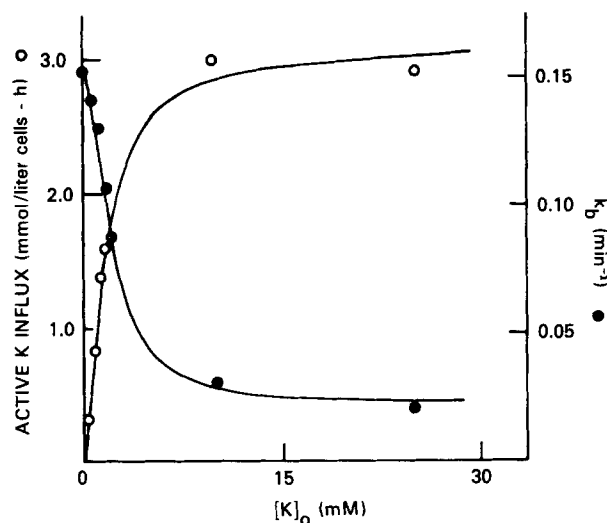
FIGURE 2. Active potassium influx and rate of ouabain binding as functions of external K in Na-containing solutions. (●) Rate constants (k_b); (○) active K influx. Cells were treated with nystatin so that $[Na]_c = 82$ mmol/liter, $[K]_c = 9$ mmol/liter. For determination of k_b, cells were preincubated for 15 min in 10^{-7} M ouabain with samples removed and washed each 3 min, and remaining pump measured. For determination of active K influx, cells which had not been exposed to ouabain were incubated in the indicated external K concentrations plus the standard sodium medium ($[Na] + [K] = 150$ mM; 10 mM Tris-HCl; 5 mM glucose) with and without 10^{-4} M ouabain. Flux experiments were carried out in triplicate. Curves were drawn from the Hill equation where, for rate of ouabain binding, $\Delta k_{b(max)} = 0.132$ min^{-1}, $K_m^G = 2.1$ mM, $n_G = 1.9$; and for active K influx, $V_{max} = 3.1$ mmol/liter of cells × h, $K_m^P = 1.7$ mM, and $n_P = 1.5$ (see Materials and Methods for meanings of symbols).

TABLE II

KINETICS OF ACTIVE K INFLUX AND OF OUABAIN BINDING WITH VARYING $[K]_o$ IN Na-CONTAINING AND Na-FREE (CHOLINE) SOLUTIONS

	$[Na]_c$	$[K]_c$	K_m^P	n_P	K_m^G	n_G
A. Na solutions						
fresh	9	80	0.72	1.1	1.3	1.5
Nystatin-treated	82	9	1.7	1.5	2.1	1.9
Nystatin-treated	100	2	1.6	1.6	1.7	2.9
B. Choline solutions						
Nystatin-treated	76	8	0.47	0.78	0.16	0.84
PCMBS-treated	39	1.4	0.73	0.82	0.096	1.7

Cells were either fresh or treated to alter $[Na]_c$ and $[K]_c$ as shown. K_m^P and K_m^G are the concentrations of K needed for half maximal saturation of K pump influx and half-maximal change in the rate of ouabain binding, respectively; n_P and n_G are the Hill coefficients for these two processes. These values were determined by fitting the experimental data to the Hill equation as given in Materials and Methods. The protocols for the experiments were the same as for Fig. 2. The solutions contained: 0-12 mM KCl; 150-138 mM NaCl; 10 mM Tris HCl, pH 7.45; 5 mM glucose. The fluxes were measured in triplicate and the k_b values in quintuplicate.

Adragna, 1971; Sachs, 1974; Gardner and Frantz, 1974). Two possible explanations suggested for this observation are (a) Na competes with K, and thus prevents the inhibition of ouabain binding by K (Sachs, 1974), or (b) Na actually promotes binding directly by occupying otherwise empty sites (Gardner and Frantz, 1974; Hobbs and Dunham, 1976). To distinguish between these possibilities it is necessary to remove as much as possible of the internal K from the red
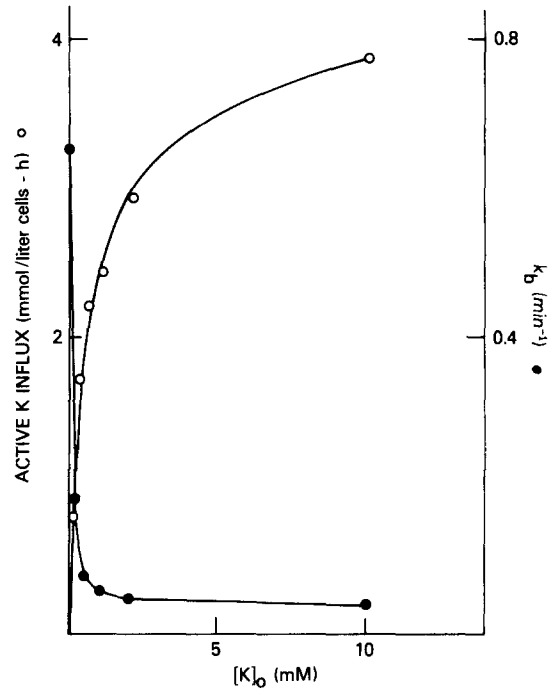

