Comparison of the Side-Dependent Effects of Na and K on Orthophosphate-, UTP-, and ATP-Promoted Ouabain Binding to Reconstituted Human Red Blood Cell Ghosts

H. HARM BODEMANN and JOSEPH F. HOFFMAN

From the Department of Physiology, Yale University School of Medicine, New Haven, Connecticut 06510. Dr. Bodemann's present address is the Medizinische Universitaetsklinik, Freiburg im Breisgau, Germany.

ABSTRACT This paper is concerned with analyzing the sidedness of action of various determinants which alter the rate of ouabain binding to human red blood cell ghosts. Thus, ouabain binding promoted by orthophosphate (P_i) and its inhibition by Na are shown to be due to inside P_i and inside Na. External K inhibits P_i-promoted ouabain binding and Na_o acts to decrease the effectiveness of K_o. Similarly, inside uridine triphosphate (UTP_i) stimulates the rate of ouabain binding which can be antagonized by either Na_i or K_o acting alone. The actions of Na_i and K_o are different when ouabain binding is promoted by P_i and UTP_i compared to inside adenosine triphosphate (ATP_i). With ATP_i, the ouabain binding rate is only affected when Na_i and K_o are both present. Possible differences in the mechanism of action of K and Na on P_i- and UTP-promoted binding are discussed in the light of their sidedness of action.

INTRODUCTION

An important key for the understanding of the function of the Na:K transport system is the side specificity of its modifying parameters. Cardiotonic steroids such as ouabain inhibit the pump by attachment to a site located on the outside surface of the pump complex (Caldwell and Keynes, 1959; Hoffman, 1966) and the rate of attachment can be influenced by the presence of nucleotide triphosphates (such as uridine triphosphate, UTP, or adenosine triphosphate, ATP) when present at the inside surface of the pump (see Bodemann and Hoffman, 1976 *a*). As shown by Matsui and Schwartz (1968) and Albers et al. (1968) ouabain binding can also be stimulated by orthophosphate (P_i) and this binding is inhibited by the presence of Na. Since these effects have been observed either in microsomal preparations or in porous ghosts systems where the membrane is permeable to the added constituents, the side dependence of the observed effects is unknown.

The work presented in this paper examines, in terms of ouabain binding rates, the sidedness of action of P_i and the side-dependent effects of Na and K on P_i as well as UTP-promoted binding using reconstituted ghosts. These effects

are compared to the action of ATP under comparable conditions. Brief accounts of this work have been presented previously (see Hoffman, 1972 and 1973).

MATERIALS AND METHODS

Except as described below, the ghost preparation, experimental procedures, and analytical techniques used in this paper are the same as those previously described (Bodemann and Hoffman, 1976 *a*). Two additional ghost preparations were used in the present work. One type utilized hemoglobin-free ghosts which had been frozen-thawed to make them permeable to all added substances. This type of porous ghosts was prepared according to the method given in Heinz and Hoffman (1965) and ouabain binding was carried out according to the procedure given in Hoffman (1969). Reconstituted ghosts were also used as made before, for studying the side-dependent effect of P_i and UTP but were prepared from energy-depleted as well as from fresh red cells. Energy depletion was carried out in the following way (see Kregenow and Hoffman, 1972): Freshly drawn, heparinized blood was centrifuged at 14,500 *g* for 3 min. The concentrated but unwashed cells were resuspended at a hematocrit of approximately 15–20% in a medium which contained 125 mM KCl + 40 mM NaCl + 10 mM Mg-glycylglycine (pH 7.4). After adding approximately 1 mg/100 ml chloramphenicol the suspension was incubated at 37°C for 20–24 h with gentle shaking. At the end of incubation, the suspension was again centrifuged and the cells washed three times at 0°C using for each wash 5-vol solution which contained 160 mM choline Cl + 10 mM Tris Cl (pH 7.4). The cells were resuspended in 170 mM Tris Cl (pH 7.4) at a hematocrit of 50% and hemolyzed as described in the previous paper (Bodemann and Hoffman, 1976 *a*). The composition of the hemolyzing solution was varied and is specified in connection with the relevant experiments. After resealing, the ghosts were again washed at 0°C three times with 5 vol of the choline Cl-Tris Cl solution used as before for each wash.

In some of the experiments dealing with the sidedness of action of P_i on ouabain binding, resealed ghosts which had been prepared as described above from energy-depleted cells were exposed to SITS (4-acetamido-4-isothiocyano-stilbene-2,2-disulfonic acid). Thus, following the procedure used for intact cells (Knauf and Rothstein, 1971), ghosts were suspended at a 30% hematocrit in 170 mM NaCl containing 0.3 mg SITS per gram of packed ghosts and incubated for 15 min at 22°C. Afterwards the ghosts were returned to 0°C and washed twice more with the choline Cl-Tris Cl solution before being used in ouabain binding studies. In other experiments, resealed ghosts, instead of being pretreated with SITS, were exposed to SITS at the same time that they were exposed to [^{3}H]ouabain, that is, during incubation at 37°C. Evidently SITS acts with sufficient rapidity that the permeability of resealed ghosts to P_i is reduced almost immediately. Measurements of $^{32}P_i$ efflux (Gardos et al., 1969) into either 160 mM sodium or choline Cl (together with 10 mM Tris Cl) under the conditions used for ouabain binding indicated that P_i permeability was reduced at least by 75% within the first 15-min incubation. In either case SITS reduces the P_i permeability of the membrane to low enough values such that the sidedness of P_i is insured during measurement of ouabain binding.

RESULTS

Concentration and Side Dependence of P_i Action

Fig. 1 shows the effect of varying concentrations of orthophosphate (P_i) on the rate of ouabain binding to porous ghosts carried out in the absence of Na and K. The concentration of P_i for half-maximum effects on the ouabain binding rate is

