Effects of Mg and Ca on the Side Dependencies of Na and K on Ouabain Binding to Red Blood Cell Ghosts and the Control of Na Transport by Internal Mg

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ABSTRACT The effect of alteration in the concentration of internal Mg on the rate of ouabain binding to reconstituted human red blood cell ghosts has been evaluated as well as the effect of Mg_i on Na:Na compared to Na:K exchange. It was found that the dependence of the rate of ATP-promoted ouabain binding on the combined presence of Na_i and K_o which occurs at high $[Mg]_i$ is lost when the concentration of Mg_i is lowered. The sensitivity of the external surface for K_o is also changed since K_o can now inhibit the ouabain binding rate in the absence of Na_i; on the other hand Na_o at low $[Mg]_i$ can stimulate ouabain binding indicating that the relative affinity of the outside surface for Na_o has either increased or that for K_o has decreased or both. Thus the effects of changes in $[Mg]_i$ result in a change in the side-dependent actions of Na and K and emphasize the possible difficulties of interpreting results obtained on systems lacking sidedness. Mg_i was found to be required for P_i-promoted ouabain binding and that the inhibitory action of Na_i increased as $[Mg]_i$ was increased. In addition, Ca was found to be most effective in inhibiting the rate of ATP-promoted ouabain binding when Na and K were present together than when either was present alone. Na:K exchange was found to be more sensitive to the concentration of Mg_i than Na:Na exchange; at low $[Mg]_i$ Na:K exchange could be stimulated without changing the extent of Na:Na exchange. These results are consistent with the idea that conformational states of the pump complex are directly influenced by $[Mg]_i$.

INTRODUCTION

This is the third paper in a series (Bodemann and Hoffman, 1976 *a*, *b*) in which various ligands of the Na:K pump are evaluated with regard to their effects on the rate of ouabain binding to human red blood cell ghosts. The previous papers have dealt with the sidedness of action of such factors as Na and K on adenosine triphosphate (ATP)-, uridine triphosphate (UTP)-, or orthophosphate (P_i)-promoted ouabain binding. The present paper extends these observations in examining the effects of alterations in concentration of Mg_i on both the rate of ouabain binding as well as the magnitude of Na:Na compared to Na:K exchange. In addition, the inhibitory action of Ca on ouabain binding as modu-

lated by Na and K are also evaluated. The results are consistent with the idea put forward by Fahn et al. (1966 *a*, *b*) that conformational states of the pump complex are directly influenced by the concentration of Mg_i. Brief accounts of this work have been presented previously (see Hoffman, 1972 and 1973).

MATERIALS AND METHODS

All experimental procedures and analytical techniques used in this paper are the same as those already described in the preceding papers (Bodemann and Hoffman, 1976 *a*, *b*). Since this paper examines the effects of varying Mg concentrations on the rate at which ATP-, UTP-, and P_i-promoted ouabain binding to human red cell ghosts occurs, it is of interest to estimate the concentration of free (ionized) Mg under the several experimental conditions used where reconstituted ghosts were studied. The hemolyzing medium usually contained 1.5 mM Tris EDTA, 2 mM ATP, and varying concentrations of $MgCl_2$ ranging from 0.4 to 4.0 mM. (The incorporation of strong and unsaturated complexing agents into ghosts is possible when osmotic hemolysis is performed strictly at 0°C [Bodemann and Passow, 1972]. If hemolysis takes place above 0°C then the ghosts in this situation fail to reseal to Na and K [Hoffman, 1962].)

Unfortunately, stability constants (reciprocals of the dissociation constants) have apparently not been determined for these specific conditions. Nevertheless, if we accept that the stability constant of MgATP is 10^4 M^{-1} and that of MgEDTA is 10^9 M^{-1} (Burton, 1959; Nørby, 1970, and Sillén and Martell, 1964) then when the ratio of Mg/EDTA is 0.8/1.5, the concentration of free Mg is approximately 10^{-9} M; when the ratio, Mg/EDTA, is 1.5/1.5 then the free Mg concentration is estimated to be about 10^{-6} M. The addition of 2 mM ATP to either of these mixtures does not appreciably alter the free Mg concentration. Since stability constants are known to be concentration dependent and since it is also not known to what extent the cellular constituents (e.g. Mg) that are liberated during hemolysis alter the calculated equilibria, it was decided to present simply the concentrations of Mg and EDTA used without attempting to calculate the concentrations of free Mg for the various conditions. On the other hand it should be remembered that intact cells contain approximately 3 mM Mg/liter cells so that if this Mg went to diffusion equilibrium during hemolysis the final concentration would be about 0.2 mM (this assumes a hemolytic ratio of cells, at their hemolytic volume, to medium of 1:13, for the conditions used above). Thus the concentration of free Mg for the condition in which 1.3 mM Mg/1.5 mM EDTA was present in the hemolysis solution approximates the 1.5 Mg/1.5 EDTA situation as calculated above.