FIGURE 3. Active potassium influx and rate constants of ouabain binding as functions of external K in Na-free solutions. (●) Rate constants (k_b); (○) active influx. Cells were treated with PCMBS so that $[Na]_c = 39$ mmol/liter cells and $[K]_c = 1.4$ mmol/liter (the remainder of intracellular cation made up by choline). The experimental protocol was identical to that in Fig. 2 except that the ouabain concentration during the preincubation was 10^{-6} M, and samples were taken every 2 min. Curves are drawn from the Hill equation where, for rate of ouabain binding, $\Delta k_{b(max)} = 0.610$ min^{-1}, $K_m^G = 0.096$ mM, and $n_G = 1.7$. For active influx, $V_{max} = 4.26$ mmol/liter of cells × h. $K_m^P = 0.73$ mM and $n_P = 0.82$.

cells so that the effects which are seen upon varying external cations will not be confused with those caused by K which has leaked from the cells. Fig. 4 shows the results of such an experiment on ouabain binding with and without external Na. Using PCMBS, internal cation concentrations were altered so that $[K]_c$ was about 1 mmol/liter cells and $[Na]_c$ was either 24 or 84 mmol/liter. For both of the internal sodium concentrations, the rate of ouabain binding in Na medium was clearly faster than in choline medium, even in the absence of K_o. It seems clear from this that sodium does indeed have a direct effect on ouabain binding independent of reducing inhibition of binding by displacement of K.

Fig. 4 also shows that, when internal K is very low ($\sim$ 1.0 mmol/liter), raising internal Na inhibits the ouabain binding rate, even in the absence of external K, an effect in contrast to that reported by Bodeman and Hoffman (1976 a), who suggested that K was necessary externally to see changes in ouabain binding caused by internal Na and K.

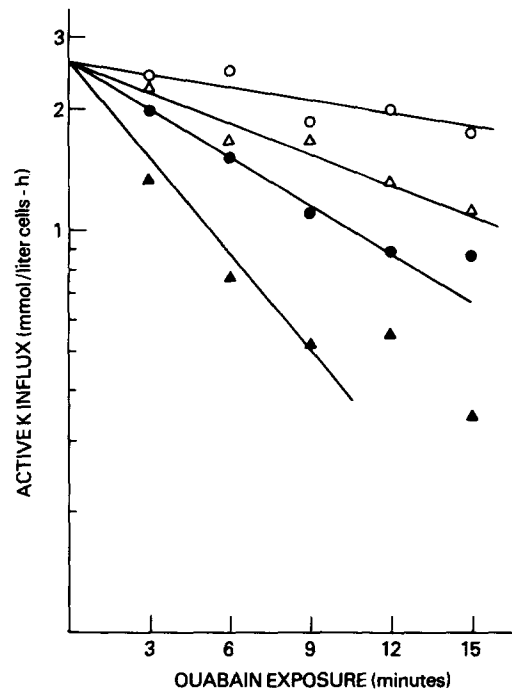Fig. 5 shows the increase in k_b promoted by Na in both fresh and sodium-

FIGURE 4. Effect of external Na on rate of ouabain binding in low K cells. Ouabain binding was indicated by the fraction of active K influx remaining during incubation in media containing 10^{-6} M ouabain. The experiment was carried out in K-free media containing 150 mM NaCl (filled symbols) or 150 mM choline Cl (open symbols). Cells with two concentrations of Na were used, altered using PCMBS: ($\bullet$, $\bigcirc$) $[Na]_c$ = 84 mmol/liter, $[K]_c$ = 1.1 mmol/liter; ($\blacktriangle$, $\triangle$) $[Na]_c$ = 24 mmol/liter, $[K]_c$ = 0.9 mmol/liter (the balance of intracellular cation was choline). The lines were fitted by eye.

loaded cells. Apart from the scatter at low external sodium concentrations for fresh cells, the curves appear to be indistinguishable. (This scatter is probably due to leakage of K from the fresh cells.) Interestingly, the shape of this curve looks little different from that obtained in the presence of saturating (for K translocation) concentrations of K_o (Hobbs and Dunham, 1976), further support for the suggestion that sodium promotes ouabain binding at sites separate from the K loading sites. To enable the comparison, the curve from this earlier publication is included in Fig. 5 as a dashed line. The rate constant for ouabain binding had been measured in media containing 11 mM $[K]_o$. The pump is saturated at this $[K]_o$ and raising $[Na]_o$ from zero to 140 mM caused no

inhibition, though the curve for the increase in k_b is nearly the same as for cells incubated in K-free solutions. (The ouabain concentrations differed under the two conditions: 10^{-7} M in the K-free solutions and 10^{-6} M in the 11 mM [K], accounting for the fortuitous similarity of the two sets of k_b.)