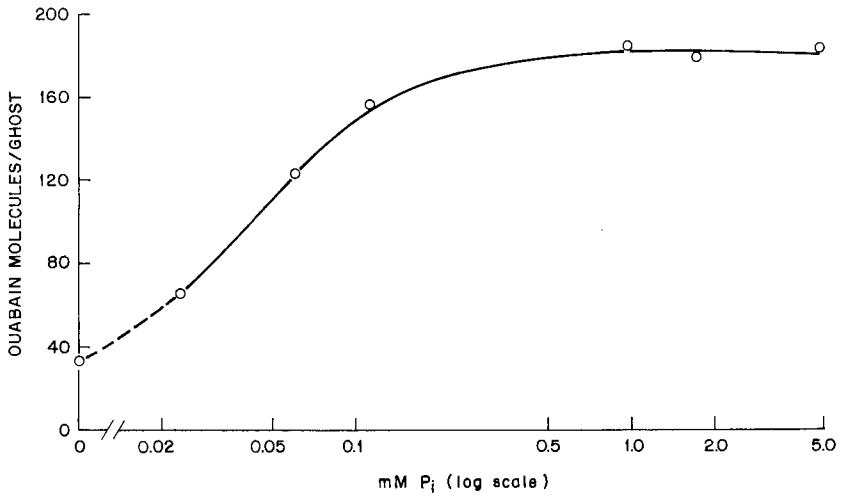

FIGURE 1. The concentration dependence of inorganic phosphate (P_i) in promoting the rate of binding of ouabain to human red cell ghosts. Hemoglobin-free, frozen-thawed ghosts were incubated for 30 min at 37°C in a medium which contained 40 mM choline Cl, 1.25 mM $MgCl_2$, 0.25 mM EDTA, 10 mM Tris Cl (final pH 7.5), 3.4×10^{-7} M [^{3}H]ouabain, and the indicated concentrations of P_i, added as H_3PO_4. Ghosts were added to paired flasks in the presence and absence of unlabeled ouabain (10^{-4} M). After incubation the ghosts were washed three times at 0°C using approximately 20 vol wash solution for each wash (5 min, 27,000 *g*). The wash solution used was the same as the incubation medium except that it was free of ouabain and P_i. After washing, portions of the packed ghosts were pipetted using an Aliquanter (Hamilton Co., Reno, Nev.) and dissolved in Nuclear-Chicago Solubilizer (NCS), diluted with toluene containing PPO (2,5-diphenyloxazole) and POPOP (1,4-bis[2-(5-phenyloxazolyl)]benzene) and counted for [^{3}H]. The number of molecules of ouabain bound per ghost was calculated as before (Bodemann and Hoffman, 1976 *a*), knowing the number of ghosts present and the specific activity of the [^{3}H]ouabain, from the difference between the counts per minute obtained from each set of paired flasks (i.e. $\pm 10^{-4}$ M unlabeled ouabain). The number of ghosts pipetted into NCS was estimated by counting, after suitable dilution with isotonic saline, using a celloscope (Particle Data Corp., Elmhurst, Ill.). The values presented represent the average of duplicate determinations. Similar results were obtained in two other experiments. The measure of the rate of ouabain binding is taken as the number bound per ghost after 30-min exposure at 37°C.

approximately 50 μM, while a maximum effect is achieved in the presence of a P_i concentration close to 0.5 mM. These values correspond well with those observed in brain microsomes by Skou et al. (1971) under similar conditions. On a comparative basis it takes about five times more P_i than ATP or UTP to get the same ouabain binding rate (see Hoffman, 1969). We have previously shown that nucleotide triphosphates, such as ATP and UTP, promote ouabain binding only when present in the intracellular medium (Bodemann and Hoffman, 1976 *a*). Similarly the sidedness of P_i in promoting glycoside binding is of interest but is complicated by the fact that reconstituted ghosts, like intact cells, are rather permeable to P_i.

This difficulty can be overcome by pretreating reconstituted ghosts with SITS, an agent known to decrease the membrane permeability to anions, such as sulfate (Knauf and Rothstein, 1971) and we have shown that SITS produces comparable inhibition of P_i permeability. The results of this type of experiment are shown in Table I, in which varying concentrations of P_i were incorporated

TABLE I

SIDEDNESS OF THE EFFECT OF P_i IN PROMOTING THE RATE OF OUABAIN BINDING TO RED CELL GHOSTS

Inside	SITS added	Outside	Molecules ouabain bound per ghost
mM		*mM*	
0.05 P_i	+	–	33,45
0.2 P_i	+	–	80,90
0.75 P_i	+	–	111,113
2.0 P_i	+	–	113,116
0 P_i	+	2.0 P_i	11,17
2.0 ATP	–	–	206,226
2.0 ATP	+	–	184,186

The reconstituted ghosts used in this experiment were made from energy-depleted red cells. The cells (50% suspension in 170 mM Tris Cl, pH 7.4) were hemolyzed with 10 vol of solution which contained 4 mM $MgCl_2$. The indicated concentrations of P_i were incorporated inside by adding appropriate amounts of a concentrated solution of H_3PO_4 (brought to pH 7.4 with Tris) to the hemolysis mixture 2 min after the cells had been hemolyzed. Then 2 min after the addition of P_i the ghosts were reversed by adding sufficient concentrated solution (3.22 M) to give a final concentration of 150 mM choline Cl and 4 mM NaCl. The ghosts were then resealed by incubation at 37°C for 60 min and washed twice (at 0°C, 34,800 *g*) with 20 vol of solution containing 160 mM choline Cl and 10 mM Tris Cl, pH 7.4. When ATP was to be incorporated inside, the procedure was altered such that the cells were hemolyzed in 2 mM Na_2ATP + 4 mM $MgCl_2$ and reversed with a solution containing only 154 mM choline Cl. These procedures provide ghosts which, while differing in their contents of P_i or ATP, have the same intracellular composition, estimated to be 4 mM NaCl, 4 mM KCl, 8 mM Tris Cl, and 150 mM choline Cl (see Bodemann and Hoffman, 1976*a*). Ouabain binding was carried out by suspending these ghosts in a medium which contained 6 mM NaCl, 1 mM KCl, 155 mM choline Cl, 10 mM Tris Cl, and 5×10^{-7} M [^{3}H]ouabain. When indicated, 0.3 mg SITS/g ghosts was present in the labeling medium. Incubation was carried out for 15 min at 37°C. The suspension was then centrifuged, the ghosts washed, and [^{3}H]content determined by counting as previously described (Bodemann and Hoffman, 1976*a*). The relative rate of ouabain binding is indicated by the number of molecules of ouabain bound per ghost during the 15-min exposure period. The results presented are typical of several similar experiments.

into ghosts before they were reconstituted. Also shown is the effect of P_i on the ouabain binding rate to reconstituted ghosts when P_i is present only in the external medium. In this way the sidedness of action of P_i on the rate of ouabain binding could be assessed. The different rates of ouabain binding are summarized in Table I as a function of $[P_i]_i$ compared to $[P_i]_o$. It is apparent that the rate of ouabain binding is not affected by P_{i_o}. The concentration of P_{i_i} which

gives half-maximum ouabain binding rate appears to be within a factor of two to that observed for ouabain binding to porous ghosts (Fig. 1) and is about 100 μM. This type of agreement is reasonable since the actual concentration of P_{ii} in the reconstituted ghosts was certainly less than the indicated concentration of incorporated P_{ii}. Table I also contains results obtained on reconstituted ghosts with incorporated ATP in order to show that SITS treatment of the ghosts per se does not change the ouabain binding rate significantly. The difference between ATP and P_i containing ghosts in the values of numbers of ouabain molecules bound is presumably referable to the differences in the hemolysis conditions which obtained during their preparation. This sidedness of action of P_i on ouabain binding has also been observed independently by Lishko et al. (1972).

Side-Specific Effects of Na and K on P_i-Promoted Ouabain Binding

The results presented in Fig. 2 show that the rate of P_i-promoted binding of ouabain to porous ghosts changes as a function of the concentration of Na in the incubation medium. The binding rates are clearly diminished by Na. It is also apparent that a higher Na concentration is required for reducing the ouabain binding rate when the concentration of P_i is increased.