RESULTS

Intracellular Mg and the Side-Specific Effects of Na and K on the Rate of Ouabain Binding to Reconstituted Ghosts

The results presented in Fig. 1 show that the rate of ouabain binding as promoted by ATP_i increases as the concentration of Mg_i is increased. For reasons mentioned in Materials and Methods only the total concentration of Mg_i is indicated in the figure although calculated estimates of the concentration of free (ionized) Mg for the EDTA buffer system employed are given in the legend.

It is also apparent that the ouabain binding rate can be influenced by Na_i depending upon the concentration of Mg_i. An inhibitory effect of Na_i on the ouabain binding rate is observed at high values of $[Mg]_i$ but in the presence of the lowest concentration of Mg_i used (i.e. 0.8 mM Mg + 1.5 mM EDTA) the

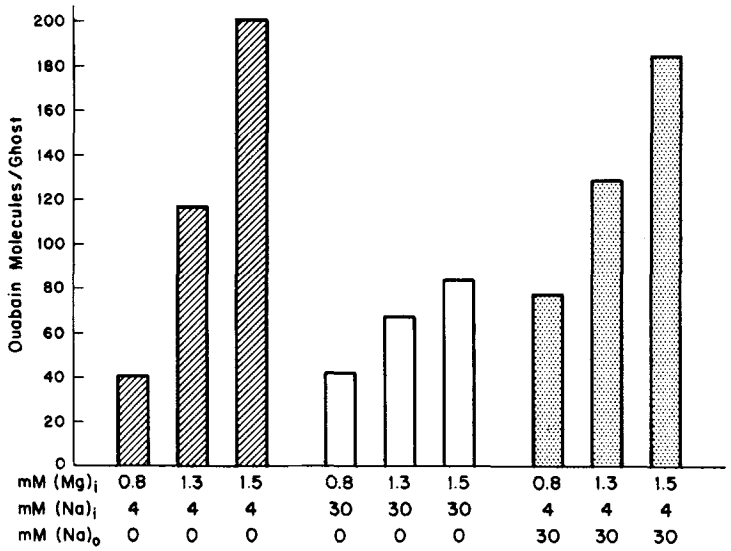

FIGURE 1. The effect of internal Mg on the rate of ATP-promoted ouabain binding to reconstituted ghosts. Ghosts were hemolyzed in a solution which contained 1.5 mM EDTA, 2.0 mM Na_2ATP, and either 0.8, 1.3, or 1.5 mM $MgCl_2$ as indicated and which had been brought to pH 7.4 with Tris. The calculated values of free Mg for these EDTA-$MgCl_2$ concentrations were 1.1×10^{-9} M, 6.5×10^{-9} M, and 1.2×10^{-6} M, respectively. After hemolysis, the ghosts were reversed by adding either concentrated choline Cl alone or together with concentrated NaCl to give a final concentration of either 140 mM choline Cl or 26 mM NaCl + 114 choline Cl. Thus, the intracellular concentrations (denoted by the subscript *i*) of the various constituents as given in the figure are defined by their concentrations in the hemolysis mixture (see Bodemann and Hoffman, 1976 *a*). Therefore, in addition to the above constituents the ghosts also contained 4 mM KCl and 7 mM Tris Cl. After resealing the ghosts were washed with 160 mM choline Cl buffered with 10 mM Tris Cl to pH 7.4. The ghosts were then incubated at 37°C for 30 min in a medium which contained the indicated concentrations of external Na (denoted by $[Na]_o$) plus choline Cl such that the sum, NaCl + choline Cl, was 150 mM together with 10 mM KCl, 10 mM Tris Cl (final pH 7.4), and 3×10^{-7} M [^{3}H]ouabain. As described previously (Bodemann and Hoffman, 1976 *a*) ouabain binding was stopped by adding iced choline Cl. The ghosts were centrifuged and washed before rehemolyzing them hypotonically in order to prepare hemoglobin-free ghosts which after solubilization in NCS (Nuclear-Chicago Solubilizer) were counted for [^{3}H]ouabain content. Protein analysis of an equivalent portion of hemoglobin-free ghosts was used to estimate the number of ghosts which were counted for [^{3}H]. The data presented represent the average of duplicate determinations. The difference between duplicate samples was less than five molecules ouabain per ghost. Several other comparable experiments gave results similar to those presented here. The measure of the rate at which ouabain binding occurred was taken as the number of molecules of ouabain bound per ghost during the 30-min exposure to ouabain.

binding rate is the same whether $[Na]_i$ is 4 or 30 mM. The inhibitory effect of Na_i on the ouabain binding rate in the presence of a relatively high concentration of Mg_i has already been described in a preceding paper (Bodemann and Hoffman, 1976 *a*) in which it was shown that this effect is a coupled action dependent upon $[K]_o$. Possible reasons for the loss of this effect at low concentrations of Mg_i will be considered later.

