Side-Dependent Effects of Internal versus External Na and K on Ouabain Binding to Reconstituted Human Red Blood Cell Ghosts

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ABSTRACT  The side-dependent effects of internal and external Na and K on the ouabain binding rate, as promoted by inside MgATP, has been evaluated utilizing reconstituted human red blood cell ghosts. Such ghost systems provide the situation where [Na]i, [K]i, [Na]o, and [K]o can each be varied under conditions in which the others are either absent or fixed at constant concentrations. It was found that, in the presence of K0', increasing either [Na]i or [K]i resulted in decreasing the rate at which ouabain was bound. Changes in [Na]i or [K]i in the absence of K0 were without effect on the ouabain binding rate. Thus, the ouabain binding rate was found to vary inversely with the rate of Na:K and K:K exchange but was independent of the rate of Na:Na exchange. The effect of K0' in antagonizing ouabain binding, as well as the influence of Na0 on this interaction, were found to require the presence of either Na or K. The results are interpreted in terms of a model relating the availability of the ouabain binding site to different conformational states of the pump complex. Differences were observed in the ouabain binding properties of red cell ghosts compared to microsomal preparations but it is not known whether the basis for the differences resides in the different preparations studied or in the lack of control of sidedness in the microsomal systems.

INTRODUCTION

It is known that in red blood cells cardiotonic steroids such as ouabain inhibit Na:K transport (Schatzmann, 1953) by binding to the outside of the cell (Hoffman, 1966). Ingram (1970) showed that there are approximately 275 ouabain binding sites per cell which presumably represent the number of Na:K pumps per cell. The studies reported in this paper represent a systematic assessment of the relative effects of Na and K in the manner in which they can influence the binding of ouabain to the plasma membrane of human red blood cells. The specific actions of Na and K can most clearly be evaluated by study of their effects in systems where the membrane's sidedness is preserved. By using reconstituted human red cell ghosts it is possible to vary the inside composition of Na and/or K independent of variations in the Na and/or K composition of the external medium. Such studies provide information not only with regard to the determinants of ouabain binding but also provide insight into the molecular
mechanism of the pump since the ouabain receptor site is a component part of the complex (see Discussion). Thus, while many different effects of Na and K have been reported utilizing porous microsomal (e.g. Albers et al., 1968; Schwartz et al., 1968; Skou, 1969) and porous red cell ghost (Hoffman, 1969) preparations, a knowledge of the side-dependent actions of these ligands as reported in this paper provided insights which would not otherwise be evident or obtainable. Some of the findings presented in this paper have been briefly reported previously (Hoffman, 1972, 1973).

METHODS

Preparation of Reconstituted Ghosts

The procedure followed was essentially the same as that previously described (Passow, 1969; Bodemann and Passow, 1972). Blood was withdrawn from healthy donors into sodium heparin (0.1 mg/ml) and used immediately. In order to wash the cells before hemolyzing them the freshly drawn blood was centrifuged at 12,000 g for 2-5 min at 0°C in 50 ml polycarbonate centrifuge tubes. The cells were suspended in about 5 vol of a solution which contained 160 mM choline Cl + 10 mM Tris CI (pH 7.40) and centrifuged again. This procedure was repeated twice taking care to aspirate theuffy coat from the surface of the packed cells at the end of each wash. The packed cells were weighed and a 50% suspension was formed by the addition of 166 mM Tris CI (pH 7.4) and cooled at 0°C. It should be noted that all solutions were adjusted to the stated pH at room temperature.

To prepare ghosts the cell suspension was hemolyzed at 0°C by rapidly mixing 1 part of suspension with 10 parts of hemolyzing solution. The hemolyzing solution contained 4 mM MgCl₂ and either 2 mM Na₂ATP or Tris ATP (Sigma Chemical Co., St. Louis, Mo.). Hemolysis was allowed to proceed for 5 min at 0°C before isotonicity was restored by the addition of a 3.2 M salt solution whose composition was varied as desired by changing the ratio of choline CI:KCl:NaCl. By changing the composition of this reversal solution, the internal concentrations of Na, K, and choline could be defined and controlled independent of each other as described before (Passow, 1960; Bodemann and Passow, 1972) and in the Results section. After 5 min the hemolysis mixture was put to incubate at 37°C for 1 h in order to reseal the ghosts with regard to their cation permeability. At the end of the resealing period the ghosts were centrifuged at 34,800 g for 10 min. After saving 5 ml of the red supernatant for analysis (see below) the ghosts were washed three times with the 170 mM choline-Tris solution referred to above.

The ghosts produced by this technique have been shown to be comprised primarily of two types which differ only with regard to their relative cation permeability. The majority of the population of ghosts (60-75%) have the ability to retain substances incorporated at hemolysis or during reversal (i.e. before resealing) and have almost completely recovered their original permeability. The rest of the ghost population cannot be reconstituted and remains leaky to incorporated substrates and to cations. While these two types of ghosts can be separated from one another by sucrose cushion centrifugation (Bodemann and Passow, 1972), pilot experiments where this procedure was used to enrich the fraction of reconstituted ghosts gave the same results as obtained with the unmanipulated population. Since it was desirable to keep the preparation time to a minimum all studies reported in this paper were carried out on the original mixed population of ghosts.

It is possible to show that the reconstituted ghosts in this preparation behave like intact cells in being able to actively transport both Na and K by demonstrating that net changes of Na and K can occur against their respective gradients. The results of such an experiment are presented in Table I. Thus over the course of 3-h incubation it is
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TABLE I

RECONSTITUTED GHOSTS PUMP AS DEMONSTRATED BY NET ACCUMULATION OF K AND NET EXTRUSION OF Na

| Ghost cation content | Medium | Minutes incubated | Ouabain-sensitive net change |
|----------------------|--------|-------------------|-----------------------------|
|                      |        | 0     | 90    | 180   | 90 min | 180 min |
| K                    |        |       |       |       |        |        |
| Without ouabain      |        | 9.50  | 10.70 | 10.89 |        | +1.45  |
|                      |        | 9.25  | 10.39 | 10.89 |        | +1.89  |
|                      |        |       |       |       | +1.17  | +1.52  |
|                      | +1 x 10^-4 M ouabain | 9.50  | 9.12  | 9.12  |        | 0       |
|                      |        | 9.17  | 9.00  | 8.82  |        | 0.17    |
|                      |        | 0     |       |       | -0.28  | -0.37  |
| Na                   |        | 14.50 | 13.12 | 12.12 |        | -2.04  |
|                      |        | 14.25 | 12.69 | 12.12 |        | -2.87  |
|                      |        |       |       |       | -1.47  | -2.25  |
|                      | +1 x 10^-4 M ouabain | 14.61 | 15.00 | 14.82 |        | 0.61   |
|                      |        | 14.25 | 15.00 | 15.25 |        | 0.25   |
|                      |        | 0     |       |       | +0.57  | +0.61  |
| Percent water (wt/wt) | ± ouabain | 95.5  | 95.7  | 95.6  |        | -       |
| ^36Cl relaxed water  | ± ouabain | 0.959 | 0.955 | 0.959 |        | -       |

Ghosts were prepared as described in the text such that they contain MgATP, and, at zero time, the indicated concentrations of K and Na (and choline) in millimoles per liter ghosts. The ghosts were suspended at a 10% hematocrit in a medium which contained 17.4 mM NaCl, 7.9 mM KCl, 131 mM choline Cl, and 5 mM Tris Cl (pH 7.4) and as indicated, 1 x 10^-4 M ouabain. The suspension was incubated at 37°C and the ghosts were analyzed after centrifugation at the indicated times for Na and K (by flame photometry) and H2O content (from wet and dry weight determinations). Cl distributions were determined by adding a trace quantity of ^36Cl to the suspension just before centrifugation for ghost analysis. One volume each of ghosts and supernatant were deproteinized by addition of 1 vol of 10% trichloroacetic acid and after centrifugation the ^36Cl was determined in the clear supernatants by liquid scintillation using Bray's (Bray, 1960) solution. Neither the ratios nor the ghost water contents changed significantly during the 180-min incubation whether with or without ouabain. The activity of the pump is apparent from the net changes in contents that occur during incubation as well as from the difference in the net changes due to the absence and presence of ouabain, given as the ouabain-sensitive net change. Three other similar experiments show the same type results.
apparent that ghosts actively transport both Na and K. This is shown by the net changes that occur and by their ouabain-sensitive components. The fact that the changes are small is due to an approximately fourfold increase in the passive permeability of this ghost system compared to intact cells. When the total net fluxes are corrected for this increased leakage, the magnitude of the cation movements by the pump becomes almost identical with that of intact cells also demonstrating a coupling ratio of 3 Na/2 K (Post and Jolly, 1957). It is because of the increased passive permeability that net gain of K and loss of Na can be shown only if the gradients are small. On the other hand, for purposes of studying the sidedness of the action of Na and K on ouabain binding it is necessary to maintain the concentration of internal and external Na and K different from each other but these differences will not be significantly affected by this increased leakage since only small changes in concentrations could occur during the time intervals used (see Results). It should be understood that the activity of the Na:K pump as determined from the unidirectional flux of either \(^{24}\text{Na}\) or \(^{42}\text{K}\) is entirely consistent with the net movement results presented in Table I. The tracer flux estimates as used below have the advantage that the pump to leak ratios for Na efflux or K influx are rather high (usually between 2 and 5) making the determination of the fractional inhibition of the pump by ouabain considerably more sensitive.