Thus, the results on the effect of Na on the rate of ouabain binding under nearly K-free conditions (Figs. 4 and 5) and with K saturating the pump (Fig. 5; Hobbs and Dunham, 1976) both indicate that Na acts directly at sites with relatively low affinity for Na (not saturated at 150 mM $[Na]_o$), sites distinct from those at which K activates translocation. A possible candidate for this class of sites is the sites from which Na is discharged following translocation outward.
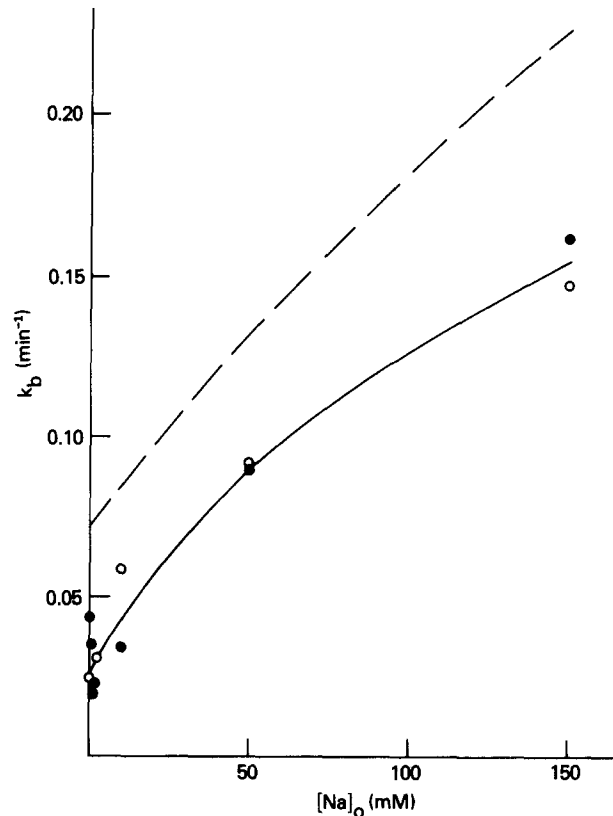

FIGURE 5.   Rates of ouabain binding measured as a function of external sodium in K-free solutions. (O) Fresh cells in which $[Na]_c = 8$ mmol/liter and $[K]_c = 89$ mmol/liter; (●) nystatin-treated cells in which $[Na]_c = 85$ mmol/liter and $[K]_c = 11$ mmol/liter. For determination of each value of k_b (rate constant, ouabain binding), aliquots of cells were preincubated for up to 16 min in 10^{-7} M ouabain, with samples removed and washed each 4 min, and the fraction of pump remaining was measured in the standard sodium medium with 10 mM K. The curve was fitted by eye. Dashed line is for similar data taken in 10^{-6} M ouabain and with a $[K]_o = 11$ mM which saturates the pump (Hobbs and Dunham, 1976); $[Na]_c = 53$ mmol/liter and $[K]_c = 43$ mmol/liter.

Glynn and Karlish (1976) observed an external site with low affinity for Na on Na-K pumps of human red cells at which the binding of Na stimulated activity of Na-ATPase. It is tempting to consider a single external site or set of sites with low affinity for Na, but it is not obvious how binding of Na could stimulate both ouabain binding and Na-ATPase at the same site.