Fig. 1 also presents results on the ouabain binding rate when 30 mM Na_o is present in the medium. Compared with the ouabain binding rate in the absence of Na_o there is a stimulation of the rate by Na_o when the concentration of Mg_i is low (0.8 mM Mg + 1.5 mM EDTA). By increasing the concentration of Mg_i the ouabain binding rate is increased, but the stimulation by Na_o is lost. Studies presented in a preceding paper (Bodemann and Hoffman, 1976 *a*) showed that, under these conditions, increases in the rate of ouabain binding by Na_o were evidently due to lowering the affinity of the external surface of the pump to K_o. It was also shown that this effect of Na_o was lost when the concentration of K_o was raised sufficiently high, within its saturation range at least in ghosts containing relatively low concentrations of Na_i, the same as obtained in the type of experiments as shown in Fig. 1. In the present experiment (Fig. 1) a high concentration of K_o was also employed (10 mM) and, as expected, no change was observed in the ouabain binding rate at high values of $[Mg]_i$ when $[Na]_o$ was increased to 30 mM. But when $[Mg]_i$ was low (0.8 mM) increasing $[Na]_o$ significantly increased (twofold) the ouabain binding rate even when $[K]_o$ was 10 mM. This result implies that changes in $[Mg]_i$ can either affect the affinity of the outside of the pump for K_o or allow for a direct effect of Na_o or both. Since the possibility of an independent action of Na_o was seen only in ghosts containing high Na_i (see Bodemann and Hoffman, 1976 *a*) and since there does not appear to be any competition between Na_i and Mg_i (see below) we favor the alternative that lowering $[Mg]_i$ decreases the affinity of K_o.

The results presented in Fig. 2 and Table I explore in further detail these changes in the effects of Na_i and Na_o that occur on the ouabain binding rate at low values of $[Mg]_i$. Again, it is apparent (Fig. 2) that despite the presence of 6 mM K_o there is no effect of increasing $[Na]_i$, and further, that increasing $[Na]_o$ even by rather small amounts markedly increases the rate of ouabain binding. It is possible, of course, that in these circumstances, that is low $[Mg]_i$, ouabain binds to membrane sites which are noninhibitory with regard to the activity of the pump. The specificity of the binding can, however, be tested by measuring the activity of the Na,K-ATPase of ghosts. The results presented in Table II show that the observed changes in the ouabain binding rates that occur with changes in $[Na]_o$ are paralleled by a corresponding change in the inhibition of the Na,K-ATPase.

The loss of an effect of Na_i at low values of $[Mg]_i$ on the rate of ouabain binding was examined further by testing the coupled action of Na_i and K_o at high concentration (4 mM Mg) and a low concentration (0.8 mM Mg + 1.5 mM EDTA) of Mg_i. In the case of ghosts containing a low concentration of Mg_i a high concentration of [^{3}H]ouabain was added to the reaction medium in order to obtain comparable binding rates at both concentrations of Mg_i. High (8 mM) and

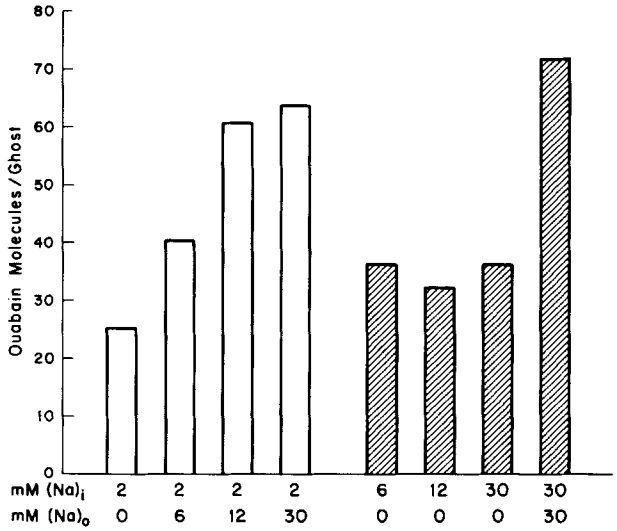

FIGURE 2. The effect of internal and external Na on the rate of ouabain binding to reconstituted ghosts containing a low concentration of free Mg. The experimental protocol was the same as outlined in the legend of Fig. 1 except that the hemolyzing solution contained 0.6 mM $MgCl_2$, 1.5 mM EDTA, 1.0 mM Na_2ATP, and 1 mM Tris ATP (final pH 7.4). (The calculated value of free Mg under these conditions was 0.7×10^{-9} M.) In addition, the incubation medium used to estimate ouabain binding contained 6 mM KCl, 10 mM Tris Cl (final pH 7.4), 5×10^{-7} M [^{3}H]ouabain, and the indicated concentrations of Na_o together with choline Cl such that their sum (Na + choline) totaled 154 mM. The results presented represent the average of duplicate determinations and are typical of at least two similar experiments. The measure of the rate of ouabain binding is taken as the number of molecules of ouabain bound per ghost during 30-min exposure to [^{3}H]ouabain.

low concentrations (0.2 mM) of K_o were present together with either high (40 mM) or low (2 mM) concentrations of Na_i. As shown in Table I the inhibition of the rate of ouabain binding is clearly dependent, at high $[Mg]_i$, on the combined presence of high $[Na]_i$ and high $[K]_o$. On the other hand, the ouabain binding rates obtained in the presence of a low concentration of Mg_i indicate that the coupled action of Na_i to K_o is completely lost. While Na_i no longer influences the rate of ouabain binding in this situation, there still is an inhibitory effect of K_o. Obviously this separate effect of K_o is reduced by the addition of Na_o in relatively small concentrations as shown before in Fig. 2. Therefore it is evident that the rates of ATP-promoted ouabain binding at low concentrations of Mg_i are insensitive to $[Na]_i$ but still respond to changes in $[Na]_o$ and $[K]_o$.