For studying the sidedness of the action of Na on the ouabain binding rate it is necessary to use reconstituted ghosts. Thus two different concentrations of Na_i were incorporated (osmotic balance maintained with choline Cl) and the ouabain binding rates as well as the Na,K-ATPase activities associated with the different quantities of bound ouabain were determined. The results are shown in Table II: Na_i diminishes the ouabain binding rates in the presence of P_{ii} regardless of

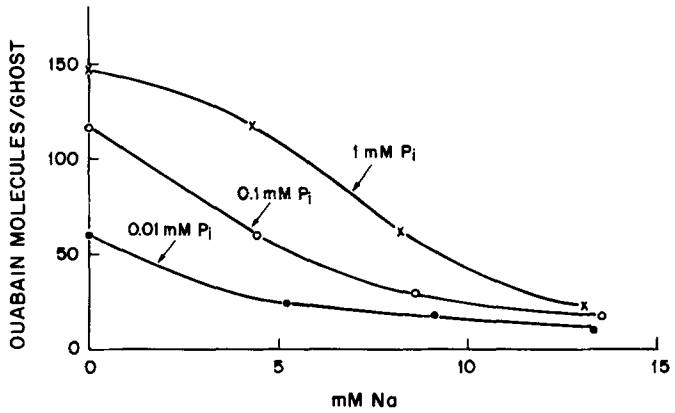

FIGURE 2. The effect of Na in inhibiting the rate of P_i-promoted ouabain binding to hemoglobin-free, frozen-thawed ghosts. The experimental procedure used was the same as that described in the legend of Fig. 1, except that the ghosts were incubated in a medium which contained the indicated concentrations of Na, where the quantity, NaCl + choline Cl, was 40 mM together with either 1.0, 0.1, or 0.01 mM P_i (added as H_3PO_4), 1.25 mM $MgCl_2$, 0.25 mM EDTA, 10 mM Tris Cl (final pH was 7.5), and 3.4×10^{-7} M [^{3}H]ouabain. The results presented are representative of several similar experiments. The measure of the rate of ouabain binding is taken as the number bound per ghost after 30-min incubation at 37°C.

TABLE II

EFFECT OF INTERNAL Na ON THE RATE OF P_i-PROMOTED OUABAIN BINDING TO RECONSTITUTED GHOSTS

Inside						
P_i	Na	Choline	SITS treated	Molecules ouabain bound per ghost	% I	No./100% I
mM	*mM*	*mM*				
1	5	150	−	112,122	53	221
1	150	0	−	4,6	3	−
1	5	150	+	130,136	57	233
1	150	0	+	6,6	3	−
1	5	150	−	−	0	−
1	5	150	+	−	−2	−

The reconstituted ghosts were made from energy-depleted cells following the procedure described in the legend of Table I except that when 150 mM NaCl was to be incorporated inside, the ghosts were reversed with concentrated NaCl rather than with choline Cl. Thus the ghosts contain in addition to the indicated constituents, 4 mM KCl, 4 mM $MgCl_2$, and 8 mM Tris Cl. Ouabain binding was carried out by incubating the ghosts at 37°C for 30 min in a medium which contained 1 mM KCl, 6 mM NaCl, 155 mM choline Cl, 10 mM Tris Cl, and 4×10^{-7} M [^{3}H]ouabain. The final pH was 7.4. When indicated SITS was present in the labeling medium. The amount of ouabain bound per ghost as well as the percent inhibition (% I) of the Na,K-ATPase due to the bound ouabain were determined 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*). Appropriate controls (bottom pair) which were carried through the incubation without exposure to ouabain were also included. The results presented represent duplicate determinations and are typical of the results obtained in several similar experiments. The rate of ouabain binding is taken as the number of ouabain molecules bound per ghost after 30-min exposure to [^{3}H]ouabain.

whether or not the ghosts had been treated with SITS. It is also apparent that the number of ouabain molecules bound result in comparable levels of inhibition of the Na,K-ATPase whether pretreated with SITS or not. Two control estimates given at the bottom of the table refer to ghosts which were incubated in the absence of ouabain in order to evaluate the effect of SITS. It is obvious that the activity of the Na,K-ATPase is not altered by pretreatment with SITS. The results presented in Fig. 3 extend the type of observations given in Table II and show the effects of different concentrations of Na_i on the ouabain binding rate. $[Na]_o$ was kept low to avoid the possible effects of external Na leaking into the ghosts and thereby altering the internal concentration. The ouabain binding rate clearly drops with increasing concentrations of Na_i. A half-maximum effect is seen to occur under those experimental conditions when $[Na]_i$ is about 10 mM. This concentration is the same as that observed for Na_i when ouabain binding was being promoted by incorporated ATP provided in this circumstance that K_o was also present (Bodemann and Hoffman, 1976 *a*). For the results presented in Fig. 3 the saturating concentration of Na_i is about 40–50 mM. This value of the Na concentration has also been obtained for P_i-promoted ouabain binding to a microsomal preparation (Hansen and Skou, 1973), when the composition of the reaction medium was similar to the composition of the intracellular fluid used in this experiment.

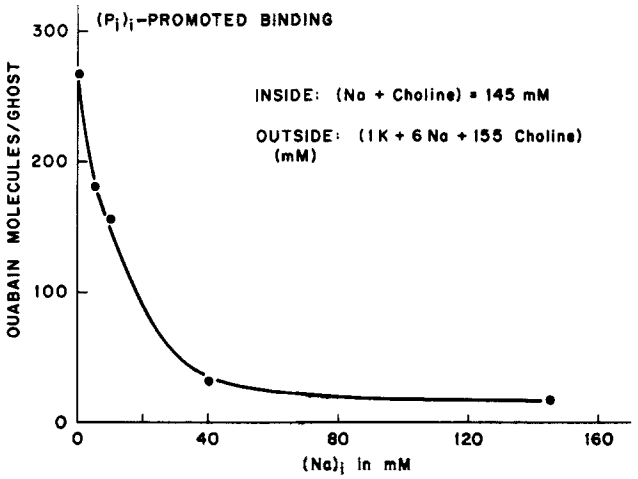

FIGURE 3. The effect of varying the concentration of internal Na on the rate of P_i-promoted ouabain binding to energy-depleted, reconstituted ghosts. Intracellular Na concentration was controlled by varying the concentration of NaCl in the reversing medium with choline Cl such that the sum, NaCl + choline Cl was kept constant at 145 mM. Concentration of P_i was 1.0 mM. Ghosts were prepared, treated, and analyzed for [^{3}H]content as described in the legend of Table I as well as in Bodemann and Hoffman (1976 *a*). Thus the ghosts are also estimated to contain 4 mM KCl, 4 mM $MgCl_2$, and 8 mM Tris Cl. Ghosts were incubated at 37°C for 20 min in a medium which contained 1 mM KCl, 6 mM NaCl, 155 mM choline Cl, 10 mM Tris Cl, and 8×10^{-7} M [^{3}H]ouabain. The final pH was 7.4. The points on the graph represent the average of duplicate determinations. Several other experiments of similar design yielded the same type of relationship as presented in this figure. A measure of the rate of ouabain binding is indicated by the number of molecules ouabain bound per ghost after 20-min exposure to [^{3}H]ouabain.

The action of Na_o on the rate of P_{if}-promoted ouabain binding was also examined, as shown in Table III, in which the $[Na]_o$ was changed independent of $[Na]_i$. The concentration of Na_i chosen was such that it fell within the saturation range (compare with Fig. 3) in order to avoid any effects of Na on the inside of the membrane by Na leakage that might occur when the ghosts were incubated in varying concentrations of Na_o. Two different concentrations of extracellular K were studied. Even though only relatively small ouabain binding rates obtain under these experimental conditions the results indicate that Na_o stimulates the rate of P_i-promoted ouabain binding when $[K]_o$ is low. This stimulation by Na_o is abolished by increasing the concentration of K_o. Thus the effects of Na_o as well as Na_i on the rate of ouabain binding are at least qualitatively similar when the characteristics of P_{if}-promoted binding are compared to that of ATP_i (see Bodemann and Hoffman, 1976 *a*). The same comparison is also true for the effects of K_o where the rates of ATP_i as well as P_{if}-promoted ouabain binding are both decreased.