Fig. 6 shows the results of an experiment designed to investigate further the relationship between the interactions of Na and K with the external aspect of the pump. Rate of ouabain binding and active K influx were measured at $[Na]_o$ between 0 and 150 mM in media containing 2 mM K, a nonsaturating K concentration at which increasing $[Na]_o$ inhibits the pump. As $[Na]_o$ is increased,
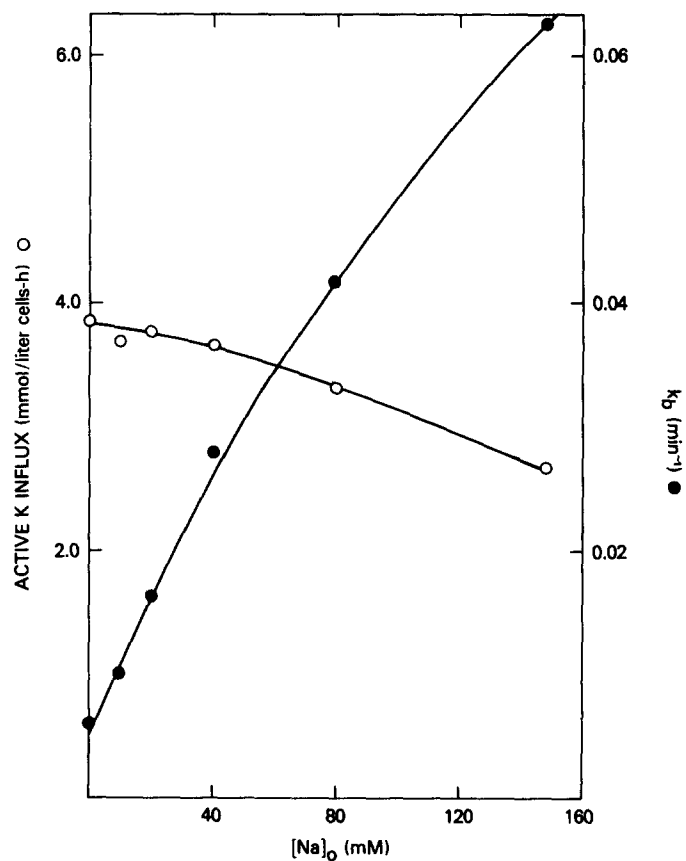
FIGURE 6. Active potassium influx and rate constant of ouabain binding as functions of external sodium in 2 mM $[K]_o$. (○) Active K influx in cells which had not been preexposed to ouabain. (●) Rate constants (k_b) in identical cells. Ouabain concentration was 10^{-7} M and cells were preincubated for up to 15 min with samples removed each 3 min for determination of fraction of pump flux remaining. Cells had been treated with nystatin so that $[Na]_c = 88$ mmol/liter and $[K]_c = 8$ mmol/liter. Experimental protocol was as described in Fig. 2 except that $[K]_o$ was held constant at 2 mM, and $[Na]_o$ was increased as shown. Flux rates were determined in triplicate, k_b values in quintuplicate. Curves were fitted by eye.
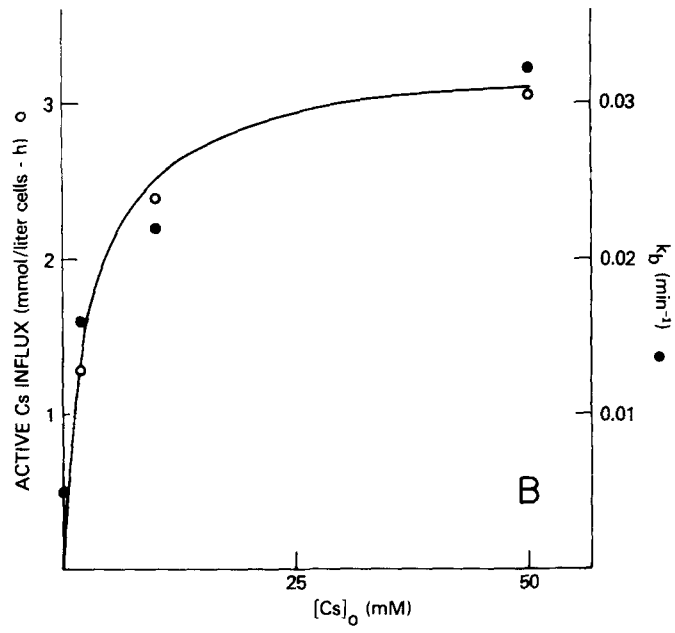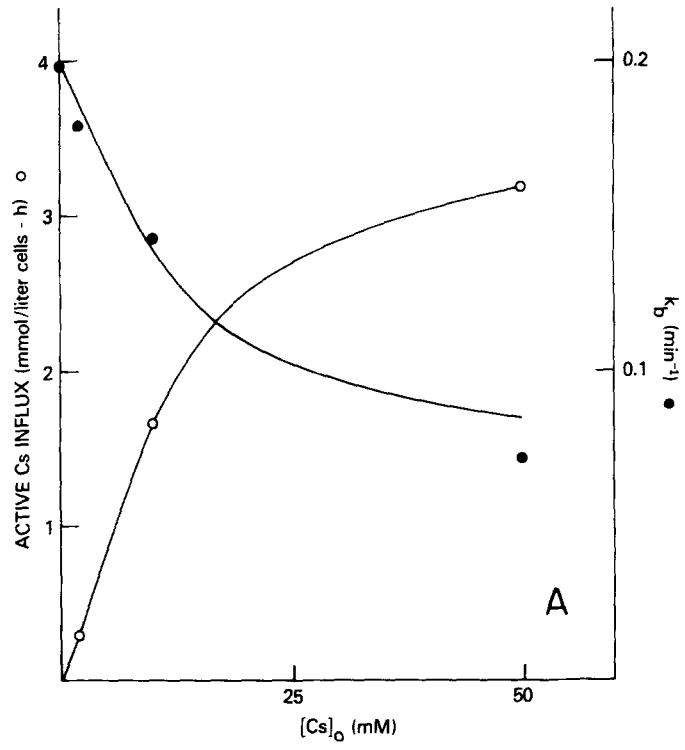
$^{i}M_{K}^{P}$ decreased and k_{b} increased, but not in the same fashion: as $[Na]_{o}$ was increased from zero to 150 mM, k_{b} increased 10-fold, but active K influx decreased by only 30%. More importantly the slopes of the curves relating k_{b} and $^{i}M_{K}^{P}$ to $[Na]_{o}$ changed in opposite directions as $[Na]_{o}$ was increased: for k_{b} the slope decreased and for $^{i}M_{K}^{P}$ it increased. The inhibition of the pump by Na is presumably by competition with K for the loading sites under these experimental conditions, whereas the promotion of ouabain binding is proposed here to be by some unspecified mechanism at the discharge sites. (Although inhibition of K influx by Na involves competition, there may be other types of inhibitory effects as well [Glynn and Karlish, 1976; Sachs, 1977].)

Effects of External Cesium

Cesium is known to compete with K for inward translocation by the pump (Sachs and Welt, 1967). Active Cs influx has a much higher apparent K_{m}^{P}, but about the same V_{max}, as for potassium influx.[1]

Figs. 7 A and B show the results of experiments which measured $^{i}M_{Cs}^{P}$ and k_{b} simultaneously as functions $[Cs]_{o}$. Fig. 7 A shows the results in media containing Na, and Fig. 7 B, in Na-free media. One striking difference between Cs and K is that, although both ions retard the k_{b} in the presence of external Na, Cs promotes ouabain binding in the absence of external Na. The shapes of the curves for $^{i}M_{Cs}^{P}$ and k_{b} are similar in Fig. 7 A, suggesting that the sites at which Cs binds for translocation and for modulation of ouabain binding are identical in the presence of Na, the same situation as observed for potassium. This suggestion is supported further by data obtained in Na-containing media shown in Table III. When the data for rate of ouabain binding was fitted to the Hill

[1] Hobbs, A. S., and P. B. Dunham. Unpublished data.