**Determination of Bound Ouabain**

The same general procedure as used by Ingram (1970) and Dunham and Hoffman (1971) to measure ouabain binding to intact cells was modified as follows for studies with reconstituted ghosts. Portions of reconstituted ghosts containing different intracellular sets of Na and K were incubated at 37°C with gentle shaking in ouabain-free media or in media containing \(^{3}\text{H}\)ouabain at concentrations which ranged between \(4 \times 10^{-8}\) and \(8 \times 10^{-7}\) M. The hematocrit was 15-20%. As an index to contaminants and to nonspecific effects, each experiment included control flasks which contained in addition to the \(^{3}\text{H}\)ouabain a high concentration of nonradioactive ouabain, usually 1 or 2 \(\times\) \(10^{-4}\) M to reduce the specific activity of \(^{3}\text{H}\)ouabain. From the difference in tritium content of ghosts incubated at high and low specific activity of \(^{3}\text{H}\)ouabain, a correction could be made for bound tritiated contaminants. All determinations of \(^{3}\text{H}\)ouabain were carried out in duplicate.

The \(^{3}\text{H}\)ouabain was obtained from the New England Nuclear, Boston, Mass. and was stated to have a specific activity of 11.7 Ci/mM. Unlabeled ouabain (Sigma) was added to reduce the specific activity to 1.0 Ci/mM. This value was used in the calculations to obtain the results presented in this paper.

At the end of the exposure to \(^{3}\text{H}\)ouabain, ouabain binding to the ghosts was stopped by pipetting one part of suspension to one part of ice-cold choline-Cl-Tris solution (see above), followed by immediate centrifugation at 34,000 g for 10 min and four washes with 10 vol of the same solution using the same conditions. The last wash was performed in special Lucite tubes (see Dunham and Hoffman, 1971) for pipetting and analyses. For liquid scintillation counting 1 ml of packed ghosts was added directly to 15-ml Bray’s (1960) solution. After stirring the mixture in the scintillation vials, the cell mass was sedimented by centrifuging the capped counting vials for 10 min at 3,600 g in an RC-3 Sorvall centrifuge (DuPont Instruments, Sorvall Operations, Newtown, Conn.) equipped with a swinging bucket rotor (see Ingram, 1970; Dunham and Hoffman, 1971). This extraction into Bray’s solution removed virtually all the \(^{3}\text{H}\)ouabain from the ghosts (Ingram, 1970). Counting efficiency was determined in each experiment by internal standards. The counting efficiency with 1 ml of ghosts in 15-ml Bray’s solution was between 8 and 11%.

In some experiments \(^{3}\text{H}\)ouabain binding was measured per milligram of membrane
protein. After ouabain was bound to the reconstituted ghosts, the ghosts were hemolyzed and washed free of hemoglobin (see Heinz and Hoffman, 1965) using an ice-cold solution which contained 15.3 mM NaCl, 1.7 mM Tris, 0.1 mM EDTA (pH 7.4). After washing and freeze-thawing, the ghosts were packed by centrifugation and 0.2 ml of membrane material containing about 3 mg of protein was solubilized in 1.5 ml Nuclear-Chicago solubilizer (NCS) (Nuclear-Chicago, Des Plaines, Ill.) and counted in 15 ml of Nuclear-Chicago Fluor PPO/POPOP standard solution (Hoffman, 1969). The counting efficiency was determined by internal standards and was about 25%. The amount of protein in the sediment was determined on separate samples using the method described by Lowry et al. (1951). The number of molecules bound per cell was calculated by the equation given by Dunham and Hoffman (1971). It was assumed that 1 mg of membrane protein represents \(2 \times 10^9\) ghosts. Consistent with previous findings (Hoffman, unpublished results) that there is an insignificant loss (less than 5%) of bound \([\alpha^3H]\)ouabain from either intact cells or porous (Hb-free) ghosts during washing or from intact cells made into ghosts by osmotic lysis, no differences were noted between the \([\alpha^3H]\)ouabain content of reconstituted ghosts or the Hb-free ghosts made from them.

**Determination of the Hemoglobin, Na, and K**

The number of ghosts and their electrolyte contents were estimated on separate samples of packed ghosts. Since hemoglobin diffuses to equilibrium during osmotic hemolysis of red cells (Hoffman, 1958; Bodemann and Passow, 1972) a comparison of the Hb content of packed resealed ghosts with a known number of intact cells gives a measure of the number of ghosts per unit volume. Thus the hemoglobin concentration was determined at 412 nm (where the ghost membrane protein has negligible absorbance) on ghosts and intact cells after hemolysis and suitable dilution. Since there are approximately \(1.1 \times 10^9\) intact cells per milliliter after packing, the number of ghosts contained in 1 ml can be calculated from the fractional Hb content of an equal volume of ghosts. A comparison of various determinations of ghost number by means of a Celloscope Counter (Particle Data, Inc., Elmhurst, Ill.) with the Hb-content technique shows that the two procedures agree to within \(\pm 10\%\). The Na and K contents of packed ghosts or intact cells were measured after appropriate dilution by flame photometry using Li as an internal standard.

**K Influx**

This was determined in order to evaluate the ouabain-sensitive K-K exchange activated by internal K. The influx of K was measured using \(^{42}\)K in the absence or in the presence of \(2 \times 10^{-4}\) M ouabain. Washed reconstituted ghosts, containing 2 mM Tris ATP, 4 mM MgCl\(_2\), 7.5 mM Tris Cl, and different concentrations of KCl and choline Cl (see above) were suspended at a hemocrit of 10% in 50-m1 medium containing 150 mM choline Cl, 10 mM KCl, 10 mM Tris Cl, and a trace quantity of \(^{42}\)K. The influx was stopped after 5- and 45-min incubation at 37°C by pipetting 10 ml of the ghost suspension into 10-ml ice-cold \(^{42}\)K-free incubation medium followed by immediate centrifugation at 34,000 g for 5 min. The ghosts were washed three times in the same medium, hemolyzed by the addition of 9 ml of H\(_2\)O, and aliquots of the hemolysis mixture counted for \(^{42}\)K activity. These same hemolysis mixtures were also used in the determinations of Hb, Na, and K as described in the preceding section. The unidirectional flux was calculated as described by Dunham and Hoffman (1971) and was referred to a constant number of cells using the Hb content rather than to a constant hematocrit.

**Na Efflux**

Ghosts were loaded during hemolysis at 0°C with \(^{24}\)Na as well as with other constituents which varied according to the particular experimental design (see Results). The rate
constant for $^{24}\text{Na}$ efflux was measured in the absence and presence of $2 \times 10^{-4}$ M ouabain as previously described (Hoffman, 1962a). Unless stated otherwise the reconstituted ghosts were suspended at a hematocrit of about 9% and incubated at 37°C in a medium which contained 150 mM NaCl, 10 mM KCl, 10 mM Tris Cl (pH 7.4). Samples were taken after 5, 35, and 65 min, respectively. The determination of $^{24}\text{Na}$ activity was made using equal volumes of the suspension mixture and the supernatant after centrifugation at the different time periods.

Estimates of Na:Na, Na:K, and K:K Exchange in Intact Cells

In the experiments reported in connection with Table III the fractional inhibition of the so-called partial reactions of the pump (Garrahan and Glynn, 1967a, b, c; Levin et al., 1968) were measured in order to compare them with the inhibition of the Na:K pump produced by previously bound ouabain. Ouabain was bound to cells (washed as described before but with 166 mM NaCl rather than with 166 mM Tris Cl) by incubating 15 g of the washed packed cells in 100 ml of medium which contained $1 \times 10^{-7}$ M ouabain, 0.1 mM KCl, 40 mM NaCl, 120 mM choline Cl, 10 mM Tris Cl (pH 7.4), and 200 mg/100 ml glucose. A control suspension contained 30 g of packed cells in 200 ml of the same medium but without ouabain. The suspensions were then incubated at 37°C for 35 min with gentle shaking. Afterwards the suspensions were centrifuged (5 min, 12,000 g) and the red cells washed three times at 0°C with 166 mM NaCl using 10 vol wash solution for each wash. The ouabain-treated and control cells were then divided into two portions each and incubated at a hematocrit of 33%, with tracer quantities of either $^{24}\text{NaCl}$ or $^{42}\text{KCl}$ for 50 min at 37°C. The incubation media contained, in all instances, 0.3 mM KCl, 40 mM NaCl, 120 mM choline Cl, 10 mM Tris Cl (pH 7.4), and 200 mg/100 ml glucose. Afterwards the cells were again washed three times at 0°C but with 166 mM choline Cl. After the last wash, 0.5-ml packed cells of each type were taken in duplicate for the determination of cell Na and K by flame photometry.