However, there is a substantive difference in the effects of Na on P_i compared to ATP-promoted ouabain binding. This becomes apparent when ouabain

TABLE III

EFFECT OF EXTERNAL K AND Na ON THE RATE OF P_i-PROMOTED OUABAIN BINDING TO ENERGY-DEPLETED RECONSTITUTED GHOSTS

External medium			Molecules ouabain bound per ghost
KCl	NaCl	Choline Cl	
mM	*mM*	*mM*	
1	0	159	36,38
1	40	119	40,40
1	145	14	60,60
15	0	145	17,18
15	40	105	16,17
15	145	0	20,21

Ghosts were prepared and analyzed using the procedures described before. Thus the ghosts were prepared to contain 2 mM P_i, 4 mM KCl, 4 mM $MgCl_2$, 50 mM NaCl, 90 mM choline Cl, and 7 mM Tris Cl. Ouabain binding was carried out by incubating the ghosts at 37°C for 30 min in a medium which contained the indicated concentrations of KCl, NaCl, and choline Cl together with 10 mM Tris Cl (final pH was 7.4) and 6×10^{-7} M [^{3}H]ouabain. The results of duplicate analyses are presented and are similar to the results obtained in another experiment of the same design. The rate of ouabain binding is indicated by the number of glycoside molecules bound per ghost after 30-min exposure to [^{3}H]ouabain.

binding to ghosts containing either ATP_i or P_i and different concentrations of Na_i is studied using a K-free medium. Table IV shows the results of this type of experiment. As already demonstrated in the preceding paper (Bodemann and Hoffman, 1976 *a*), Na_i does not affect the rate of ATP-promoted ouabain binding in the absence of K_o. On the other hand, the rate of P_i-promoted ouabain binding is markedly decreased by Na_i despite the absence of K_o. A higher concentration of [^{3}H]ouabain was chosen in the case of P_i-promoted ouabain binding in order to overcome the pronounced effect of Na_i. Thus, unlike ATP_i the effect of Na_i in P_{ii}-promoted ouabain binding is not linked to the presence of K_o. And this difference most likely accounts for the different action of Na on ATP compared to P_i-promoted ouabain binding seen when porous ghost systems were studied.

Comparison of the Effects of Na and K on the Rate of Ouabain Binding to Porous Ghosts Using Different Promoting Agents

It has been found previously that ATP promotes similar rates of ouabain binding to porous ghosts over a broad concentration range down to less than 10 μM of ATP (Hoffman, 1969). The reaction medium for these binding experiments while containing Na were K free. On the other hand, since Hegyvary and Post (1971) presented evidence that K alters the affinity of the enzyme for ATP binding, it became of interest to study the action of K on the rate of ouabain binding to porous ghosts in the presence of different K and ATP concentrations. The results are shown in Fig. 4. The concentration of K in the reaction medium

TABLE IV

A COMPARISON OF THE RELATIVE EFFECTS OF INTERNAL Na ON THE RATE OF P_i- AND ATP-PROMOTED OUABAIN BINDING TO ENERGY-DEPLETED, RECONSTITUTED GHOSTS IN THE ABSENCE OF EXTERNAL K

Inside			
Substrate	NaCl	External [^{3}H]ouabain	Molecules ouabain bound per ghost
mM	*mM*	*M*	
2 P_i	1	2×10^{-7}	106,108
	15		56,58
	120		12,12
2 ATP	1	5×10^{-8}	116,123
	15		117,117
	120		116,120

Ghosts were prepared and analyzed according to the procedures referred to before (see legend of Table I). The ghosts were hemolyzed in a medium which contained either 4 mM $MgCl_2$ or 2 mM ATP + 4 mM $MgCl_2$. For ghosts containing 1 mM NaCl, Tris ATP was used; otherwise, Na_2ATP was used. 2 mM P_i was added after hemolysis and the intracellular Na was controlled by varying the concentration of NaCl in the reversing medium with choline Cl, keeping the sum, NaCl + choline Cl, constant at 140 mM. The ghosts also contained 4 mM KCl and 8 mM Tris Cl. The ghosts were incubated at 37°C for 15 min in a medium which contained 120 mM NaCl, 40 mM choline Cl, 10 mM Tris Cl, and the indicated concentrations of [^{3}H]ouabain. Final pH was 7.4. The results presented are duplicate analyses and are essentially the same as the results obtained in another similar experiment. The rate of ouabain binding is indicated by the number of molecules of ouabain bound per ghost after 15-min exposure to [^{3}H]ouabain.

was increased in exchange for Na in the presence of four different ATP concentrations. While the rate of ouabain binding is not changed in the absence of K, it is apparent that when the ATP concentration is decreased 100-fold not only does K now have an effect but it is obvious that K becomes more effective in slowing the ouabain binding rate the lower the ATP concentration. While it can be supposed that K acts by altering the membrane's affinity for ATP, the possibility cannot be eliminated that the observed effects result from the combined actions of Na and K. However, it has not as yet been possible to prepare reconstituted ghosts where the $[ATP]_i$ can be controlled at low enough levels (due to the presence of adenylate kinase [cf. Glynn and Hoffman, 1971]) that the sided effects of Na and K can be assessed (see next section).

One way to circumvent this limitation is to use a nucleotide triphosphate which will promote ouabain binding but is not broken down by the Na,K-ATPase. Thus ouabain binding experiments have been carried out using UTP, which has previously been shown to be just as effective as ATP in promoting ouabain binding in the absence of K (Hoffman, 1969). Competition experiments (unpublished observations) between UTP and ATP indicated that the affinity of the human red cell Na, K-ATPase for UTP may be at least 100 times less than for ATP, a result similar to that obtained by Siegel and Goodwin (1972) for a

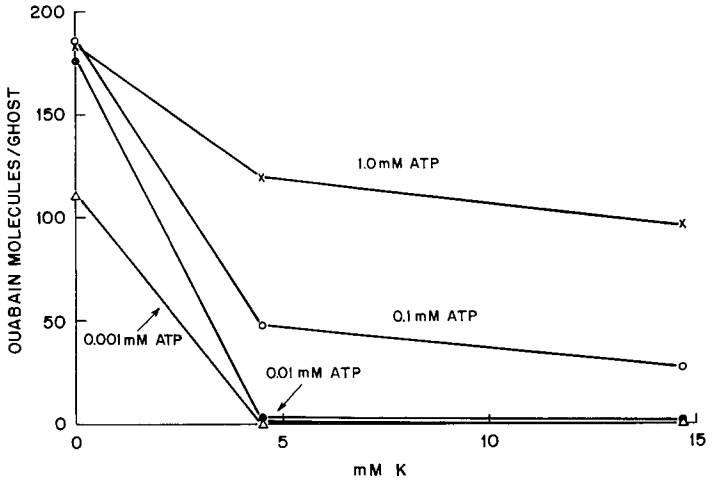

FIGURE 4. The effect of K in inhibiting the rate of ATP-promoted ouabain binding to hemoglobin-free, frozen-thawed ghosts. The experimental procedure used was the same as that described in the legend of Fig. 1 except that the ghosts were incubated in a medium which contained the indicated concentrations of K, where the quantity, NaCl + KCl, was 40 mM together with either 1.0, 0.1, or 0.01 mM Na_2ATP, 1.25 mM $MgCl_2$, 0.25 mM EDTA, 10 mM Tris Cl (final pH 7.5), and 3.4×10^{-7} M [^{3}H]ouabain. The results presented are typical of the results obtained in several similar experiments. The measure of the rate of ouabain binding is taken as the number of molecules bound per ghost after 30-min incubation at 37°C.