FIGURE 7. Active cesium influx and rate constant for ouabain binding as functions of external [Cs] in (A) Na-containing media and (B) Na-free media. (●) Rate constants (k_{b}); (○) active Cs influx. Cells were treated with nystatin so that $[Na]_{c} = 88$ mmol/liter and $[K]_{c} = 8$ mmol/liter. The curves were drawn from the Hill equation (see Materials and Methods) using the values for the constants given below except that for clarity the curve for ouabain binding has been omitted from (B).

Curve	Constants	Values of constants	
		A. Na	B. Choline
k_{b}	$\Delta k_{b(max)}$	0.127	
	K_{m}^{G}	10.8	
	n_{G}	1.44	
Cs influx	V_{max}	3.52	3.26
	K_{m}^{P}	10.8	2.85
	n_{P}	1.44	0.97

equation, K_m^G and n_G (the Hill coefficient for Δk_b) were the same as the constants for active Cs influx. When Na is removed K_m^P declined as expected but K_m^G did not (in some experiments it increased). One possibility which would account for this observation is that, in addition to the loading sites, cesium can also occupy another class of sites at which it promotes ouabain binding in the absence of sodium; these are probably the same sites at which Na promotes ouabain binding. This suggestion is supported by the data shown in Fig. 8, where k_b was measured as a function of external Cs in both the absence and presence of external Na (50 mM). The results show generally the same effects shown in Fig. 7. In addition, at high Cs concentrations, the k_b is not significantly different in the presence and absence of Na_o, as indicated by the similarity of the asymptotes

TABLE III

EFFECTS OF EXTERNAL CESIUM ON THE RATE CONSTANT
FOR OUABAIN BINDING AND ON ACTIVE Cs INFLUX IN
FRESH AND Na-LOADED CELLS

Principal external cation	K_m^P	n_P	K_m^G	n_G
Na	10.8	1.44	9.5	1.50
	(1)	(1)	(4)	(4)
Choline	3.2	0.91	11.1	0.77
	(2)	(2)	(2)	(2)

The cells had been pretreated with nystatin to give values of $[Na]_c$ and $[K]_c$ which were, in four experiments, between 8 and 88 mmol/liter. K_m^P and K_m^G are the concentrations of Cs needed for half-maximal saturation of active Cs influx and half-maximal change in ouabain binding rate, respectively. n_P and n_G are the Hill coefficients for these two processes. These values were determined by fitting the experimental values to the Hill equation, as described in Materials and Methods. The solutions contained: 150-100 mM NaCl or choline chloride; 10 mM Tris·HCl, pH 7.45; 5 mM glucose. $[Cs]_o$ was varied from 0 to 50 mM, replacing Na or choline. The experimental protocol was as described in Fig. 2. The fluxes were measured in triplicate and the k_b values in quintuplicate in each of up to four experiments (number of experiments for each value is shown in parentheses).

of the two curves; apparently Cs_o and Na_o are mutually competitive with regard to modulation of k_b. In this regard it should be noted that sodium does not alter V_{max} for the Cs pump influx (Sachs, 1977).

Effects of External Lithium

Lithium acts like K, just as Cs does, on the exterior of the Na-K pump in red cells (Sachs and Welt, 1967; Beaugé and del Campillo, 1976); just as with Cs, the apparent K_m^P for Li is higher than for K. However, as Table V shows, Li_o promotes ouabain binding, though not as effectively as sodium. (When replacing 75 mM of a 150 mM NaCl solution with LiCl, the ouabain binding rate was reduced approximately 25%.)