For measuring the outflux of $^{24}\text{Na}$ and $^{42}\text{K}$ from these prelabeled cells, the packed cells were resuspended, at a hematocrit of 10%, in a medium which contained either 160 mM NaCl + 10 mM Tris Cl (pH 7.4) or 148 NaCl + 12 mM KCl + 10 mM Tris Cl (pH 7.4), and the outflux measured over a period of 60 min as described above using reconstituted ghosts. The outflux of $^{24}\text{Na}$ into the K-free, NaCl solution was taken as a measure of Na:Na exchange (Garrahan and Glynn, 1967a-d; Levin et al., 1968). The outflux of $^{24}\text{Na}$ into the KCl-NaCl solution was taken as representing Na:K exchange. And the outflux of $^{42}\text{K}$ into the KCl-NaCl solution was taken as a measure of K:K exchange (Glynn et al., 1970). In each type of flux study the outflux was measured in duplicate, from control cells in the presence and absence of a maximally inhibiting concentration of ouabain ($1.5 \times 10^{-4}$ M) and from the cells which had been previously exposed to ouabain. Thus, the percent inhibition of the outflux due to previously bound ouabain could be calculated as that fraction of the total ouabain-sensitive flux of the control cells, which was assumed to represent 100% inhibition in each case.

An essential feature in the design of this type of experiment is that the Na and K content of control and ouabain-exposed cells do not differ significantly at the beginning of the flux measurement. If the electrolyte contents of the two sets of cells were different then the resultant fluxes would in turn reflect these differences and invalidate the above method used to estimate the percent inhibition due to previously bound ouabain. Problems of this sort were avoided by using a solution which contained a minimal concentration of K and a relatively low concentration of Na during the cells' exposure to ouabain to diminish any subsequent differences due to ouabain binding. As a result of this procedure the control and ouabain-exposed cells which were prepared for this experiment contained 10.6 ± 0.3 mM Na/liter cells and 85.8 ± 0.7 mM K/liter cells.
ATPase Activity

The specificity of [3H]ouabain binding was assayed in several experiments by measuring the remaining Na,K-ATPase activity using the method described by Heinz and Hoffman (1965). In this procedure 0.05 ml of packed Hb-free ghosts (frozen-thawed) were mixed with 0.95 ml of medium containing 40 mM NaCl, 1.25 mM MgCl₂, 0.25 mM EDTA, 10 mM Tris Cl (pH 7.4), 20 mM KCl, 0.1 mM [γ-32P]ATP. Splitting of [γ-32P]ATP was allowed to take place for 10 min at 37°C. After incubation at 37°C for 10 min the reaction was stopped by rapid mixing of the sample with 1 ml of ice-cold 6% perchloric acid. The remaining [γ-32P]ATP was absorbed by treatment of the sample with an excess of activated charcoal. After centrifugation at 3,600 g for 10 min, 100 μl of the clear supernatant were taken for counting by Cerenkov radiation, in 15-ml H₂O using a liquid scintillation counter. The increase in the content of 32P in the supernatant after charcoal treatment that occurred during the 10-min incubation indicates the ATPase activity relative to the concentration of [γ-32P]ATP present. Thus the Na,K-ATPase activity of control ghosts, ghosts containing various quantities of bound [3H]ouabain, and ghosts incubated in the presence of 1 × 10⁻⁴ M ouabain was determined. This provided an additional way of determining the percent inhibition produced by the bound [3H]ouabain. All determinations were carried out at least in triplicate.

Rationale of Experimental Design

This paper is concerned with the way different ligands affect the rate of ouabain binding and how this rate is related to pump activity. The first relationship can be assessed by following either the time-course of ouabain binding or by measuring the amount of ouabain bound in a given time interval and both approaches have been used in the present work. When the amount of ouabain bound in a given time interval was studied, care was taken in selecting the particular concentration of ouabain used such that the qualitative comparison of the relative rates was optimized; the comparisons being in this type circumstance little affected by deviations from linearity in individual uptake rates. The second relationship can be established by comparing the changes in pump activity (known either from the present or related work) that take place under the same circumstances that the relative ouabain binding rate was measured. Since, in this regard, the important aspect of each experiment is the observed pattern of effects of the various ligands under each set of conditions, the consistency of the results obtained (see below) provide at least a qualitative basis for evaluating the relationship between ouabain binding rate and pump activity. This kind of reproducibility of the results allowed the different types of measurements to be carried out in separate and shorter experiments, where, because of the long-term instability of the ghost systems, the accuracy of the measurements could be increased.

RESULTS

Before considering the side-dependent actions of Na and K on ouabain binding, it is of interest to further characterize the reconstituted ghost system used, first, with respect to the sidedness of action of nucleoside triphosphates in promoting ouabain binding and second, the inhibitory effects of already bound ouabain on the different types of alkali metal flux components which are ouabain sensitive.

The results presented in Table II show the sidedness of action of nucleoside triphosphates in their promotion of ouabain binding to ghosts. Thus, ATP or uridine triphosphate (UTP) incorporated inside were found to be almost equally effective in increasing the rate at which ouabain binds to ghosts; in contrast, these nucleotides when present only in the external medium failed to stimulate
significantly the rate of ouabain binding above control levels. Although the percent inhibition of the Na-K pump was not estimated in the experiment presented in Table II, the number of ouabain molecules bound to ghosts containing incorporated ATP or UTP obtained here would result in an approximate 50% inhibition of the pump (see later). In addition, it is known from other studies (Hoffman, 1969) that ouabain binding to porous ghosts promoted by ATP or UTP as carried out in this experiment results in inhibition of the Na,K-ATPase proportionate to the amount bound. This type of sidedness emphasizes the asymmetry of action not only of the nucleotides but also of the ouabain since it is already known (Caldwell and Keynes, 1959; Hoffman, 1966) that ouabain or cardiotonic steroids inhibit the pump by binding only to the outside of the membrane. Presumably the pump complex is stimulated to change its conformation by inside nucleotide such that glycoside can then bind to the outside (Hoffman, 1969, 1972).

With regard to the inhibitory action of ouabain it is possible that ouabain bound to different sites on the membrane inhibits different types of fluxes. This kind of consideration is important since in attempting to define the separate actions of Na and K on ouabain binding, it would be difficult to know which flux components were being affected if different types of binding sites were involved. If separate sites existed then it might be expected that prebound ouabain would

| Incorporated Constituents | Medium additions | Molecules ouabain bound per ghost in 40 min | Average number ouabain bound per ghost due to substrate addition |
|---------------------------|-----------------|--------------------------------------------|-------------------------------------------------------------|
| mM | mM | molecules ouabain bound per ghost in 40 min | | |
| 4.0 MgCl₂ alone | None | 24,24 | 24,24 |
| 2.0 ATP | 0.5 UTP | 42,43 | 18 |
| 2.0 ATP | None | 28,28 | 4 |
| 0.5 UTP | None | 146,157 | 128 |
| 0.5 UTP | None | 126,131 | 105 |

Different constituents were incorporated into ghosts, prepared as described in Materials and Methods, by hemolyzing cells (50% hematocrit, suspended in 170 mM Tris-Cl at pH 7.4) with 10 vol of a medium containing 4 mM MgCl₂ or 4 mM MgCl₂ with either 2 mM Na₂ATP or 0.5 mM Na₃UTP. Sufficient concentrated (3.22 M) choline Cl and NaCl were then added to the hemolysis mixture such that the final concentrations were 121 and 24 mM, respectively. The ghosts were then ressealed by incubation at 37°C for 60 min. After washing, ouabain binding was measured by suspending the reconstituted ghosts in a medium which contained 160 mM NaCl, 1 mM KCl, 10 mM Tris Cl (pH 7.4), and the indicated medium additions. The concentration of [³H]ouabain was 1.5 × 10⁻⁷ M and the extent of ouabain binding to the ghosts was determined after 40-min incubation at 37°C. The intracellular concentrations of Na and K (estimated by flame photometry) were 24 and 4 mM, respectively. The results of duplicate analyses are presented. See text for details. The measure of the rate of ouabain binding is taken as the number of molecules of ouabain bound per ghost after 40-min exposure to [³H]ouabain.
inhibit the different types of fluxes to different extents when these were subsequently measured. The results of such an experiment are shown in Table III. It is clear that the fractional inhibition of each type of flux (K:K, Na:Na, or Na:K) was the same under all the conditions measured. Since ouabain was initially bound to the cells when the pump was operating in the Na:Na mode the results also indicate that the flux mode does not discriminate binding sites. While these results imply that there is only one type of binding site the possibility cannot be excluded that different types of binding sites exist each with the same affinity for ouabain. But even if this were the case there still would be no uncertainty in

| Type flux measured | Flux medium composition | Control (no ouabain) | Control + ouabain | Ouabain (prior exposed) | Percent inhibition |
|--------------------|------------------------|----------------------|-------------------|-------------------------|-------------------|
| Na:Na              | 160 NaCl               | 0.325                | 0.147             | 0.202                   | 69                |
| Na:K               | 12 KCl + 148 NaCl      | 0.485                | 0.152             | 0.247                   | 71.5              |
| K:K                | 12 KCl + 148 NaCl      | 0.0403               | 0.0371            | 0.0380                  | 72                |