microsomal preparation of stomach Na,K-ATPase. It has been previously shown that there is essentially no hydrolysis of UTP by human red cell ghosts nor can UTP_i be utilized to drive the Na:K pump (Hoffman, 1962). Fig. 5 shows the rates of ouabain binding to porous ghosts in the presence of either 0.66 mM ATP or UTP as a function of the K concentration in the reaction medium. In the absence of K, the rates of ATP- and UTP-promoted ouabain binding are quite similar. However, the rates of UTP- and ATP-promoted ouabain binding become completely different when K is present in the medium. At 5 mM K, UTP-promoted ouabain binding is almost completely inhibited under conditions where appreciable ATP-promoted binding still occurs. This effect of K on UTP-promoted ouabain binding resembles the effect of K as seen in Fig. 4 when binding was studied using 10 μM ATP.

The action of Na on the rate of UTP-promoted ouabain binding to porous ghosts, in the absence of K, has also been examined and compared with ATP-promoted binding (Fig. 6). As reported previously there is little effect of Na on the rate of ATP-promoted ouabain binding (see Bodemann and Hoffman, 1976 *a*) while the rate of ouabain binding promoted by UTP is markedly stimulated. Considering the extreme sensitivity that K has on UTP binding (see Fig. 4) it seems possible that the effect of Na, instead of representing a direct or independent action, is associated with the presence of small amounts of K contained by the ghosts (that is not removed during their preparation) sufficient to account for the differences seen in the rates of UTP- and ATP-promoted ouabain

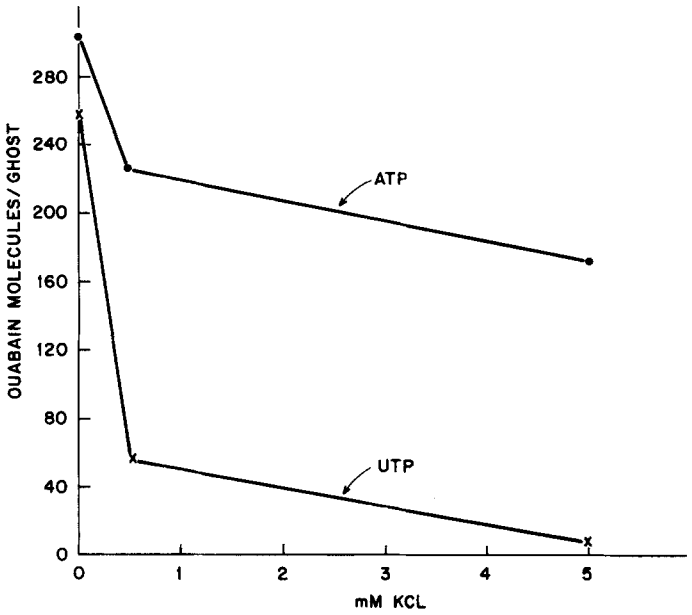

FIGURE 5. Comparison of the effect of K on the rate of ATP- and UTP-promoted ouabain binding to hemoglobin-free, frozen-thawed ghosts. The ghosts were prepared, treated, and analyzed as already described in the legend of Fig. 1. The incubation medium contained the indicated concentrations of K, 0.5 mM EDTA, 3 mM $MgCl_2$, 10 mM Tris Cl, and either 0.66 mM Na_3UTP or 0.66 mM Na_2ATP + 0.66 mM NaCl together with 1×10^{-7} M [^{3}H]ouabain. Choline Cl was also present in the medium such that the quantity, KCl + choline Cl, was kept constant at 35 mM. The final pH was 7.4. Incubation was carried out at 37°C for 30 min. The data points in the figure are the average of duplicate determinations. The results presented are representative of two other similar experiments. The measure of the rate of ouabain binding is taken as the number of molecules bound per ghost after 30-min exposure to [^{3}H]ouabain.

binding. This would also account for the differences seen in the rates of binding in the absence of Na. Thus Na may act to reduce the affinity of the system to K and therefore stimulate UTP-promoted ouabain binding. This type of explanation is more attractive than, say, a direct effect of Na since as previously discussed (Bodemann and Hoffman, 1976*a*) no effect of Na, at least with regard to ATP, has been observed in the absence of K.

Fig. 7 compares directly the relative effects of Na,K, or Na + K in their ability to alter the rate of ouabain binding to porous ghosts as promoted by either P_i or UTP or ATP or UTP + P_i. It is apparent that UTP dominates the binding when P_i is also present since there is no inhibition by Na. On the other hand, P_i tends to overcome the effects of K compared to when UTP is present alone. Since the type of results seen in Fig. 7 represent the interplay of the different affinities of the system to the various constituents present at any one time, some insight is provided by taking into account the sidedness of action of the agents involved.

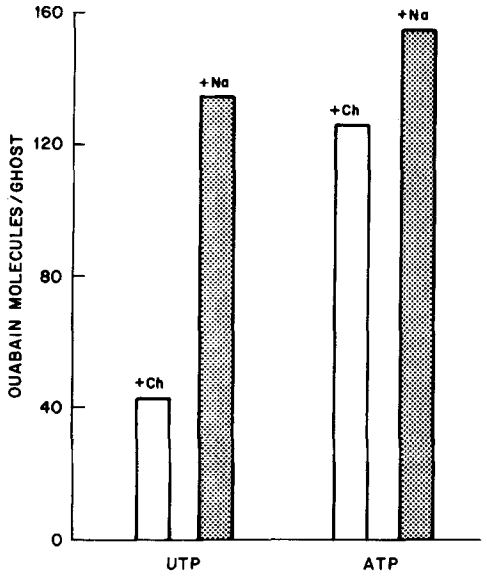

FIGURE 6. The effect of Na on the rate of ATP- and UTP-promoted ouabain binding to hemoglobin-free, frozen-thawed ghosts. The ghosts were prepared, treated, and analyzed according to the procedures referred to in the legend of Fig. 1. The ghosts were incubated at 37°C for 25 min in a medium which contained either 30 mM NaCl or 30 mM choline Cl (Ch), 3 mM $MgCl_2$, 1 mM EDTA, 5 mM Tris Cl, 2.5×10^{-8} M [^{3}H]ouabain, and either 0.33 mM Na_3UTP or 0.5 mM Na_2ATP (in order to have the Na concentration the same in the choline medium). The final pH was 7.4. The results presented represent the average of duplicate determinations and are typical of the results obtained in two similar experiments. The measure of the rate of ouabain binding is indicated as the number of molecules bound per ghost after 25-min exposure to [^{3}H]ouabain.