This information about the sidedness of action of Na and K on the ouabain binding rate as a function of $[Mg]_i$, makes it possible to interpret rationally results obtained in systems lacking sidedness in ways which would otherwise be puzzling if not misleading. This is illustrated by the results presented in Fig. 3. In these experiments the effect of Na, in the presence of K, was tested on the rate of ATP-promoted ouabain binding to porous ghosts carried out at two

TABLE I

EFFECT OF VARYING THE INTERNAL Mg CONCENTRATION ON THE ACTIONS OF Na_i AND K_o IN ALTERING THE RATE OF ATP-PROMOTED OUABAIN BINDING TO RECONSTITUTED GHOSTS

Incorporated inside	Concentration [^{3}H]ouabain	Internal NaCl	External KCl	Molecules ouabain bound per ghost
	M	*mM*	*mM*	
4.0 mM $MgCl_2$ (no EDTA)	5×10^{-8}	2	8.0	159,165
			0.2	161,187
		40	8.0	18,20
			0.2	106,108
0.8 mM $MgCl_2$ + 1.5 mM EDTA	3×10^{-7}	2	8.0	45,45
			0.2	179,203
		40	8.0	44,44
			0.2	191,204

This experiment was carried out as outlined in the legend of Fig. 1 except that the hemolyzing medium contained in addition to the indicated concentrations of $MgCl_2$ and EDTA, 1 mM Na_2ATP and 1 mM Tris ATP. After hemolysis and resealing the ghosts are estimated to also contain 4 mM KCl, 7 mM Tris Cl, and 140 mM sum of (NaCl + choline Cl). Exposure to [^{3}H]ouabain was carried out using a medium which also contained the indicated concentrations of KCl plus choline Cl such that the sum, NaCl + choline Cl, was 140 mM together with 20 mM NaCl and 10 mM Tris (final pH 7.4). Incubation was carried out for 30 min at 37°C. The results presented are representative of several other experiments of similar design in which similar results were observed. The measure of the rate of ouabain binding is taken as the number of molecules bound per ghost during 30-min exposure to [^{3}H]ouabain.

TABLE II

EFFECT OF EXTERNAL Na ON THE RATE OF ATP-PROMOTED OUABAIN BINDING TO RECONSTITUTED GHOSTS CONTAINING A LOW CONCENTRATION OF FREE Mg

Na_o	Molecules ouabain bound per ghost	Inhibition Na,K-ATPase	Molecules bound at 100% inhibition
mM		%	
0	73,75	28	264
12	123,123	54	228

The experiment was carried out as described in the legend of Fig. 1. The hemolyzing solution contained 0.6 mM $MgCl_2$, 1.5 mM Tris EDTA, 1 mM Na_2ATP, and 1 mM Tris ATP (final pH 7.4). The ghosts were reversed by addition of choline Cl and are therefore estimated to contain, in addition to the constituents present in the hemolyzing solution, 4 mM KCl, 8 mM Tris Cl, and 140 mM choline Cl. Ouabain binding was estimated after 60-min incubation at 37°C with the ghosts suspended in a medium which contained either 154 mM choline Cl (Na free) or 12 mM NaCl + 142 mM choline Cl together with 6 mM KCl, 10 mM Tris Cl (pH 7.4), and 6×10^{-7} M [^{3}H]ouabain. The amount of ouabain bound per ghost as well as its concomitant inhibition of the Na,K-ATPase were determined, with inclusion of suitable controls, on the ghosts made hemoglobin free after incubation in the presence and absence of [^{3}H]ouabain as described in Bodemann and Hoffman (1976 *a*). The rate of ouabain binding is indicated by the number of ouabain molecules bound per ghost after 60-min incubation in the presence of ouabain.