The plan of this experiment was to first bind ouabain to cells and then, after washing the cells free of bulk ouabain, to determine to what extent the various types of ouabain-sensitive fluxes were inhibited by the previously bound ouabain. The details of the experimental protocol are given in Materials and Methods. Control and ouabain-exposed cells were labeled with either \( ^{23} \text{Na} \) or \( ^{42} \text{K} \) and the outward rate constants \( k_{\text{Na}} \) and \( k_{\text{K}} \) for Na and K were determined, respectively, in units of reciprocal hours. The percent inhibition of the flux due to previously bound ouabain is taken as that portion of the total ouabain-sensitive flux obtained from the control cells for that particular flux process. The measure of Na:Na exchange was taken as the ouabain-sensitive \( ^{23} \text{Na} \) outflux that occurs when the medium contains Na but no K. Na:K exchange was taken as that ouabain-sensitive component of \( ^{23} \text{Na} \) outflux that occurs in the presence of external K. The ouabain-sensitive outflux of \( ^{42} \text{K} \) that occurs in the presence of external K is taken to represent K:K exchange. Fluxes were measured over different time intervals during 60-min incubation at 37°C. The values given are averages of duplicate flux measurements. The standard error of the mean of the measurements was less than \( \pm 0.003 \) for \( k_{\text{Na}} \) and \( \pm 0.0004 \) for \( k_{\text{K}} \). The same type results showing the same consistency in the fractional inhibitions were obtained in two other experiments using either intact cells or reconstituted ghosts.

relating binding to a particular flux component since they would all be affected proportionately.

Effect of \( \text{Na}_o \) in the presence of \( \text{K}_o \)

Fig. 1 shows that the rate at which ouabain binds to reconstituted ghosts is increased as [\( \text{Na}_o \)] is increased, under conditions where [\( \text{K}_o \)] as well as [\( \text{Na}_i \)] and [\( \text{K}_i \)] are held constant. This effect of \( \text{Na}_o \) on reconstituted ghosts is the same as has been observed with intact red cells (Schatzmann, 1965; Ingram, 1970; Beauge and Adragna, 1971; Sachs, 1974) and is evidently due to the competition of \( \text{Na}_o \) with \( \text{K}_o \) in antagonizing the effect of \( \text{K}_o \) in reducing the rate at which ouabain binds (Glynn, 1957; Hoffman, 1966) rather than an independent action of \( \text{Na}_o \) as suggested by Gardner and Conlon (1972) (see Sachs, 1974). Thus, as
Figure 1. The effect of external Na on the rate of ouabain binding to reconstituted ghosts. Ghosts containing the indicated concentrations of NaCl, KCl, and Tris Cl also contained 2 mM Na₂ATP + 4 mM MgCl₂. The concentrations of these substances were in general not measured directly but since, as pointed out in Materials and Methods, they were present either at the time of hemolysis or added at reversal before resealing, the indicated concentrations are assumed to represent their distribution at the time of resealing. Analyses of ghosts after washing at least for Na and K indicated that these assumed values were close to the measured concentrations (millimole per liter ghosts). The concentration of ATP is known to decrease (Glynn and Hoffman, 1971) during the span of incubation but does not become rate limiting during this time either for ouabain binding or for cation transport. After washing, the reconstituted ghosts were suspended in media which contained 10 mM KCl + 10 mM Tris (pH 7.4) plus the indicated concentrations of either NaCl or choline Cl to maintain the total concentration 170 mM. The concentration of [³H]ouabain was the same in all media and was approximately $8 \times 10^{-8}$ M. Incubation was carried out at 37°C at a hematocrit of about 15%. Analyses of [³H]ouabain content were performed in duplicate. The results presented are typical of several similar experiments.

shown in Table IV, the stimulatory effect of Na₀ on the rate of ouabain binding that is observed when [K₀] is low (1 mM) is prevented when the concentration of K₀ is increased to 15 mM. Further support for the idea that Na₀ acts primarily by changing the affinity of the outside of the membrane for K₀ comes from experiments (presented later in connection with Table VI) which show that in the absence of K₀, Na₀ is without effect on the ouabain binding rate (see also Sachs, 1974). These results are also compatible with the idea that the effects of
TABLE IV
MODULATION BY EXTERNAL K OF THE EFFECT OF EXTERNAL Na ON THE RATE OF OUABAIN BINDING TO RECONSTITUTED GHOSTS

| Extracellular | K (mM) | Na (mM) | Choline (mM) | Molecules ouabain bound per ghost in 60 min |
|---------------|--------|---------|--------------|--------------------------------------------|
|               | 1      | 3       | 161          | 191,197                                    |
|               | 1      | 40      | 124          | 240,246                                    |
|               | 1      | 150     | 14           | 318,323                                    |
|               | 15     | 3       | 147          | 131,135                                    |
|               | 15     | 40      | 110          | 132,140                                    |
|               | 15     | 150     | 0            | 156,144                                    |

The ghosts contained (in millimolar) 5 KCl, 15 NaCl, 135 choline Cl, 8 Tris Cl, 2 Na₂ATP, and 4 MgCl₂ and these internal concentrations were set as described in the legend of Fig. 1. The external media contained the indicated concentrations of KCl, NaCl, and choline Cl together with 5 mM Tris Cl (final pH was 7.4). Ghosts were incubated in the presence of 8 x 10⁻⁶ M [³H]ouabain, at 37°C for 60 min (hematocrit approximately 15%) before washing and analyzing for [³H]ouabain. The results of duplicate samples are presented and 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 ouabain bound per ghost after 60-min exposure to [³H]ouabain.

Naₐ as well as Kₒ on the rate of ouabain binding are referable to the inverse of their actions on the Na:K pump. This is because the Na:K pump is known to require Kₒ for its activation (Harris and Maizels, 1952; Glynn, 1956) and Naₐ is known to competitively inhibit the pump's activation by Kₒ (Post et al., 1960; Garrahan and Glynn, 1967 a-c; Sachs, 1967). This would mean that the rate at which ouabain binds to the membrane is inversely related to the rate at which the Na:K pump was operating, a conclusion which is considered in more detail below. It can also be mentioned here, that since alterations in [Naₐ] will be seen to have opposite effects from changes in [Naₐ], that when ouabain binding rates are observed to be increased by increasing concentrations of Naₐ, this is most likely due to Na acting on the outside and not on the inside of the membrane.¹

¹ While the foregoing results have been discussed in terms of a single action of Naₐ, it is possible that there might also be a direct effect of Naₐ in addition to the effect Naₐ has on altering the affinity of the membrane for Kₒ. This separate action of Naₐ is suggested by the fact that Naₐ had no effect when [Kₒ] was 15 mM as in Table IV but had a marked effect when [Kₒ] was 10 mM as in Fig. 1. Both of these concentrations of Kₒ appear to be well above the concentration of Kₒ needed to saturate Na:K exchange. On the other hand, the ghosts used in the experiments presented in Table IV contained approximately 15 mM Naₐ while the ghosts used in the experiments given in Fig. 1 contained 150 mM Naₐ. Separate experiments showed that the pump flux of Na in ghosts containing a low [Naₐ], as in Table IV, was reduced by increasing [Naₐ], when [Kₒ] was low but not when [Kₒ] was high. On the other hand, comparable measurements have not as yet been performed on ghosts containing high [Naₐ] so the action of Naₐ in this regard must remain unspecified. Therefore increased or high concentrations of Naₐ could either change the membrane's affinity for Kₒ or provide for a direct action of Naₐ or both but the conclusion that the effects of Naₐ on ouabain...
Effects of Na\textsubscript{i} in the presence of K\textsubscript{o}

If there is a correlation between the conditions which activate the Na:K pump and the ouabain binding rate, then it should be possible to demonstrate an effect of Na\textsubscript{i} since the pump is known (Glynn, 1962; Whittam, 1962) to require Na\textsubscript{i} for its operation. The results presented in Fig. 2 show such an effect since it is binding are opposite from the effects of Na\textsubscript{o} would not be affected. While the effects of different concentrations of Na\textsubscript{i} had not been evaluated systematically it should be emphasized again that the effects of Na\textsubscript{i} in altering the rate of ouabain binding are dependent upon the presence of K\textsubscript{o}. To the extent that Na\textsubscript{i} is able to modify any action of Na\textsubscript{o} or K\textsubscript{o} on the external surface would be interesting in view of the results of Garay and Garrahan (1973) indicating lack of transmembrane effects of monovalent cation concentration on the affinities of transported ions.