Comparison of the Side-Specific Effects of Na and K on the Ouabain Binding Rate Carried Out with UTP and P_i

Table V shows the rates of ouabain binding to reconstituted ghosts containing UTP_i and different concentrations of Na_i and the effect of alterations in the concentration of K_o. It is apparent that Na_i as well as K_o reduce the binding rates. These results are similar to the effects seen with ATP_i but they differ qualitatively in the sense that changes in the concentration of K_o influence more significantly the UTP-promoted binding rates than changes in the concentration of Na_i. Another similarity to ATP-promoted ouabain binding is observed when the concentration of K_i is increased. Fig. 8 shows that the rates of UTP-promoted ouabain binding are decreased as the concentration of K_i is increased (see Figs. 4 and 7, Bodemann and Hoffman, 1976*a*) even though changes in $[K]_i$ are not as effective as changes in $[K]_o$ in reducing the binding rate (cf. Table VI). To clarify further the effects of Na and K as seen on the rates of UTP compared with ATP-promoted ouabain binding to porous ghosts, the action of Na_i and K_o was studied with respect to the rates of ouabain binding in reconstituted ghosts containing either UTP_i or P_{ii} or the combination UTP_i + P_{ii}. Table VI shows

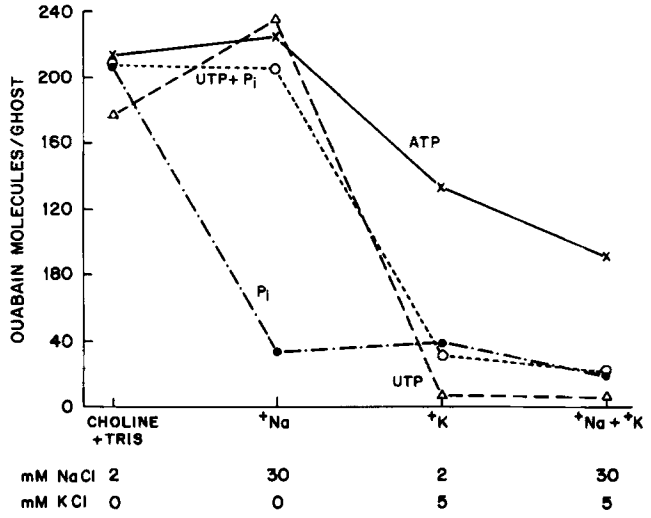

FIGURE 7. A comparison of the relative effects of Na and K on the rate of P_i-, UTP-, and ATP-promoted binding to hemoglobin-free, frozen-thawed ghosts. The experimental protocol was essentially the same as that described in the legend of Fig. 1. Ghosts were incubated at 37°C for 30 min in a medium which contained the indicated concentrations (in millimolar) of Na and K together with sufficient choline Cl that the total concentration of NaCl + KCl + choline Cl was equal to 35 mM. The medium also contained 3 mM $MgCl_2$, 0.5 mM EDTA, 5 mM Tris Cl, 1×10^{-7} M [^{3}H]ouabain, and, when present, 1 mM P_i, 0.66 mM Na_3UTP, or 0.66 mM Na_2ATP. The points in the figure represent the average of duplicate determinations. Similar results have been obtained in another experiment of the same design. The measure of the rate of ouabain binding is taken as the number of ouabain molecules which are bound per ghost after 30-min exposure to [^{3}H]ouabain.

that similar to results already presented (Table IV), Na_i inhibits P_i-promoted ouabain binding regardless of the presence or absence of K_o. The inhibitory action of K_o on P_i-promoted ouabain binding is also independent of Na_i. Comparing the uncoupled effects of K_o and Na_i it is evident that the isolated effect of Na_i is stronger than that of K_o. A further decrease of the ouabain binding rate is observed when a high concentration of Na_i is combined with a high concentration of K_o.

In UTP-promoted ouabain binding the effects of Na_i and K_o are also at least partly uncoupled. This is different from what has been observed in ATP-promoted ouabain binding (see Bodemann and Hoffman, 1976 *a*) but resembles P_i-promoted ouabain binding as already noted. However, after evaluation of the separate effects of Na_i and K_o it is evident, now, that the effect of K_o is stronger than that of Na_i. In other words, no separate effects of Na_i and K_o are observed in ATP_i-promoted ouabain binding; in UTP_i-promoted binding, the effect of K_o is more pronounced than Na_i which is different again from P_{ii}-promoted binding where the effect of Na_i dominates. These separate effects of Na and K may account for the differences seen in the rates of ouabain binding to porous ghosts promoted by P_i, UTP, or low concentrations of ATP (Fig. 4). On the other hand,

TABLE V

EFFECTS OF INTERNAL Na AND EXTERNAL K ON THE RATE OF UTP-PROMOTED BINDING TO ENERGY-DEPLETED RECONSTITUTED GHOSTS

Inside		Outside		Molecules ouabain bound per ghost
NaCl	Choline	KCl	Choline	
mM	*mM*	*mM*	*mM*	
3	140	10	0	50,52
13	130	10	0	27,29
143	0	10	0	17,19
143	0	1.5	8.5	126,126
143	0	0.1	10	320,336

The ghosts were prepared and analyzed using the procedures referred to before. Thus, the intracellular concentrations were defined by having 4 mM $MgCl_2$ and 1 mM Na_3UTP present in the hemolysis solution and the indicated concentrations of NaCl and choline Cl present in the reversing solution. In addition, the ghosts contained 4 mM KCl and 8 mM Tris Cl. Ouabain binding was carried out at 37°C for 30 min in a medium which contained 150 mM NaCl, 10 mM Tris Cl, 1×10^{-7} M [^{3}H]ouabain, and the indicated concentrations of KCl and choline Cl. The results of duplicate analyses are presented and are similar to the results obtained in another experiment of the same type. The rate of ouabain binding is indicated by the number of ouabain molecules bound per ghost after 30-min exposure to [^{3}H]ouabain.

UTP-promoted ouabain binding is slowed even further when a high concentration of K_o is combined with a high concentration of Na_i. Table VI also presents results on the rates of ouabain binding when the ghosts contain P_{ii} + UTP_i. It is apparent that in this situation the differences in the side-specific effects of Na_i and K_o are partly compensated. A pronounced decrease in the ouabain binding rate is observed in the presence of 40 mM NaCl and 6 mM KCl. Since this decrease can evidently be explained by summing the separate effects of Na_i on P_{ii}-promoted binding with the effect of K_o on UTP_i-promoted ouabain binding the coupled or combined action of Na_i and K_o as seen in ATP_i-promoted ouabain binding is mimicked when the ghosts contain UTP_i + P_{ii}.