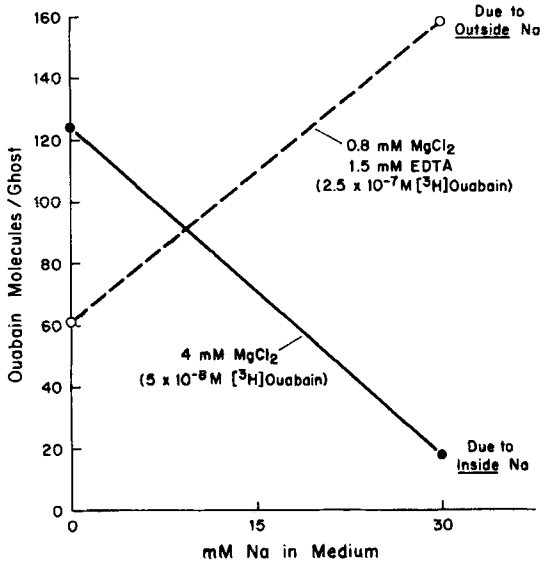

FIGURE 3. Alterations of the effect of Na on the rate of ATP-promoted ouabain binding to ghosts by varying the Mg concentration. Hemoglobin-free, frozen-thawed ghosts were incubated for 30 min at 37°C in a medium which contained either 30 mM choline (Na-free medium) or 30 mM NaCl together with 6 mM KCl, 1.0 mM Tris ATP, 5 mM Tris Cl, and as indicated, either 4.0 mM $MgCl_2$ or 0.8 $MgCl_2$ + 1.5 mM EDTA. The final pH was 7.4. [^{3}H]ouabain was also present as indicated, but note that different concentrations were used to adjust for the differences in the relative rates of ouabain binding that were obtained at the different Mg concentrations. The experiment and analyses were carried out in accordance with the methods described in a previous paper (Bodemann and Hoffman, 1976 *b*). The results presented are the average of duplicate measurements and are representative of the results obtained in two other similar experiments. The measure of the rate of ouabain binding is taken as the number bound per ghost after 30 min-exposure to [^{3}H]ouabain at 37°C.

different concentrations of Mg (the low concentration being buffered, as before, with EDTA). Again the concentration of [^{3}H]ouabain was increased at the low Mg concentration in order to keep the rates of ouabain binding comparable. The results show that the addition of Na *decreases* the rate of ouabain binding when the concentration of Mg is high but *increases* the rate, when Mg is low. From the sidedness of action as already developed, it is clear that when the addition of Na is seen to decrease the rate it is due to Na acting on the inside and is coupled to the presence of K on the outside. It is also clear, that at the low Mg concentration, when the addition of Na increased the rate, it is due to Na acting on the outside presumably decreasing the membrane's affinity for external K.

Mg and P_i-Promoted Binding

Table III contains the rates of P_i-promoted ouabain binding to porous (part A) and to reconstituted ghosts (part B) in the presence of different concentrations of Mg and Na. The results obtained using either porous or reconstituted ghosts were the same in the sense that increasing the concentration of Mg increased the

TABLE III

RATE OF P_i-PROMOTED OUABAIN BINDING TO HUMAN RED CELL GHOSTS AS AFFECTED BY VARYING THE Mg AND Na CONCENTRATIONS

Part A, Frozen-Thawed Ghosts

Incubation Medium					Molecules ouabain bound per ghost
EDTA	KCl	NaCl	Choline Cl	$MgCl_2$	
mM	*mM*	*mM*	*mM*	*mM*	
1.5	6	0	30	0.8	−4,−8
				1.3	27,49
				1.5	133,136
		30	0	0.8	−1,−1
				1.3	9,18
				1.5	30,34

Part B, Reconstituted Ghosts

	Inside				Molecules ouabain bound per ghost
Outside choline	EDTA	Na	Choline Cl	Mg	
mM	*mM*	*mM*	*mM*	*mM*	
160	1.5	0.5	140	0.8	26,26
				1.3	102,187
				1.5	221,226
		50	90	0.8	4,6
				1.3	15,16
				1.5	30,30

For the experiment presented in part A, hemoglobin-free, frozen-thawed ghosts were incubated for 60 min at 37°C in a medium which in addition to the constituents given in the table contained 1.0 mM H_3PO_4, 10 mM Tris Cl, and 1×10^{-6} M [^{3}H]ouabain. The final pH was 7.4. The experiments presented in parts A and B were carried out on different batches of ghosts prepared in different ways. For the experiment presented in part B, reconstituted ghosts were prepared to contain, in addition to the constituents listed, 2 mM P_i, 4 mM KCl, and 8 mM Tris Cl. Ouabain binding was carried out by suspending these ghosts in a medium which contained 160 mM choline Cl, 10 mM Tris Cl, and 6×10^{-7} M [^{3}H]ouabain. The final pH was 7.4. Both types of experiments and analyses were carried out in accordance with the methods given previously by Bodemann and Hoffman (1976 *a*, *b*). The results presented in both parts A and B are representative of the results obtained in two similar types of experiments. In each case the measure of the rate of ouabain binding is taken as the number of molecules of ouabain bound per ghost after 60-min (part A) and 30-min (part B) exposure at 37°C to [^{3}H]ouabain.

rate of ouabain binding and this stimulation was accompanied by an inhibitory action of Na on the binding rate. In other words, to the extent that P_i-promoted ouabain binding can be stimulated, it utilizes, as shown in part B, inside Mg and is inhibited by Na_i. However, in the presence of low concentrations of Mg_i, where the inhibitory effect of Na_i on ATP_i-promoted ouabain binding has disappeared (Table I), no significant rate of P_i-promoted ouabain binding can be observed. While it is known that P_i-promoted ouabain binding requires the presence of Mg (Matsui and Schwartz, 1968) the difference in whether or not binding occurs is presumably associated with the difference in the concentration dependence of ouabain binding on P_{ii} compared to ATP_i (see Bodemann and Hoffman, 1976 *b*).