**Figure 2.** The effect of internal Na on the rate of ouabain binding to reconstituted red cell ghosts. The ghosts contained varying concentrations of NaCl and choline Cl together with 5 mM KCl, 8 mM Tris Cl, 2 mM Na\textsubscript{2}ATP, and 4 mM MgCl\textsubscript{2}. The inside NaCl concentration was varied at the expense of choline Cl such that the quantity, NaCl + choline Cl, was kept at 152 mM. Intracellular concentrations were estimated as described in the legend of Fig. 1. For all cases, ouabain uptake was measured at 37°C at the indicated times suspending the ghosts (hematocrit was approximately 15%) in a medium which contained 145 mM NaCl + 10 mM KCl + 10 mM Tris Cl at pH 7.4. The medium also contained 4 x 10\textsuperscript{-8} M [\textsuperscript{3}H]ouabain. The results presented are typical of those obtained in several similar experiments. All analyses were carried out in duplicate.
apparent that increasing [Na], at constant Na0 and K0, decreases the rate at which ouabain binds to ghosts. Thus the effect of NaI on ouabain binding is opposite to that of Na0. The concentration of NaI which appears to inhibit maximally (approximately 35-40 mM, estimated from the results of several experiments of the type presented in Fig. 2) the ouabain binding rate corresponds at least qualitatively to the concentration of NaI necessary to saturate or run the pump at its maximum (Post and Jolly, 1957). On the other hand, the concentration of NaI required to reduce the ouabain binding to one-half its maximum rate is consistently lower (about 10 mM) for the experimental situation presented in Fig. 2 than the apparent K1/2 for activation of the pump, i.e. approximately 20 mM (Hoffman, 1962 a). The difference is presumably referable to the concentration of KI present since we have found in other experiments of this type (not presented) that intermediate concentrations of NaI became less effective in inhibiting the rate of ouabain binding the higher the [K]I. This result is consistent with the competitive interaction of KI with NaI on the efflux of Na via the pump (Hoffman, 1962 a; Garay and Garrahan, 1973; Knight and Welt, 1974).

Table V shows that the change in the number of ouabain molecules bound to

| TABLE V |
|---|
| INHIBITION OF THE Na,K-ATPase BY OUABAIN WHICH WAS BOUND AS A FUNCTION OF INTRACELLULAR Na |

| Exp. | Na | K | Choline | Molecules ouabain bound per ghost in 50 min | Percent inhibition | Molecules ouabain bound per ghost at 100% inhibition |
|---|---|---|---|---|---|---|
| A | 4 | 4 | 146 | 104,105 | 55.9 ± 0.8 | 187 |
| | 8 | 4 | 142 | 73,75 | 38.2 ± 0.8 | 194 |
| | 16 | 4 | 134 | 24,25 | 13.1 ± 0.9 | 187 |
| | 40 | 4 | 110 | 11,11 | -- | -- |
| B | 4 | 4 | 146 | 124 | 52 | 238 |
| | 8 | 4 | 142 | 73 | 31 | 235 |

Ghosts contained the indicated concentrations of NaCl, KCl, and choline Cl together with 2 mM Na2ATP, 4 mM MgCl2, and 10 mM Tris Cl (pH 7.4). The composition of the suspension medium was (mM): 20 NaCl, 6 KCl, 134 choline Cl, and 10 Tris Cl at a final pH of 7.4. The medium also contained 4 × 10^-4 M [3H]ouabain. Ghosts were incubated (hematocrit was approximately 15%) at 37°C for 30 min. At the end of the incubation the ghosts were washed and the amount of [3H]ouabain bound per ghost was determined in the usual way. In addition, a portion of each type of ghost preparation was rehemolyzed and hemoglobin-free ghosts were made in order to estimate the percent inhibition of the Na,K-ATPase which was due to the bound ouabain, as described in Materials and Methods. Reconstituted control ghosts, which had not been exposed to ouabain, were carried through the entire procedure in order to establish the zero and 100% inhibition limits (by incubation in the presence and absence of 1 × 10^-4 M ouabain) of the Na,K-ATPase so that the fractional inhibition caused by the bound [3H]ouabain could be evaluated. The variance is given as the standard error of the mean (n = 5). The number of ouabain molecules bound per ghost at 100% inhibition is obtained by dividing the number of ouabain molecules bound per ghost by the percent inhibition of the Na,K-ATPase. The results of two experiments (A and B) presented are typical of several other similar experiments. The relative rate of [3H]ouabain binding is indicated by the number of molecules which are bound after 30-min exposure.
reconstituted ghosts when \([Na]_i\) is varied results in a parallel change in the inhibition of the Na-K-ATPase when measured subsequently on porous ghosts made from them. Thus, in both experiments shown, increased \([Na]_i\) decreased proportionately the number of molecules of glycoside bound and the percent inhibition of the Na-K-ATPase. That the binding of ouabain to the ghosts was specific in the sense that all of the bound ouabain was inhibitory is shown by the fact that the calculated number of molecules bound at 100% inhibition is the same for the different binding conditions in each experiment.

If the effect of Na~ on ouabain binding is related to its action on the pump then its effect should also be dependent upon the simultaneous presence of K0, paralleling the pump's obligatory requirement for K0 and Na~ (Glynn, 1962; Whittam, 1962). To test this interdependence the rate of ouabain binding was measured using ghosts which were prepared to contain either low (1 mM) or high (40 mM) \([Na]_i\) and which were suspended in either low (0.2 mM) or high (6 mM) \([K]_o\). The particular concentrations of Na~ and K0 chosen were high enough to provide a maximum stimulation of the Na:K pump. The results are shown in Fig. 3. The only condition in which the ouabain binding rate is inhibited is when the conditions for pump activation are satisfied. Neither Na~ or K0 alone is sufficient to alter the rate of ouabain binding indicating that the action of either Na~ or, for that matter, K0, depends upon their coupled action on the pump. In addition, it is also apparent that there is a reciprocal relationship between the pumping rate and the rate of ouabain binding: the fastest rates of binding are associated with minimal pump rates.

It is interesting to note in this connection that the effect of K0 in antagonizing the binding of glycosides to the membranes, as mentioned earlier, is also dependent upon the presence of Na~. This result implies that K0 in altering the rate of ouabain binding does so by producing a particular conformation of the pump complex rather than a direct interaction.

\textit{Na:Na Exchange and the Rate of Ouabain Binding}

From the foregoing results it might be expected that since Na:Na exchange represents a partial reaction of the pump and that it is ouabain sensitive (Garrahan and Glynn, 1967 \textit{a,b,c}; Levin et al., 1968) the rate of ouabain binding could also be affected by the rate at which Na:Na exchange can take place. On the other hand, the fact that ouabain binding was only altered by K0 when Na~ was present in the experiments presented in Fig. 3, even though Na~ was always present in the external medium would imply that the rate of ouabain binding was independent of the rate of Na:Na exchange. The experiments to be presented show the latter supposition to be correct. The evidence presented in Fig. 3 is insufficient to prove the point since the conditions in terms of the Na concentrations on the two sides of the membrane were not optimal for the occurrence of Na:Na exchange (see Garrahan and Glynn, 1967 \textit{a,b}; Sachs, 1970).

\textit{Independence of Ouabain Binding Rate and Na:Na Exchange}

The design of the following experiments was to study the rate of ouabain binding to ghosts when Na:Na exchange was activated by either (a) increasing
H. H. BODEMANN AND J. F. HOFFMAN Na and K Sidedness and Ouabain Binding. I

INSIDE: (Na\textsubscript{i} + Choline + 5K + 8 Tris) = 157 mM
OUTSIDE: (K\textsubscript{o} + Choline + 20 Na + 10 Tris) = 171 mM

\[ [\text{Na}]_{i} \text{holding } [\text{Na}]_{o} \text{ constant (Table VI, part A, exp. A), or (b) increasing } [\text{Na}]_{i} \text{ holding } [\text{Na}]_{o} \text{ constant (Table VI, part A, exp. B), or (c) changing the intracellular concentration of ADP holding both internal and external Na constant at values which support the occurrence of Na:Na exchange. The results presented in Table VI (part A) indicate that ouabain binds to ghosts at a rate which is independent of either the internal (exp. A) or external (exp. B) concentration of Na. It is known from previous work (Garrahan and Glynn, 1967a, b; Levin et al., 1968; Sachs, 1970) that for the conditions under which exp. A was carried out Na:Na exchange either does not occur or is inhibited when [Na]_{i} is either very low or very high (see Garrahan and Glynn, 1967c), but is almost optimally activated at the intermediate concentration of Na\textsubscript{i} used (15 mmol/liter ghosts). Alternatively when [Na]_{i} is maintained at approximately this optimal value, Na:Na exchange can then be activated by increasing the concentration of Na\textsubscript{o}.}

\[ [\text{Na}]_{i} \text{ holding } [\text{Na}]_{o} \text{ constant (Table VI, part A, exp. A), or (b) increasing } [\text{Na}]_{i} \text{ holding } [\text{Na}]_{o} \text{ constant (Table VI, part A, exp. B), or (c) changing the intracellular concentration of ADP holding both internal and external Na constant at values which support the occurrence of Na:Na exchange. The results presented in Table VI (part A) indicate that ouabain binds to ghosts at a rate which is independent of either the internal (exp. A) or external (exp. B) concentration of Na. It is known from previous work (Garrahan and Glynn, 1967a, b; Levin et al., 1968; Sachs, 1970) that for the conditions under which exp. A was carried out Na:Na exchange either does not occur or is inhibited when [Na]_{i} is either very low or very high (see Garrahan and Glynn, 1967c), but is almost optimally activated at the intermediate concentration of Na\textsubscript{i} used (15 mmol/liter ghosts). Alternatively when [Na]_{i} is maintained at approximately this optimal value, Na:Na exchange can then be activated by increasing the concentration of Na\textsubscript{o}.}
TABLE VI
INDEPENDENCE OF Na:Na EXCHANGE AND THE RATE OF OUABAIN BINDING TO RECONSTITUTED GHOSTS