DISCUSSION

From the work presented in this paper it is clear that P_i promotes ouabain binding only when it is present on the inside of the membrane (Table I). There is evidence that P_i in this situation is not only incorporated into a phosphoprotein *(E-P)* associated when the Na,K-ATPase but this *E-P* is indistinguishable from a phosphoprotein generated from ATP (Post et al., 1973). The formation of this phophoenzyme may be required in order for P_i to promote ouabain binding. If this is so, then it would imply that the E_2-*P* form of the pump complex is involved in the binding of ouabain (Albers et al., 1968). In addition, since it has also been shown (Table II, Fig. 3) that it is Na_i which inhibits the action of P_i, Na_i could be acting by holding the pump complex in either an E_1 or E_1-*P* form (Albers et al., 1968; Sen et al., 1969). This is consistent with the finding of Post et al. (1973) that the formation of *E-P* by P_i is inhibited by Na. In any event it

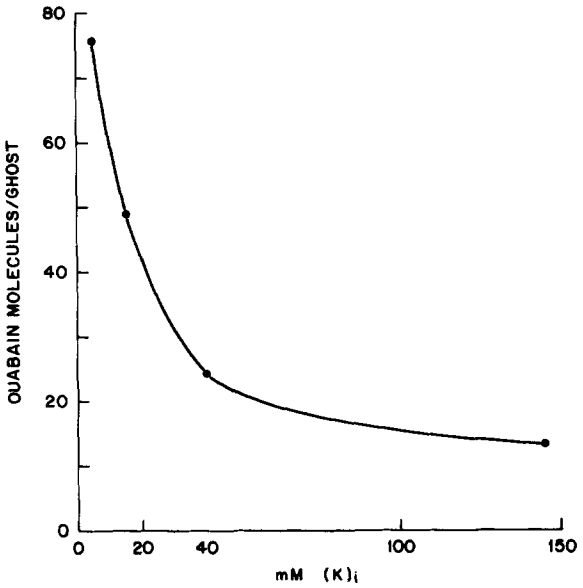

FIGURE 8. The effect of varying the internal concentration of K on the rate of UTP-promoted ouabain binding to energy-depleted, reconstituted ghosts. Intracellular K concentration was controlled by varying the concentration of KCl in the reversing medium with choline Cl such that the quantity, KCl + choline Cl, was kept constant at 150 mM. Ghosts were prepared, treated, and analyzed for [^{3}H] content as described in the legend to Table I as well as in Bodemann and Hoffman (1976 *a*). Thus the ghosts were also made to contain 0.5 mM Na_3UTP, 4 mM $MgCl_2$, and 8 mM Tris Cl. Incubation of the ghosts was carried out at 37°C for 40 min in a medium which contained 6 mM KCl, 20 mM NaCl, 134 mM choline Cl, 10 mM Tris Cl, and 3×10^{-7} M [^{3}H]ouabain. The points on the graph represent the average of duplicate determinations. The same relationship shown here was also obtained in another experiment. A measure of the rate of ouabain binding is indicated by the number of molecules ouabain bound per ghost after 40-min exposure to [^{3}H]ouabain.

appears that there is a substrate-dependent modification of the pump which occurs at the inner surface of the membrane which induces an alteration of the conformation of the complex such that ouabain binds at a greater rate at the outer surface. As developed in the previous paper (Bodemann and Hoffman, 1976 *a*) a model was proposed in which the ouabain binding site oscillated between two forms, *A* and *B*, in which ouabain could only combine with the *B* form. In terms of this model, the $A \rightleftharpoons B$ equilibrium would be shifted to the right by P_i and to the left upon the addition of Na.

The effect of Na has also proven useful for comparing P_i-promoted binding with binding promoted by nucleotide triphosphates. It has been shown in the preceding paper (Bodemann and Hoffman, 1976 *a*) that Na_o stimulates ATP-promoted ouabain binding evidently by decreasing the affinity of the system to K_o and that Na_i slows ouabain binding but only in the presence of K_o. Thus the difference in the action of Na_i between the P_i- and ATP-promoted systems is that

TABLE VI

RELATIVE EFFECTS OF INTERNAL Na AND EXTERNAL K IN ALTERING THE RATE OF P_i- AND UTP-PROMOTED OUABAIN BINDING TO ENERGY-DEPLETED, RECONSTITUTED GHOSTS

Inside				
Substrate	Na	Outside K	Molecules ouabain bound per ghost	Ouabain binding
	mM	*mM*		%
1.1 mM P_i	2	0.1	343,347	100
		6	194,196	56
	40	0.1	122,122	35
		6	35,39	11
0.25 mM UTP	2	0.1	328,334	100
		6	110,110	33
	40	0.1	211,213	64
		6	11,13	4
1.1 mM P_i + 0.25 mM UTP	2	0.1	353,367	100
		6	200,200	56
	40	0.1	273,275	76
		6	42,46	12

The ghosts were prepared, treated, and analyzed according to the procedures described in the legend of Table I and in Bodemann and Hoffman (1976 *a*). Thus the ghosts were made to contain the indicated concentrations of Na together with either P_i or UTP or the combination, P_i + UTP. The internal Na concentration was controlled by varying the concentration of NaCl in the reversing medium with choline Cl such that the sum, NaCl + choline Cl, was kept constant at 140 mM. In addition, the ghosts are estimated also to contain 4 mM $MgCl_2$, 4 mM KCl, and 8 mM Tris Cl. Incubation of the ghosts was carried out at 37°C for 30 min in a medium which contained the indicated concentrations of KCl together with sufficient choline Cl to make the total, KCl + choline Cl, equal to 140 mM; additional medium constituents were 20 mM NaCl, 10 mM Tris Cl, and 3×10^{-7} M [^{3}H]ouabain. Final pH was 7.4. The values presented represent duplicate analyses. Similar results to those presented here have also been obtained in several additional experiments. A measure of the rate of ouabain binding is indicated by the number of molecules ouabain bound per ghost after 30-min exposure to [^{3}H]ouabain.

inhibition by Na_i of P_i-promoted binding is not dependent upon K_o where inhibition of ATP-promoted binding is. The difference in action of K_o in these two circumstances is presumably related to the differences in the way the phosphoenzyme is formed. Since there is a mutual interdependence between the formation of *E-P* from P_i and ouabain (Albers et al., 1968) in this instance the ouabain complex containing *E-P* is presumably less sensitive to K_o in the sense that it is not dissociated by K_o. In the ATP-promoted system, K_o, as discussed by Bodemann and Hoffman (1976 *a*), could either change the particular phosphoenzyme form and/or alter the affinity of the complex for ATP. In addition, if Na in this situation inhibits the formation of *E-P* from P_i more effectively than K, as reported by Post et al. (1973), then one could also envisage the mechanism by which K in the absence of Na alters the rate of ouabain binding (Fig. 7, Table

VI). If this latter argument is correct it would be predicted that increasing the concentration of P_i (in the absence of K) would overcome the inhibitory effect of Na (on the formation of *E-P*) and a suggestion that this might be the case is seen in Fig. 2. On the other hand it cannot be excluded that a configurational shift of *E-P* in the presence of P_i is induced by either K_o or by Na_i acting alone. Thus, it has been suggested that Na and ouabain have their highest affinities for different conformations of the enzyme, which are in equilibrium with each other (Tobin and Sen, 1970; Tobin et al., 1973).

The mechanism of action of K in inhibiting the rate of UTP-promoted ouabain binding (Table V, Fig. 8) can be considered from several points of view. One possibility, as already mentioned, is that K acts to decrease the affinity of the pump complex for UTP similar to that found for ATP (Hegyvary and Post, 1971). If ouabain binding is promoted by UTP adsorbing to its active site on the inside of the pump complex without phosphorylation then ouabain binding could result from a change in conformation associated with the formation of an *E*-UTP complex analogous to the *E*-ATP form as discussed by Bodemann and Hoffman (1976 *a*). If the action of UTP results in phosphorylation rather than just attachment then ouabain binding would be associated with the formation of an $E_1\begin{matrix}\diagup P \\ \diagdown \text{UDP}\end{matrix}$ complex. In either case, K would act to dissociate UTP or its products from *E*, a result presumably brought about by K_o. On the other hand there is no direct evidence indicating either the chemical nature of interaction of UTP with the enzyme complex of red cells or the associated effects of K_o or Na_i. Nevertheless, the kinetics of ouabain binding may serve as an indirect measure of the extent of formation of an enzyme-UTP complex.