Fig. 9 shows the results of an experiment comparing stimulation of Na efflux and stimulation of ouabain binding rate when varying external Li. Like Cs, and unlike K or Na, Li increases the rate of ouabain binding while stimulating the pump. The two curves are similar, suggesting that, as for K and Cs, Li modifies
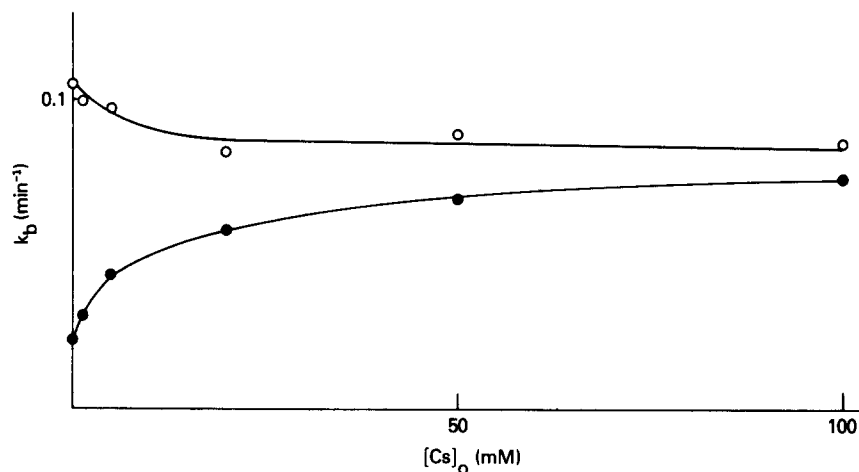
FIGURE 8. Rate constants for ouabain binding as functions of external [Cs] in sodium-containing and sodium-free solutions. (O) Rate constants (k_b) in the presence of 50 mM $[Na]_o$. The curve was drawn from the Hill equation with $\Delta k_{b(max)} = 0.0216$ (min^{-1}), $K_m^G = 5.06$ mM, $n_G = 1.1$. (●) Rate constants in absence of Na_o with $\Delta k_{b(max)} = 0.0647$ (min^{-1}), $K_m^G = 15.8$ mM, $n_G = 0.7$. Cells had been treated with nystatin so that $[Na]_c = 38$ mmol/liter and $[K]_c = 50$ mmol/liter.
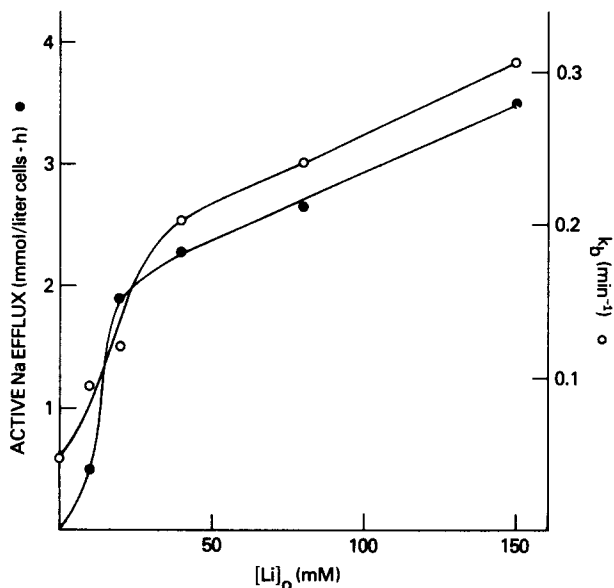

FIGURE 9. Active sodium efflux and rate constants of ouabain binding as functions of external lithium in sodium-free solutions. (●) Rate constants (k_b); (O) ouabain-sensitive sodium efflux. Cells had been loaded with Na (and ^{24}Na) using nystatin so that $[Na]_c = 119$ mmol/liter and $[K]_c < 1$ mmol/liter. For measurement of k_b, cells were preincubated in 5×10^{-7} M ouabain for 6 min and then washed, and the residual pump was measured by ^{24}Na efflux into 40 mM Li in the standard choline solution. For measuring Na efflux cells were incubated in the indicated lithium solutions. Values shown are the means of two samples (20 and 40 min).

ouabain binding at the pump's loading sites. There are insufficient data, however, to determine whether additional sites might be required as well.

Effects of Divalent Cations

Gardner and Frantz (1974) found, in their studies on fresh red cells, that divalent as well as monovalent cations were able to affect ouabain binding. We examined this effect in sodium-loaded cells using Mg and Ca. Table IV shows that both Mg and Ca increase the ouabain binding rate, Mg about twice as effectively as Ca at the same (23 mM) concentrations. In addition, 115 mM Mg solutions produce nearly the same ouabain binding rate ($k_b = 0.11$ min^{-1}) as 150 mM Na (0.13 min^{-1}).

TABLE IV

EFFECTS OF EXTERNAL LITHIUM, CALCIUM, AND MAGNESIUM ON RATE CONSTANTS OF OUABAIN BINDING (k_b)

External solution	k_b (min^{-1})
mM	
150 Na	0.174
75 Na, 75 Li	0.137
75 Choline, 75 Li	0.0587
150 Choline	0.0142
120 Choline, 23 Mg	0.0832
120 Choline, 23 Ca	0.0415

Cells were preincubated for 15 min in 10^{-7} M ouabain plus the indicated solutions, then ^{42}K influx was measured in the standard sodium-containing medium in order to determine k_b's. Cells had been treated with nystatin so that [Na]$_c$ = 49 mmol/liter and [K]$_c$ = 53 mmol/liter cells.

Affinity Sequence for Ouabain Binding

For forms of the pump associated with the various alkali metal ions, the sequence of rates of ouabain binding (called for simplicity affinity sequence) appears to be: E-Na $\geq$ E-Li $>$ E-Cs $>$ E-K. This conclusion must be drawn by comparing forms of the pump saturated to the same extent by various ions, and the ions have different affinities for the sites on the pumps. So the rate of binding is determined not only by the rate at which a particular form of the pump (e.g., E-Na or E-K) binds ouabain but also the fraction of the total pumps which are combined with the species of ion in question. Ideally the rates of ouabain binding of the different ion-pump forms would be compared at saturation with the ions, but saturation with neither Li nor Na was achieved. We can speculate that E-Li has a lesser affinity for ouabain than E-Na because the rate of binding is lower at 150 mM Li than at 150 mM Na (Table V), and the increase in k_b upon increasing [Li]$_o$ is less marked (Fig. 9) than for [Na]$_o$ (Fig. 5). But the conclusion is not secure, so we state the sequence as E-Na $\geq$ E-Li. The actions of Cs and K on k_b saturate with the experimental conditions employed and the positions of E-Cs and E-K in the affinity sequence are unequivocal. A direct comparison of k_b's for pumps associated with the four species of ion is shown in Table V.