Regulation of Na:Na and Na:K Exchange by $[Mg]_i$

The rate constants for ouabain-sensitive Na efflux from reconstituted ghosts into a K-containing or a K-free medium are shown in Table IV as a function of the concentration of Mg_i. Both fluxes, which represent Na:K and Na:Na exchange, respectively, are clearly stimulated by Mg_i. However, the stimulation which occurs in Na:Na exchange is less pronounced than that seen in Na:K exchange indicating that Na:K exchange is more sensitive than Na:Na exchange to the Mg_i concentration at least for the ranges studied. For instance, by raising

TABLE IV

EFFECT OF VARYING INTERNAL Mg ON Na:Na VERSUS Na:K EXCHANGE IN ATP CONTAINING RECONSTITUTED GHOSTS

	Hemolysis solution			Rate constants for Na efflux (h^{-1})				Rate constants for ouabain-sensitive Na efflux	
				12 K medium		K-free medium			
Exp.	Mg	EDTA	ATP	Alone	Ouabain	Alone	Ouabain	12 K medium	K-free medium
	mM	*mM*	*mM*						
A	0.8	1.5	2.0	0.704	0.354	0.675	0.344	0.350	0.331
	2.5	1.5	2.0	1.084	0.311	0.827	0.306	0.773	0.521
B	0.8	1.5	3.0	0.668	0.288	0.443	0.314	0.380	0.129
	2.5	1.5	3.0	1.105	0.223	0.524	0.283	0.882	0.241
C	0.8	1.5	3.0	0.421	0.210	0.306	0.194	0.211	0.112
	1.5	1.5	3.0	0.694	0.215	0.341	0.216	0.479	0.124
	3.5	–	3.0	0.710	0.070	0.296	0.085	0.640	0.211

Ghosts were hemolyzed in solutions containing the indicated concentrations of $MgCl_2$, EDTA, and Na_2ATP together with a trace quantity of $^{24}NaCl$. The preparation of the ghosts after hemolysis was carried out as described in the legend of Fig. 1. Therefore the ghosts contained, in addition to the above constituents, 4 mM KCl, 135 mM choline Cl, and 7 mM Tris. Final pH of the hemolyzing solution was 7.4. The efflux of ^{24}Na was measured, as described previously (Bodemann and Hoffman, 1976 *a*), into a medium which was either K free or contained 12 mM KCl. The K-free medium contained 160 mM NaCl + 10 mM Tris Cl. The 12 mM K medium contained, in addition 10 mM Tris Cl, 148 mM NaCl in experiment A or 6 mM NaCl + 142 mM choline Cl for experiments B and C. The concentration of ouabain when present was 1×10^{-4} M. The final pH of all media was 7.4. The efflux of ^{24}Na was measured at 37°C and samples were taken after 10-, 40-, and 70-min incubation. The outward rate constants given in the table represent the average of duplicates of the two 30-min time periods. The ouabain-sensitive ^{24}Na efflux which occurred in the K-free media was taken as a measure of Na:Na exchange; that which occurred in the 12 mM K media was taken to indicate the extent of Na:K exchange.

the $[Mg]_i$ from 0.8 to 2.5 mM for experiments A and B, the average increase in Na:K exchange is 126% compared to 72% for Na:Na exchange (the rate constants at 2.5 mM Mg_i are known from separate experiments to represent essentially maximum values for the particular conditions under which these determinations were made). It is thus possible to develop the circumstance (exp. C) in which no stimulation of Na:Na exchange occurs under conditions where the rate of Na:K exchange is increased. Variations in conditions were not explored to see if Na:Na exchange could take place when Na:K exchange was completely inhibited.