Another possible mechanism for the action of K in inhibiting the UTP-promoted ouabain binding rate is that K in interacting with E_2-*P*, to promote say, dephosphorylation, forms a K-*E* complex, which as indicated by Post et al. (1973) may be slow in dissociating. For the matter at hand, if K-E were formed (whether or not phosphorylation had also occurred) the UTP-promoted ouabain binding rate would be correspondingly inhibited because the formation of either a *E*-UTP or $E\begin{matrix}\diagup P \\ \diagdown \text{UDP}\end{matrix}$ complex would also be inhibited. Thus, a role for K_i as well as K_o becomes evident since either source of K would favor the *E* form unassociated with UTP. It should also be kept in mind that there is probably more than one nucleotide triphosphate binding site associated with Na,K-ATPase or ouabain binding activity of red cells (Hoffman and Proverbio, 1974; see also Post et al., 1972). This would complicate the action of K since interaction with both UTP binding sites would have to be taken into account.

It should again be emphasized that the study of the sidedness of action of Na and K as well as P_i and UTP provides information about the asymmetry of the pump complex with regard to the binding of ouabain (Bodemann and Hoffman, 1976 *a, b*). Limitations associated with the analysis of porous systems becomes somewhat relieved as side assignments of various determinants become evident, as illustrated by the results presented in this paper.

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REFERENCES

ALBERS, R. W., G. I. KOVAL, and G. I. SIEGEL. 1968. Studies on the interaction of ouabain and other cardioactive steroids with sodium-potassium-activated adenosine triphosphatase. *Mol. Pharmacol.* **4**:324–336.

BODEMANN, H. H., and J. F. HOFFMAN. 1976 *a*. Side-dependent effects of internal versus external Na and K on ouabain binding to reconstituted human red blood cell ghosts. *J. Gen. Physiol.* **67**:497–525.

BODEMANN, H. H., and J. F. HOFFMAN. 1976 *b*. 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. *J. Gen. Physiol.* **67**:547–561.

CALDWELL, P. C., and R. D. KEYNES. 1959. The effect of ouabain on the efflux of sodium from a squid giant axon. *J. Physiol. (Lond.).* **148**:8–9P.

GARDOS, G., J. F. HOFFMAN, and H. PASSOW. 1969. Flux measurements in erythrocytes. *In* Laboratory Techniques in Membrane Biophysics. H. Passow and R. Stämpfli, editors. Springer-Verlag, Berlin. 9–20.

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

HANSEN, O., and J. C. SKOU. 1973. A study on the influence of the concentration of Mg^{++}, P_i, K^+, Na^+ and Tris on (Mg^{++} + P_i)-supported *g*-strophanthin binding to (Na^+ + K^+)-activated ATPase from ox brain. *Biochim. Biophys. Acta.* **311**:51–66.

HEGYVARY, C., and R. L. POST. 1971. Binding of adenosine triphosphate to sodium and potassium ion-stimulated adenosine triphosphatase. *J. Biol. Chem.* **246**:5234–5240.

HEINZ, E., and J. F. HOFFMAN. 1955. Phosphate incorporation and Na,K-ATPase activity in human red blood cell ghosts. *J. Cell. Comp. Physiol.* **65**:31–44.

HOFFMAN, J. F. 1962. Cation transport and structure of the red-cell plasma membrane. *Circulation.* **26**:1201–1213.

HOFFMAN, J. F. 1966. The red cell membrane and the transport of sodium and potassium. *Am. J. Med.* **41**:666–668.

HOFFMAN, J. F. 1969. The interaction between tritiated ouabain and the Na-K pump in red blood cells. *J. Gen. Physiol.* **54**:343s–350s.

HOFFMAN, J. F. 1972. Sidedness of the red cell Na:K pump. *In* Roles of Membranes in Secretory Processes. L. Bolis, R. D. Keynes, and W. Wilbrandt, editors. North-Holland, Amsterdam. 203–214.

HOFFMAN, J. F. 1973. Molecular aspects of the Na^+, K^+-pump in red blood cells. *In* Organization of Energy-Transducing Membranes. M. Nakao and L. Packer, editors. University of Tokyo Press, Tokyo. 9–21.

HOFFMAN, J. F., and F. PROVERBIO. 1974. Membrane ATP and the functional organization of the red cell Na:K pump. *Ann. N.Y. Acad. Sci.* **242**:459–460.

KNAUF, P. A., and A. ROTHSTEIN. 1971. Chemical modification of membranes. I. Effects of sulfhydryl and amino reactive reagents of anion and cation permeability of the human red blood cell. *J. Gen. Physiol.* **58**:190–210.

KREGENOW, F. M., and J. F. HOFFMAN. 1972. Some kinetic and metabolic characteristics of calcium-induced potassium transport in human red cells. *J. Gen. Physiol.* **60**:406–429.

LISHKO, V. K., M. K. MALYSHEVA, and T. I. GREVIZIRSKAYA. 1972. The interaction of the (Na^+,K^+)-ATPase of erythrocyte ghosts with ouabain. *Biochim. Biophys. Acta.* **288**:103–106.

MATSUI, H., and A. SCHWARTZ. 1968. Mechanism of cardiac glycoside inhibition of the $(Na^+ + K^+)$-dependent ATPase from cardiac tissue. *Biochim. Biophys. Acta.* **151**:655–663.

POST, R. L., HEGYVARY, C., and S. KUME. 1972. Activation by adenosine triphosphate in the phosphorylation kinetics of sodium and potassium ion transport adenosine triphosphatase. *J. Biol. Chem.* **247**:6530–6540.

POST, R. L., S. KUME, and F. N. ROGERS. 1973. Alternating paths of phosphorylation of the sodium and potassium ion pump of plasma membranes. *In* Mechanisms in Bioenergetics. G. F. Azzone, L. Ernster, S. Papa, E. Quagliariello, and N. Siliprandi, editors. Academic Press, New York. 203–218.

SEN, A. K., T. TOBIN, and R. L. POST. 1969. A cycle for ouabain inhibition of sodium- and potassium-dependent adenosine triphosphatase. *J. Biol. Chem.* **244**:6596–6604.

SIEGEL, G. J., and B. GOODWIN. 1972. Sodium-potassium-activated adenosine triphosphatase: potassium regulation of enzyme phosphorylation. Sodium-stimulated, potassium-inhibited uridine triphosphate hydrolysis. *J. Biol. Chem.* **247**:3630–3637.

SKOU, J. C., K. W. BUTLER, and O. HANSEN. 1971. The effect of magnesium, ATP, P_i, and sodium on the inhibition of the $(Na^+ + K^+)$-activated enzyme system by *g*-strophanthin. *Biochim. Biophys. Acta.* **241**:443–461.

TOBIN, T., T. AKERS, S. I. BASKIN, and T. M. BRODY. 1973. Calcium ion and sodium- and potassium-dependent adenosine triphosphatase: its mechanism of inhibition and identification of the E_1-*P* intermediate. *Mol. Pharmacol.* **9**:336–349.

TOBIN, T., and A. K. SEN. 1970. Stability and ligand sensitivity of ^{3}H-ouabain binding to $(Na^+ + K^+)$-ATPase. *Biochim. Biophys. Acta.* **198**:120–131.