   This line of reasoning does not take into account the affinity for ouabain of the form of the pump uncombined with external alkali metal ions. This form of the pump would be present during some (unknown) fraction of the time even at high concentrations of ions if the pump is turning over. One cannot assume that the uncombined pump cannot bind ouabain, but if the following two assumptions can be made, then binding by the uncombined pump may not be critical to the arguments above: (a) the affinity for ouabain of the uncombined form of the pump does not vary greatly with the species of ion most recently released from a binding site; (b) the pump is uncombined a small fraction of the time with high concentrations of alkali metal ions (that fraction can vary as long as it is small).

TABLE V

RATE CONSTANTS FOR OUABAIN BINDING WITH ALKALI METAL CATIONS

Alkali metal ion	k_b (min^{-1})
mM	
none (choline 150)	0.023
K 10	0.004
Cs 10	0.050
Cs 50	0.056
Cs 158	0.056
Li 10	0.038
Li 50	0.090
Li 150	0.132
Na 150	0.176

Cells were preincubated for 15 min in media containing the cations at the concentration indicated plus ouabain at 10^{-7} M (in all of the media choline made up the balance of the tonicity). Five samples were removed at 3-min intervals to determine fraction of pump remaining from which rate constants for ouabain binding (k_b) were calculated. Cells had been pretreated with nystatin so that $[Na]_c = 32$ mmol/liter and $[K]_c = 61$ mmol/liter.

DISCUSSION

   The main conclusion to be drawn from this study is that external alkali metal ions can exert effects on the rate of ouabain binding to the Na-K pump of red cells at two classes of sites: (a) the loading sites from which K ions (or congeners of K) are translocated inwardly and (b) another class of sites postulated to be those from which Na is discharged after outward translocation. We have shown that the kinetics for activation of the pump and for inhibition of ouabain binding rate are similar when outside K is increased in Na-containing solutions. This is consistent with the hypothesis that, under these conditions, one class of sites, the K loading sites, modulates both processes. On the other hand, there are conditions in which the kinetics of these two processes are different. For example, increasing the sodium concentration in the medium has similar effects

on ouabain binding (a nearly linear increase in the range from 0 to 150 mM) whether the pump rate is saturated with external K (Fig. 5; Hobbs and Dunham, 1976), partially saturated (Fig. 6), or when K-free solutions are used (Fig. 5). This must mean either that Na can displace K from an external site on the pump with little or no effect on pump rate but a dramatic one on ouabain binding rate, or, more likely, that Na binds to sites which have little or no affinity for K, perhaps the Na discharge sites.

Inasmuch as the Hill coefficients were > 1 for the effect of K on the rate of ouabain binding, two external binding sites for K per pump must be invoked for modulating ouabain binding as well as for activating the pump. These two sets of sites are probably identical, as proposed by Sachs (1974), since in sodium media the apparent K_m's are not significantly different for transport and for ouabain binding. In the absence of sodium, however, the apparent K_m^G (for potassium's effect on ouabain binding) becomes much lower than K_m^P (for activation of the pump), suggesting that, whereas one K ion per pump may decrease the ouabain binding rate (Na-free), two are still necessary for optimal activation of translocation. An alternative possibility is that, in the absence of Na, K now interacts with the Na discharge sites and there inhibits ouabain binding, rather than promoting it as Na does, and perhaps as other species of ions do as well. Our scheme differs from Sachs' model (1974), which predicts promotion of binding when one K ion occupies the external aspect of the pump. (If this occurred, one would expect K_m^G to be higher, not lower, than K_m^P in Na-free solutions.) Our suggestion is in agreement with the more recent model of Kropp and Sachs (1977) which specifies a lower affinity of the E_K form of the pump (one K ion bound per pump) for ouabain than the uncombined form of the pump, and the E_K^K form having a still lower affinity. Our scheme also differs from Sachs' in sodium's role in influencing rates of steroid binding. He found no change in the ouabain binding rate on addition of external sodium to low sodium, K-free cells. Fig. 5 in the present report shows an increase in the binding rate in an experiment similar to that reported by Sachs. It is unclear why this difference has occurred—whether it is due to the lower ouabain concentration and longer incubation times which he used, or some other unknown variable. It is worth noting that Kropp and Sachs (1977) do report variable effects of external Na in the absence of K_o.

As suggested above, another class of sites for monovalent cations which does not activate translocation must be proposed to explain the effect of external sodium on ouabain binding. At an intermediate external K concentration (2 mM), where external Na inhibits active K influx, this inhibition, and concommitant increase in ouabain binding, do not follow similar curves (Fig. 6). Again the simplest interpretation is that Na inhibits the pump by competitive interaction with K at the loading sites and promotes ouabain binding by unspecified means at the discharge sites.

There is no compelling reason that the Na binding sites be Na discharge sites, but there is no attractive alternative. The apparent affinity of these sites for Na is relatively low compared to the affinity of Na at its intracellular loading sites (Fig. 5; Hobbs and Dunham, 1976), but correspondingly the apparent affinity

for K at its intracellular discharge sites is far lower ($\sim$ 2 orders of magnitude) than the affinity for K at its external loading sites (Robinson et al., 1977). If the external sites at which Na promotes ouabain binding are the discharge sites, this would mean that distinct sites or channels for Na and K coexist, though they may be interconvertible.