Effect of Ca on MgATP-Promoted Ouabain Binding

Previous work has shown that Na:K exchange (and Na,K-ATPase) is inhibited by Ca_i (see Hoffman, 1962). In contrast, ouabain binding to porous ghosts promoted by MgATP is essentially insensitive to added Ca and Ca cannot replace Mg in stimulating the rate of ouabain binding (Hoffman, 1969). While it was desirable in the context of the present experiments to evaluate the action of Ca in the light of the effects of Na_i and K_o on the rate of ouabain binding promoted by ATP, this was not feasible since the ATP-dependent Ca pump (Schatzmann, 1966; Schatzmann and Vincenzi, 1969) would extrude any incorporated Ca presumably before its effect on ouabain binding could be measured. Therefore, the effect of Ca was examined in porous ghosts in the presence of MgATP, Na, and/or K as presented in Table V. Changes in the ouabain binding rates due to the presence of Na and/or K were partly compensated by using different concentrations of [^{3}H]ouabain. Inhibition of the binding rate by K and even more by the combination, Na + K, is clearly recognizable particularly when the different concentrations of [^{3}H]ouabain are taken into account. Only a small inhibitory effect of Ca on the ouabain binding rate is observed in the presence of Na alone, in agreement with previous studies (Hoffman, 1969; Tobin et al., 1973). However, in the absence of Na, the inhibitory effect of Ca on the ouabain binding rate was greatly increased. The binding rate in the presence of K but in the absence of Na was also inhibited, due presumably to K:K exchange (see Bodemann and Hoffman, 1976 *b*), but even with this complication, Ca further reduced the ouabain binding rate seen with K alone. The effect of Ca in inhibiting ouabain binding is also apparent when both Na + K are present, the

TABLE V

EFFECT OF Ca ON THE RATE OF ATP-PROMOTED OUABAIN BINDING TO GHOSTS

Medium additions			Molecules ouabain bound per ghost	
NaCl	KCl	[^{3}H]ouabain	Control	+0.6 Ca
mM	*mM*	*M*		*mM*
0	0	3.7×10^{-5}	149,151	67,71
40	0	2.5×10^{-5}	138,140	108,111
0	6	1×10^{-4}	88,88	51,55
40	6	2.5×10^{-4}	78,78	10,12

Hemoglobin-free, frozen-thawed ghosts were incubated for 15 min at 37°C in a medium which, in addition to the constituents shown, contained 1 mM Tris ATP, 0.25 mM EDTA, and 2 mM $MgCl_2$. The final pH was 7.4. While the concentrations of Na and K are given in the table, choline Cl was added such that the sum of NaCl + KCl + choline Cl was 46 mM in all cases. Ca when present was added as $CaCl_2$. The concentration of [^{3}H]ouabain was varied in an attempt to keep the ouabain binding rate in the presence of the different concentrations of Na, K, and Ca approximately comparable. The experiment and analyses were carried out in accordance with the methods given in a previous paper (Bodemann and Hoffman, 1976 *b*). The results presented in this table are duplicate analyses and are representative of similar results obtained in three other experiments of the same design. The measure of the rate of ouabain binding is taken as the number of ouabain molecules bound per ghost after 15-min exposure to

extent of the inhibition being much greater in this situation than when Na alone (or K alone) is present or when there is no Na or K. Thus, the inhibitory action of Ca is seen to be most marked when the ouabain binding rate has already been reduced by the combined action of Na + K.

DISCUSSION

Much of the evidence reported in this paper is consistent with the suggestion made by Fahn et al. (1966 *a,b*) that the formation of the enzyme conformation E_1 and its transition to E_2 (see Albers et al., 1968) depends upon the Mg concentration present in the system. To the extent that ouabain binding can be used as an indirect measure of this dependence (see Bodemann and Hoffman, 1976 *a*), correlations can be developed between certain conformational states and the sidedness of action of Na and K. For the purposes of this paper it can therefore be assumed that the formation of $E_1 \cdot ATP$ or $E_1 \lessgtr^{P}_{ADP}$ (see Bodemann and Hoffman, 1976 *a*) is associated with a low Mg requirement whereas a high concentration of Mg is needed for the formation of E_2P. As reported previously (Bodemann and Hoffman, 1976 *a*) and also shown in Table II, the rate of ouabain binding, as promoted by ATP_i at high concentrations of Mg_i, can be directly antagonized by the combined action of Na_i and K_o. However, when $[Mg]_i$ is low (see Fig. 1, Table II) the coupling between Na_i and K_o is lost and the ouabain binding rate is independent of changes in $[Na]_i$. Presumably the reason for this is that since only E_2P is sensitive to K (Post et al., 1969; Post et al., 1972) the low concentration of Mg_i inhibits its formation and therefore slows the cycling of the pump complex, which under ordinary conditions (high $[Mg]_i$) would respond to the combined presence of Na_i and K_o. Since the rate of ouabain binding appears to be inversely related to the rate of Na:K transport (Bodemann and Hoffman, 1976 *a*) inhibition of the latter by reducing $[Mg]_i$ essentially prevents seeing any dependence on $[Na]_i$. The increased sensitivity of the outside of the pump to the action of Na_o when $[Mg]_i$ is low (Fig. 2) presumably indicates that the affinity for K_o is also reduced, perhaps reflecting that the drag on the transition of the E_1 form to E_2P is also reduced. In any event, the dissociation of either E_1ATP or $E_1 \lessgtr^{P}_{ADP}$ by K_o as discussed previously (Bodemann and Hoffman, 1976 *a*) would account for the diminished ouabain binding rate in this situation.