Part A

| Cation concentration | Internal | External | Molecules ouabain bound per ghost in 15 min |
|----------------------|----------|----------|------------------------------------------|
| Exp. Na | K | Na | K | |
| Exp. | Na | K | Molecules ouabain bound per ghost in 15 min |
| A | 1–2 | 4 | 120 | 0 | 117,123 |
| 15 | 4 | 120 | 0 | 116,117 |
| 120 | 4 | 120 | 0 | 116,120 |
| B | 13.8 | 1.95 | 6.4 | 0 | 0.15* | 118,119,120* |
| 13.8 | 1.95 | 144 | 0 | 0.025* | 133,138,140* |

Part B

| Cation concentration | Rate constants for Na efflux (h⁻¹) ± SEM |
|----------------------|-----------------------------------------|
| Internal | External | Na | K | Na | K | Alone | Ouabain | ^*10⁻⁴ |
| mmol/liter ghosts | mmol/liter ghosts | |
| 14.9 | 1.5 | 0 | 0 | 0.0654±0.0007 | 0.0333±0.0004 | 0.0321 |
| 14.9 | 1.5 | 6.4 | 0 | 0.0453±0.0007 | 0.0334±0.0004 | 0.0121 |
| 18.9 | 1.5 | 0 | 0 | 0.0662±0.0011 | 0.0827±0.0008 | 0.0355 |
| 18.9 | 1.5 | 6.4 | 0 | 0.0428±0.0018 | 0.0348±0.0005 | 0.0080 |
| 14.8 | 1.5 | 0 | 0 | 0.0676±0.0044 | 0.0389±0.0003 | 0.0337 |
| 14.8 | 1.5 | 144 | 0 | 0.0992±0.0003 | 0.0476±0.0008 | 0.0516 |

* Determinations after 30-min incubation period.
Part A refers to the rate at which ouabain is bound to ghosts as the internal (exp. A) or external (exp. B) Na concentration is increased. Part B refers to estimates of Na:Na exchange (taken as the ouabain-sensitive portion of the Na outflux, ^*10⁻⁴) determined on intact red cells under conditions comparable to part A (exp. B), at two different concentrations of internal and external Na. The procedure used for the experiments dealing with ghosts is described in Materials and Methods. The experiments dealing with intact red cells (part B) were carried out by John R. Sachs. The internal concentrations of Na and K were altered (with choline being used to maintain isotonicity) by application of the p-chloromercuribenzenesulfonic acid (PCMBS) technique as described in Sachs (1974). Na efflux was measured using ^*¹⁴Na, as described in Sachs and Welt (1967) by suspending the cells in a medium which contained in addition to NaCl, 5.4 mM MgCO₃, 27.3 mM glycyglycine, 10 mM glucose, 20-μg/100 ml crystalline bovine serum albumin. Where the Na concentration in the medium was varied, NaCl was replaced by an equal volume of a solution containing a mixture of 75% isoosmotic MgCl₂ and 25% isoosmotic sucrose. The efflux was measured at 37°C at pH 7.4. SEM refers to standard error of the mean when n = 4.

That this is so in the present experiments can be seen from the results of flux measurements presented in part B (Table VI) which were carried out on intact red cells using the same conditions under which the ouabain binding rate (exp. B, Table VI) was determined. In this circumstance Na:Na exchange is activated when [Na]₀ is increased from 6.4 to 144 mM and is not associated with the change
in efflux that occurs when the medium is Na-free (Garrahan and Glynn, 1967a, b; Levin et al., 1968; Sachs, 1970). Thus, independent of the rate of Na:Na exchange or the manner of its activation the ouabain binding rate remains essentially unaltered. It should be stated, however, that the small difference in the amount of ouabain bound when the Na concentration was changed as seen in exp. B (part A) probably reflects an interaction of Na with K as previously discussed.

The third approach used for evaluating the relationship between the ouabain binding rate and Na:Na exchange is based on the finding that the rate of Na:Na exchange is proportional to the intracellular concentration of ADP (Glynn and Hoffman, 1971). The intracellular ADP can be regulated by incorporating into the ghosts an ATP regenerating system consisting of creatine phosphate and creatine phosphokinase. Two types of ghosts were prepared in a manner completely comparable to those used by Glynn and Hoffman (1971) such that one type contained 2 mM ATP, 2.4 mM MgCl₂, 10 mM NaCl, 7.5 mM Tris CI, creatine phosphokinase (160 mg/ml ghosts) and the other type contained, in addition, 5 mM creatine phosphate in order to maintain the ADP level low enough to inhibit Na:Na exchange. Both types of ghosts were reversed with choline CI rather than with KCl as used by Glynn and Hoffman (1971) to keep [K] low consistent with the present experiments. When the two types of ghosts were incubated in high NaCl (160 mM) Na:Na exchange could be shown to occur only in those ghosts which contained ADP and not in those ghosts which were low in ADP due to the incorporation of creatine phosphate. The average number of ouabain molecules bound per ghost after 30-min incubation at 37°C was 143 and 168, respectively, which provides additional evidence that the ouabain binding rate is independent of Na:Na exchange.

**Effects of Kᵢ in the Presence of Kₒ**

If the effects of Kᵢ were analogous to the action of Naₒ as discussed above, it might be expected that increasing [Kᵢ] would accelerate the rate of ouabain binding since the rate of Na:K exchange would be slowed by [Kᵢ] as a result of lowering the affinity of the pump for Na (Hoffman, 1962a; Garay and Garrahan, 1973; Knight and Welt, 1974; Simons, 1974). Fig. 4 shows that this expectation is not fulfilled, that the ouabain binding rate decreases rather than increases as [Kᵢ] is raised. The concentration of Kᵢ which reduces the binding rate by one-half is about 15 mM, somewhat higher than the approximate 10 mM concentration of Naₒ needed to produce the same effect (Fig. 2). To compare more directly the relative effectiveness of [Naᵢ] with [Kᵢ] in inhibiting the rate of ouabain binding, experiments were carried out in which the sum, [Na + Kᵢ], was held constant but the ratio, [Na/Kᵢ], was varied. The total concentration of Naᵢ + Kᵢ was kept low (isotonicity was maintained with choline CI) in order to make the comparison around the concentration which gives half-maximal effects. It is apparent from the results presented in Fig. 5 that Naᵢ is a more potent inhibitor of the ouabain binding rate than Kᵢ since the concentration of Kᵢ necessary to obtain an equivalent effect is about three times the concentration of Naᵢ.

One explanation for the inhibitory effect of Kᵢ on ouabain binding is that Kᵢ
The effect of internal K on the rate of ATP-promoted ouabain binding to reconstituted ghosts. The ghosts contained varying concentrations of KCl and choline Cl together with 4 mM NaCl, 4 mM MgCl₂, 2 mM Na₂ATP, and 8 mM Tris Cl. The KCl concentration was varied at the expense of choline Cl such that the sum, KCl + choline Cl, was held constant at 150 mM as indicated. Intracellular concentrations were estimated as described in the legend of Fig. 1. Ouabain binding was measured at 37°C at the indicated times after suspending the ghosts in a medium which contained 145 mM NaCl, 10 mM KCl, 10 mM Tris Cl, and 4 × 10⁻⁸ M [³H]ouabain. The final pH was 7.4. The results presented are typical of those obtained in two other experiments of the same design. The points in the figure represent the average of duplicate determinations.

Figure 4.