The opposite effects of Cs on ouabain binding, depending upon the presence of Na, are surprising. Although Cs activates its own ouabain-sensitive influx whether or not Na is present, Cs inhibits ouabain binding in Na-media and enhances it in Na-free solutions. These observations are consistent with the finding that, in Na media, the concentration of Cs necessary for half-maximal reduction in k_b is higher than the concentration giving half-maximal activation of active Cs influx. (With K, the two half-maximal concentrations were the same in Na media.) One interpretation of these results with Cs is that Cs is affecting ouabain binding at two classes of sites: the loading sites, where the effect is inhibition of binding, and the discharge sites, at which binding is promoted. This proposal of a Na-like action of Cs at the discharge sites is supported by the results in Fig. 8, where the rate of ouabain binding extrapolates to the same value in both Na-free and Na-containing solutions, showing that Cs displaces Na. The promotion by Cs of binding in Na-free media (rather than inhibition), even though Cs is occupying the loading sites for translocation (where the effect is inhibitory), suggests a predominance of the discharge sites over the loading sites in the control of ouabain binding. The particular significance of this could constitute a basis for speculation about the configuration of the external aspect of the pump, but such speculation would for the present be idle.

Lithium can act both as an analog for K at the external aspect of the pump (Sachs and Welt, 1967; Beaugé and del Campillo, 1976) and as an analog for Na intracellularly (Dunham and Senyk, 1977). We find that Li in Na-free solutions behaved similarly to Cs in that it stimulated both the pump and ouabain binding (Fig. 9). As with Cs, the stimulation of the pump is a K-like action and the promotion of ouabain binding is a Na-like effect. (It is unlikely that, in activating Na efflux, external Li is promoting the pump in its Na-Na exchange mode because at low $[K]_o$ and low $[Li]_o$, Li "coactivated" active K influx, a K-like action of Li, promoting Na-K exchange [Sachs and Welt, 1967].)

The sequence of affinities of various external pump forms for ouabain, $E\text{-Na} \geq E\text{-Li} > E\text{-Cs} > E\text{-K}$, is exactly the reverse of the sequence found by Post et al. (1972) for the effects of these ions on dephosphorylation of the Na,K-ATPase. In other words, the more readily a species of ion promotes dephosphorylation, the slower is the rate of ouabain binding when that ion is combined with the external aspect of the pump. This is in accord with studies which suggest that ouabain binds preferentially to the so-called E_2-P form of the Na,K-ATPase (Albers et al., 1968; Post et al., 1969) and suggests that, in the absence of an external monovalent cation (e.g., K) to drive dephosphorylation ($E_2 - \text{P}$ $\rightarrow E + \text{P}_i$), the ouabain binding sites are more available to ouabain. This is similar to the scheme proposed by Bodemann and Hoffman (1976 a), which features the rate of turnover of the pump as the determinant of the rate of ouabain binding, although finding no effect of Na_o in the absence of K_o, they

suggested that during Na-Na exchange the pump must remain in the $E_1 - P$ form. This latter assumption is unnecessary under our experimental conditions, since Na_o was seen to promote ouabain binding independent of the presence of external K. (In some of the results of Bodemann and Hoffman there was an effect of Na on ouabain binding with both saturating $[K]_o$ and with $[K]_o < 0.2$ mM [Fig. 1 and Table VI, respectively; Bodemann and Hoffman, 1976 a], but their conclusions emphasized the other results. Bodemann and Hoffman [1976 c] present data suggesting that the concentration of cellular Mg may control the effect of external Na on ouabain binding.)

These formulations cannot explain all the facts at hand, however. Even when external Na does not inhibit inward K transport (e.g., at saturating $[K]_o$) and therefore presumably does not inhibit dephosphorylation, Na enhances ouabain binding, suggesting involvement of additional sites for Na. Furthermore, when external sodium inhibits inward K transport, the kinetics of this inhibition and of the concommitant stimulation of ouabain binding are not the same (Fig. 6). These observations indicate, as suggested above, that stimulation of ouabain binding by Na occurs at sites distinct from the presumed two sites for K translocation; these Na sites need also have little to do with dephosphorylation. It appears that pump turnover, and presumably the accompanying dephosphorylation, may be only one of several factors which controls the availability of the ouabain binding site.

The limited data on the effect of divalent cations contained in this paper do not contradict the suggestion of Gardner and Frantz (1974) that there is also an external divalent cation site which affects ouabain binding. However, Gardner and Frantz proposed only one external monovalent cation site mediating such effects, and clearly a more complex model is necessary to account for the information available. External Mg, unlike Na, does not raise the apparent K_m for external K activation of the pump (Sachs and Welt, 1967), so Mg has little effect on the K loading sites. Therefore, the effect of Mg on ouabain binding is probably mediated at another locus, perhaps the sodium sites. Cs inhibits ouabain binding in Mg-substituted solutions (data not shown) as it does in Na media; this is consistent with the possibility that Mg has an affinity for the Na discharge sites where it promotes binding.

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