The shift in the side specificity of the actions of Na and K in being limited to the outside surface at low concentrations of Mg provides the basis for a different explanation of the results obtained by Skou et al. (1971). They studied the Na,K-ATPase activity of brain microsomes in which similar types of maneuvers in the incubation conditions were carried out in the presence of *g*-strophanthin. They found that the level of inhibition was diminished (and therefore less glycoside bound) when the Mg concentration was lowered, but could be increased back to the original level by increasing the concentration of Na. On the other hand, the amount of Mg had to be increased in the presence of K to maintain the same level of ouabain inhibition (Skou, 1974). Instead of invoking an antagonism between Mg and Na to explain these results it would appear more reasonable on

the basis of the present findings (cf. Fig. 3) that what occurred at low Mg, was that inhibition by Na_i was lost in conjunction with a concomitant stimulation by Na_o. The unlikelihood of a direct interaction between Na and Mg has also been discussed elsewhere with regard to the effects of changes in $[Na]_i$ at constant $[Mg]_i$ (Bodemann and Hoffman, 1976 *a*). In addition, we have found in separate experiments that Na:K transport, an exchange which requires Mg (Table IV), is not stimulated by Na_o at low concentrations of Mg_i.

P_i-promoted ouabain binding is not observed at low concentrations of Mg (Table III). If we accept the idea that P_i-promoted ouabain binding indicates that a phosphorylated intermediate is involved (e.g. E_2P) and is always diminished by Na_i (see Bodemann and Hoffman, 1976 *b*), then ATP-promoted ouabain binding can be taken to reflect binding to an E·ATP-type complex when the concentration of Mg_i is low. Disappearance of inhibition of ATP-promoted ouabain binding by Na_i and loss of P_i-promoted ouabain binding may indicate that a phosphorylated intermediate need not be present for binding to occur.

ATP binding to a Na,K-ATPase has been studied under conditions where no hydrolysis of the nucleotide triphosphate occurs (Hansen et al., 1971; Hegyvary and Post, 1971). As demonstrated by Hegyvary and Post (1971) ATP binding, in the absence of Mg, was slightly stimulated by Na, while the addition of K alone dissociated ATP from its binding site. Addition of Na reversed this action of K and allowed ATP to be bound once again. If the rate of ATP-promoted ouabain binding at low concentrations of Mg is taken as a measure of the amount of E·ATP present then the formation of this complex would appear to be regulated exclusively by Na_o and K_o.

The fact that the fractional activation of Na:Na exchange is greater than Na:K exchange at low values of $[Mg]_i$, as indicated in Table IV, is consistent with Na:Na exchange being associated with transphosphorylation of the E_1-type conformation (e.g. $\dot{E}$·ATP, $E\begin{smallmatrix}\nearrow P \\ \searrow ADP\end{smallmatrix}$, E_1P) as discussed by Glynn and Hoffman (1971) since the low Mg_i concentration would inhibit the transition of the E_1 type to the E_2 forms presumably required for the pump complex to cycle through in carrying out Na:K exchange. On the other hand, since the rate of ouabain binding is unaffected by the rate of Na:Na exchange (Bodemann and Hoffman, 1976 *a*) in contrast to Na:K exchange as already mentioned, the effects of Mg_i must be associated with the formation of the E_1-type conformations in order to affect binding. Thus it appears that the rates of formation of the E_1 types and not their transition times limit the ouabain binding rate.

With regard to the action of Ca on the ouabain binding rate (Table V), the most dramatic effect is seen when Na and K are present together. This is so even though Ca reduces the binding rate when Na or K are present individually. These effects of Ca obtained on human red cell ghosts are evidently different from the way Ca affects ATP-promoted ouabain binding to microsomal Na,K-ATPase prepared from pig heart (Schön et al., 1970), rat brain (Tobin et al., 1973, 1974), or ox brain (Hansen, 1974). In these latter instances Ca appears to act like Na since, in the presence of Mg and ATP, the addition of Ca increases the extent of ouabain binding. Perhaps this type of action of Ca in microsomal

Na,K-ATPase is a consequence of a possible contamination of these preparations with K as suggested previously (Bodemann and Hoffman, 1976 *a*). In any case, the difference in these effects again emphasizes that there are differences in the observed characteristics between microsomal preparations and porous ghost systems. Just why the ATP-promoted ouabain binding rate is maximally affected by Ca when both Na and K are present (Table V) is not clear, particularly since it has been shown by Knauf et al. (1974) that Ca in this circumstance prevents K from dephosphorylating the Na-dependent phosphorylated form of the red cell Na,K-ATPase. This result would imply that K in interacting with the pump complex in the presence of Ca (and Na) either forces a conformation in which the ouabain binding site is occluded (e.g. shifting the $A \rightleftharpoons B$ equilibrium, as described by Bodemann and Hoffman (1976 *a*) to the left) or perhaps changes the dissociation constant of the ouabain binding site as suggested by Tobin et al. (1974) and Hansen (1974). This latter type of experiment has not as yet been carried out using porous red cell ghosts but the chances that this kind of effect is operative is minimal since in red cells only changes in rate and not in equilibrium have so far been observed with ouabain binding (Hoffman, 1966; Ingram, 1970).

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