The effect of internal K on the rate of ATP-promoted ouabain binding to reconstituted ghosts. The ghosts contained varying concentrations of KCl and choline Cl together with 4 mM NaCl, 4 mM MgCl₂, 2 mM Na₂ATP, and 8 mM Tris Cl. The KCl concentration was varied at the expense of choline Cl such that the sum, KCl + choline Cl, was held constant at 150 mM as indicated. Intracellular concentrations were estimated as described in the legend of Fig. 1. Ouabain binding was measured at 37°C at the indicated times after suspending the ghosts in a medium which contained 145 mM NaCl, 10 mM KCl, 10 mM Tris Cl, and 4 × 10⁻⁸ M [³H]ouabain. The final pH was 7.4. The results presented are typical of those obtained in two other experiments of the same design. The points in the figure represent the average of duplicate determinations.

The most likely explanation for the action of Kᵢ in inhibiting the ouabain

might be acting to accelerate the rate of Na:K exchange by increasing the affinity of the pump for Na. This type of an effect could only be expected to occur at low values of Kᵢ, since as referred to before, high concentrations of Kᵢ are inhibitory to the pump. Since activation of the Na:K pump at low concentrations of Kᵢ has been observed in goat (Sachs et al., 1974) and human (Garay and Garrahan, 1973) red cells it was of interest to see if any similar type of effect was operative in human red cell ghosts under the same conditions in which the ouabain binding rate was measured. But, as seen in Table VII, the pump flux of Na was not increased at low values of [Kᵢ] but was decreased, as expected from previously discussed results, when [Kᵢ] was high (see below). Therefore, the effect of Kᵢ in decreasing the ouabain binding rate is an independent effect evidently unrelated to the rate of Na:K exchange.

The most likely explanation for the action of Kᵢ in inhibiting the ouabain
Figure 5. Comparison of the relative effects of internal Na and K on the rate of ATP-promoted ouabain binding to reconstituted ghosts. The ghosts were prepared to contain the indicated concentrations of KCl, NaCl, and choline Cl as described in the legend of Fig. 1, together with 2 mM ATP, 4 mM MgCl₂, and 8 mM Tris Cl as described before. These ghosts were incubated at 37°C in a medium which contained 145 mM NaCl, 10 mM KCl, 10 mM Tris Cl, and 4 x 10⁻⁶ M [³H]ouabain. The final pH was 7.4 and the suspensions were sampled for [³H]ouabain binding determinations at the indicated times. The points in the figure represent the average of duplicate analyses and the same pattern of results was obtained in another experiment of a similar type.

Table VII
EFFECT OF INTERNAL K ON THE OUABAIN-SENSITIVE EFFLUX OF ⁴⁰Na FROM RECONSTITUTED GHOSTS

| Inside | Rate constants for Na efflux |
|-------|----------------------------|
| KCl mM | NaCl mM | Choline Cl mM | Alone h⁻¹ | Ouabain h⁻¹ |
| 5 | 4 | 145 | 0.555 | 0.146 | 0.409 |
| 15 | 4 | 135 | 0.580 | 0.169 | 0.411 |
| 40 | 4 | 110 | 0.536 | 0.127 | 0.409 |
| 150 | 4 | 0 | 0.313 | 0.147 | 0.165 |

The indicated concentrations of KCl, NaCl, and choline Cl together with 2 mM ATP, 4 mM MgCl₂, and 8 mM Tris Cl were set according to the procedures referred to in the legend of Fig. 1. The ghosts were incubated at 37°C in a medium which contained 10 mM KCl, 150 mM NaCl, and 10 mM Tris Cl in the presence and absence of 2 x 10⁻⁴ M ouabain. The final pH was 7.4. The ouabain-sensitive outward rate constant (⁴⁰Na) for Na was evaluated at 30- and 60-min incubation as described in Materials and Methods. The values for ⁴⁰Na as presented in the table are the average of duplicate determinations at the two time periods and are representative of the results obtained in another experiment of the same design.
binding rate is that it is directly associated with the transport of K through the K:K exchange pathway (Glynn et al., 1970). That this is so is seen from measurements of the ouabain-sensitive K influx under the same conditions as the effect of Kᵢ was characterized with respect to ouabain binding. As shown in Fig. 6, K influx is increased by increasing concentrations of Kᵢ with half-maximal activation being achieved at approximately 15 mM [Kᵢ], in good agreement with the ouabain binding result discussed before (Fig. 4). This effect of Kᵢ in activating K:K exchange and inhibiting the rate of ouabain binding is dependent upon the concentration of Naᵢ. Thus, as seen in Table VIII, the ouabain binding rate is sensitive to the concentration of Kᵢ when [Naᵢ] is low (4 mM), but the effect of Kᵢ is essentially nullified when [Naᵢ] is raised to 36 mM. These effects of Kᵢ and Naᵢ on K:K exchange have been independently evaluated by Simons (1974) and while the results of the two studies are consistent with each other, the analysis by Simons (1974) details the characteristics of K:K exchange as antagonized by Naᵢ. It should also be noted that the decrease in the rate of ouabain binding at high [Naᵢ] compared to low [Naᵢ] reflects, in addition to the resultant inhibition of K:K exchange, the effects of activation of Na:K exchange.

Since K:K exchange requires the presence of K on both sides of the membrane in analogy with the requirements of the Na:K pump (as depicted in Fig. 3) a
MODULATION BY INTERNAL Na OF THE EFFECT OF INTERNAL K ON THE RATE OF ATP-PROMOTED OUABAIN BINDING TO RECONSTITUTED GHOSTS

| Intracellular | Molecules ouabain bound per ghost in 30 min |
|---------------|------------------------------------------|
| K (mM) | Na (mM) | Choline (mM) | |  |
| 5 | 4 | 136 | 153,159 |
| 37 | 4 | 104 | 157,159 |
| 109 | 4 | 32 | 94,100 |
| 5 | 36 | 104 | 84,96 |
| 37 | 36 | 72 | 97,101 |
| 109 | 36 | 0 | 109,113 |

The ghosts were prepared, treated, and analyzed according to the procedures referred to before in the legend to Fig. 1. Thus the ghosts were prepared to contain the indicated concentrations of KCl, NaCl, and choline Cl together with 2 mM Na$_2$ATP, 4 mM MgCl$_2$, and 8 mM Tris Cl. The external medium contained 150 mM NaCl, 10 mM Tris Cl, and $1 \times 10^{-7}$ M [H]$^+$ouabain and the incubation was carried out at 37°C for 30 min. The final pH of the medium was 7.4. The results of duplicate analyses are presented and are typical of the results obtained in two other experiments of the same design. The measure of the rate of ouabain binding is taken as the number of molecules of ouabain bound per ghost after 30-min exposure to [H]$^+$ouabain.

DISCUSSION

The main findings of this work are, first that Na, as well as K, decrease the rate of ATP-promoted ouabain binding to red cell ghosts and, secondly that these actions are dependent upon the presence of K$_o$. The relative effects of Na and K in regulating the rates of ouabain binding are directly associated with their effects in activating either Na:K exchange (Fig. 3) or K:K exchange (Fig. 7), the relationship being in both instances that the ouabain binding rate is inversely proportional to the rate of translocation. It is particularly significant that these
effects require K_o, given that there appears to be only one type of ouabain binding site involved regardless of the mode of transport being carried out (Table III), and since the ouabain binding rate is independent of any activity of the pump apparatus related to Na:Na exchange (see Table VI). Thus, any explanation of the effects of Na_i and K_i must include a role for K_o other than simply stimulating a turnover of the pump machinery. But before discussing these aspects it is worthwhile to consider other characteristics of ouabain binding both with regard to the types of determinates involved as well as the sidedness of their action.

As discussed previously (Hoffman, 1966) the sites which bind ouabain are thought to be located on the outside surface of the pump complex. Occupancy of a site by a glycoside results in inactivation of that site (presumably on a basis of one site per pump) since there is a direct correlation between the number of glycosides bound and the fractional inhibition of the transport (Hoffman, 1969; Ingram, 1970; Dunham and Hoffman, 1971). Ouabain once bound to a site is
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essentially irreversible since its dissociation takes place only at very slow rates (Ingram, 1970; Dunham and Hoffman, 1971). On the other hand, Glynn (1957) showed that glycoside binding to red cell membranes can be directly antagonized by \( K_o \) and it is of course of interest that it is only the rate of binding and not the equilibrium that is evidently influenced by \( K_o \) (Hoffman, 1966; Ingram, 1970). \( Na_o \) acts to increase the rate of ouabain binding (Fig. 1, Table IV) by decreasing the affinity of the membrane for \( K_o \) (see also Beauge and Adragna, 1971; Sachs, 1974). Obviously, \( Na_o \) has no effect on glycoside binding in the absence of \( K_o \) as already discussed. And it should be kept in mind that glycoside binding rates are markedly increased by the presence of nucleoside triphosphates (Matsui and Schwartz, 1968; Hoffman, 1969) these effects being exerted from the inside and not from the outside surface of the pump (Table II).

It is convenient to consider a model for the ouabain binding site (see Hoffman, 1972) in which the site itself exists in either one of two forms, \( A \) or \( B \). The two forms differ from each other depending upon their reactivity with ouabain such that ouabain binds only to the \( B \) form; there the \( B \) form is open to the outside while the \( A \) form is occluded. Combination of ouabain \((G)\) with the \( B \) form can be described by the reaction:

\[
B + G \rightarrow GB,
\]

in which the pump inactivation is associated with the formation of \( BG \) and, as mentioned before, \( BG \) is stable since its dissociation rate is extremely slow. Thus, to the extent that the set of the equilibrium, \( A \leftrightarrow B \), can be influenced by various conditions the rate of ouabain binding is thereby defined. Since it is known that the ouabain binding site \((B)\) is a component part of the pump complex (Dunham and Hoffman, 1970; Ruoho and Kyte, 1974) it is apparent that changes in the conformation of the pump affect the equilibrium (or configuration) of \( A \leftrightarrow B \). Even though quantitative relationships between changes in pump conformation and \( A, B \) configuration cannot be defined at the present time, there are certain qualitative and obvious relationships that are apparent. For instance, the \( A \leftrightarrow B \) equilibrium is shifted to the right, favoring the \( B \) form, by intracellular nucleoside triphosphates (Table II) and it has been discussed previously (Hoffman, 1969) that this can occur without phosphorylation of the pump complex; \( K_o \) in antagonizing ouabain binding would shift the equilibrium to the left but this action of \( K_o \) is more complicated than this since \( Na_o \) or \( K_i \) must also be considered. By thinking in terms of the "induced fit" concept it is possible to understand how combination of the pump with nucleotide triphosphates can result in a change in conformation (see Hoffman, 1961).

An important conclusion from the work presented in this paper is that in order for \( K_o \) to antagonize ouabain binding it is necessary that either \( Na_o \) or \( K_i \) must also be present (see Figs. 3 and 7). This is to say that in the absence of \( Na_o \) or \( K_i \) there is no competition between \( K_o \) and ouabain binding and there is no effect of \( Na_o \) on the action of \( K_o \).

To explain this type result using the \( A \leftrightarrow B \) equilibrium model it is attractive to consider that the particular set of the equilibrium is directly associated with some specific conformation of the pump complex. Using the terminology introduced by Albers et al. (1968) the \( B \) form could be tentatively identified as associated
with \( E_P \), the form of the phosphoprotein which in ordinary cycling of the pump is thought to provide a configuration which is oriented to the outside and to which ouabain most easily is complexed (Albers et al., 1968; Post et al., 1969). The fact that \( E_P \) is K sensitive (presumably this K is \( K_0 \)) and ADP insensitive (Post et al., 1969) provides a role for \( K_0 \) in the present studies but only provided \( N_a \) or \( K_i \) is also present. If this is so then, since under these conditions translocation of the ions is also occurring in either the Na:K or K:K mode, the pump is cycling through its various "E" forms and the \( A \rightleftharpoons B \) equilibrium is shifted to the left providing for the inverse relationship between transport and binding as already discussed. To explain the lack of correlation between the rate of ouabain binding and Na:Na exchange it could be that if the phosphorylated form of the enzyme is \( E_P \), which presumably would also be oriented to the outside in order to accommodate Na:Na exchange (see Glynn and Hoffman, 1971) with \( E_P \) being ADP sensitive and K insensitive (Post et al., 1969), the \( A \rightleftharpoons B \) equilibrium is not shifted without \( K_0 \) and therefore ouabain would bind at a rate independent of \( N_a \) or \( N_i \) (see Table VI).

When ouabain binding is promoted by nucleotide triphosphates (NTP) in the absence of phosphorylation the binding rate can only be changed by \( K_0 \) again when \( N_a \) or \( K_i \) is also present. This would imply (see Hoffman, 1969) that NTP combines with \( E \) to form \( E-NTP \) (see Hegvarya and Post, 1971; Fukushima and Tonomura, 1973; Mårdh and Zetterquist, 1974) and the formation of this complex shifts the \( A \rightleftharpoons B \) equilibrium to the right. \( K_0 \) would antagonize this by decreasing the affinity of \( E \) for NTP, thereby shifting the \( A \rightleftharpoons B \) equilibrium to the left. The particular form of \( E \) is not specified in this situation since it is not known what form \( E \) would take given the presence of \( K_0 \). It is also possible that \( E \) in combining with NTP may form a phosphorylated complex, \( E_P \), where the nucleotide diphosphate is still attached (see Fukushima and Tonomura, 1973; Mårdh and Zetterquist, 1974) and where the addition of K would reverse its formation. In either way the alteration of the binding rate by \( K_0 \), given \( N_a \) or \( K_i \), by either ATP or NTP (such as UTP [see Bodemann and Hoffman, 1976 a]) can be understood in terms of the \( A \rightleftharpoons B \) equilibrium model. Since the pump cannot actively transport using UTP as a substrate (see Hoffman, 1962 b), the dependence of the effect of \( K_0 \) on either \( N_a \) or \( K_i \) need not be correlated with the turnover of the pump through complete cycles.

There is another instance in which the rate of onset of inhibition of the pump by an inhibitor is inversely related to the pump's activity, and this is the finding by Skou (1974) that inhibition of ox brain Na,K-ATPase by N-ethylmaleimide is greater the lower the rate of Na,K-ATPase activity.

The question can be raised as to whether some of the effects of \( N_a \) might be due to the presence of orthophosphate (\( P_i \)) present on the inside as a result of the inevitability of ATP breakdown during incorporation. That any of the effects could be due to \( P_i \) seems unlikely since the rate of ouabain binding is decreased by \( N_a \) in the absence of \( K_0 \) in \( P_i \)-promoted ouabain binding. This is considered in more detail in a separate paper (Bodemann and Hoffman, 1976 a). Alternatively, \( N_a \) might exert its effect by direct antagonism with \( M_g \). But since
raising $[Na]$ is known to activate Na:K exchange, and since raising $[Na]$ in the absence of $K_a$ has no effect on the rate of ouabain binding, any effects due to changes in $[Mg]$ by $Na_i$ would appear to be minimal (see Bodemann and Hoffman, 1976b).

Perhaps the most surprising effects reported in this paper are those seen in association with changes in $[Na]$. This is because of the fact that not only is there an effect of Na per se but its effect is just opposite to what would be expected from results obtained on microsomal preparations (Matsui and Schwartz, 1968; Lindenmayer et al., 1968). There is a clear difference in the effects of Na on ouabain binding to porous human red cell ghosts compared to microsomal preparations of Na,K-ATPase. Ouabain binding to porous ghosts is little affected by the presence of Na (Hoffman, 1969) whereas ouabain binding to microsomal preparations shows a marked dependence on the addition of Na. The lack of an effect of Na on porous ghosts is consistent with the results presented in this paper since ouabain binding was unaltered by the presence or absence of Na:Na exchange. Sachs (1974) has also shown in intact human red cells that ouabain binding in the absence of K is the same whether or not Na is present. On the other hand, when ouabain binding to porous human red cell ghosts is studied in the presence of K as well as Na, the rate of ouabain binding (relative to the rate in the absence of K) is reduced. On the basis of the results presented in this paper, opposite effects are obtained with Na$_i$ compared to Na$_o$ given the presence of K$_o$. The results on porous ghosts, interpreted in the light of the present findings, would imply that any observed rate of ouabain binding is a resultant of the relative effects of the two ions on the inside compared to the outside. Thus Na$_o$ would increase the rate of ouabain binding by decreasing the effectiveness of K$_o$ in preventing binding, and, while Na$_o$ would tend to decrease the ouabain binding rate, K$_i$ would tend to counteract this just as Na$_o$ would tend to counteract K:K exchange, thereby yielding the overall result. The results with microsomal preparations (which to our knowledge are completely porous systems in the sense that no ion gradients are known to exist and the distribution of any added ion is the same inside as outside) either represent systems with quite different inherent properties with regard to the actions of Na on ouabain binding, or, more interestingly, there is the possibility that these preparations still contain small quantities of K. If this were so then at least it would be possible to understand the differences displayed by microsomal preparations compared to red cell ghosts. That is, in microsomal preparations the effect of Na in increasing the rate of ouabain binding would be the result of Na$_o$ counteracting an effect of K$_o$.

Finally, it should be emphasized that the effects of Na and K as reported in this paper very much depended on their sidedness. Since the observed actions of Na and K varied from what might be expected from the study of preparations which lack sidedness, e.g. microsomal preparations, a serious doubt can be raised concerning the locus of action with regard to side of any added ligand without independent supporting evidence. Thus there is a built-in indeterminacy of sided effects which should limit interpretations based on results obtained in the absence of a knowledge of side-dependent effects. It follows from
this type of consideration that reaction sequences derived from studies on such preparations could be misleading if not limited with regard to establishing relationships between transphosphorylation events and translocation of Na and K.

This work was supported by National Institutes of Health Grants, HE 09906, AM 05644, and National Science Foundation Grant GB 18924. Dr. Bodemann was supported by a fellowship provided by the Deutsche Forschungsgemeinschaft (BO 425/1,2).

Received for publication 25 March 1975.

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