[\textsuperscript{3}H]Ouabain Binding and Na\textsuperscript{+},K\textsuperscript{+}-ATPase in Resealed Human Red Cell Ghosts

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\textbf{Abstract} The interaction of the cardiac glycoside [\textsuperscript{3}H]ouabain with the Na\textsuperscript{+},K\textsuperscript{+} pump of resealed human erythrocyte ghosts was investigated. Binding of [\textsuperscript{3}H]ouabain to high intracellular Na\textsuperscript{+} ghosts was studied in high extracellular Na\textsuperscript{+} media, a condition determined to produce maximal ouabain binding rates. Simultaneous examination of both the number of ouabain molecules bound per ghost and the corresponding inhibition of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase revealed that one molecule of [\textsuperscript{3}H]ouabain inhibited one Na\textsuperscript{+},K\textsuperscript{+}-ATPase complex. Intracellular magnesium or magnesium plus inorganic phosphate produced the lowest ouabain binding rate. Support of ouabain binding by adenosine diphosphate (ADP) was negligible, provided synthesis of adenosine triphosphate (ATP) through the residual adenylate kinase activity was prevented by the adenylate kinase inhibitor Ap5A. Uridine 5'-triphosphate (UTP) alone did not support ouabain binding after inhibition of the endogenous nucleoside diphosphokinase by trypan blue and depletion of residual ATP by the incorporation of hexokinase and glucose. ATP acting solely at the high-affinity binding site of the Na\textsuperscript{+},K\textsuperscript{+} pump ($K_m \sim 1 \mu M$) promoted maximal [\textsuperscript{3}H]ouabain binding rates. Failure of 5'-adenylyl-\textbeta\textgamma-imidophosphate (AMP-PNP) to stimulate significantly the rate of ouabain binding suggests that phosphorylation of the pump was required to expose the ouabain receptor.

\textbf{Introduction} Previous investigations from this laboratory on human and sheep red blood cells established a one-to-one correlation between the number of molecules of [\textsuperscript{3}H]ouabain bound and the fractional inhibition of the active K\textsuperscript{+} influx component of the Na\textsuperscript{+},K\textsuperscript{+} pump (Joiner and Lauf, 1978b). The rate of [\textsuperscript{3}H]ouabain binding was influenced by changes of the intracellular Na\textsuperscript{+} and K\textsuperscript{+} concentrations, which suggested a conformational dependence of the ouabain receptor similar to or affected by the Na\textsuperscript{+},K\textsuperscript{+} pump (Joiner and Lauf, 1978c). Although others have examined the effect of various ligands on ouabain binding in resealed human erythrocyte ghosts (Lishko et al., 1972; Bodemann and Hoffmann, 1976a–c), no study has yet determined directly the...
specificity of ouabain for the Na\textsuperscript{+},K\textsuperscript{+} pump in these model membranes. Moreover, results obtained in resealed ghosts correlating the rate of ouabain binding with the turnover of the Na\textsuperscript{+},K\textsuperscript{+} pump (Bodemann and Hoffman, 1976a) contradict those obtained in intact human and sheep erythrocytes (Joiner and Lauf, 1978c), as well as those reported for purified membrane enzyme preparations (Wallick and Schwartz, 1974; Lindenmayer et al., 1974).

We examined the \[^3H\]ouabain binding process in resealed human erythrocyte ghosts under conditions similar to those used by Joiner and Lauf (1978a, b) in intact cells. We found that binding of one molecule of \[^3H\]ouabain was sufficient to inhibit one Na\textsuperscript{+},K\textsuperscript{+}-ATPase complex in resealed ghosts. Ouabain binding specifically required ATP and was not supported by other ligands such as Mg\textsuperscript{2+}, inorganic P (P\textsubscript{i}), ADP, 5\textsuperscript{'}-adenylyl-\beta\textsuperscript{-}\gamma\textsuperscript{-}imido-phosphate (AMP-PNP), or uridine 5\textsuperscript{'}-triphosphate (UTP) under the high-sodium conditions used. This work has been reported briefly elsewhere (Shoemaker and Lauf, 1979, 1980).

\section*{METHODS}

All solutions were prepared with deionized water and reagent grade salts and, when buffered, adjusted to appropriate pH values at 0, 37°C, or room temperature. The solutions used contained 1 mg\% chloramphenicol, B grade (Calbiochem-Behring Corp., San Diego, CA), and were: Tris-buffered saline (TBS) 0, 37, and RT: 165 mM NaCl, 2 mM TrisCl, 295 \pm 5 mosmol, pH 7.2, at 0, 37, and 22.5°C, respectively; 10 TCl: 10 mM TrisCl, pH 7.6, at 0°C; TCE: 10 mM TrisCl, 0.0625 mM EGTA, pH 7.6, at 0°C; HS-IA (red ghost hemolysing solution): 2 mM ATP, 4 mM Mg\textsubscript{2}SO\textsubscript{4}, pH 3.6-4.0; HS-IB (pink ghost and white ghost primary hemolysing solution): 4 mM Mg\textsubscript{2}Cl\textsubscript{2}, 0.1 mM EGTA, pH 3.6-4.0; HS-II (white ghost secondary hemolysing solution): 4 mM Mg\textsubscript{2}Cl\textsubscript{2}, 0.1 mM EGTA, pH 4.0; HS-IA (red ghost hemolysing solution): 4 mM Mg\textsubscript{2}Cl\textsubscript{2}, 0.1 mM EGTA, pH 3.6-4.0; HS-IB (pink ghost and white ghost primary hemolysing solution): 4 mM Mg\textsubscript{2}Cl\textsubscript{2}, 0.1 mM EGTA, pH 3.6-4.0; HS-II (white ghost secondary hemolysing solution): 4 mM Mg\textsubscript{2}Cl\textsubscript{2}, 0.1 mM EGTA, pH 4.0; CHB (column hemolysis buffer): 5 mM Mg\textsubscript{2}Cl\textsubscript{2}, 15 mM HEPES, 0.1 mM EGTA, pH 6.0; HBS 0, 37, and RT: 150 mM NaCl, 10 mM HEPES, pH 7.4, at 0, 37, and 22.5°C, respectively. Restoring solutions: a 2-M NaCl solution together with various amounts of Tris-OH was used to bring the ghost preparations to isotonicity and pH 7.2.

\textit{Inhibitor, Nucleoside Phosphate, and Enzyme Solutions}

Ouabain octahydrate, 10\textsuperscript{-8} M, (Sigma Chemical Co., St. Louis, MO) was dissolved in TBS 37 or in deionized water. Two 5-mCi batches of \[^3H\]ouabain (New England Nuclear, Boston, MA) had specific activities of 20.6 and 22.1 Ci/mmoll, determined by the method of Joiner and Lauf (1978a). 1-mCi/ml stock solutions of \[^3H\]ouabain were prepared by evaporation of benzene:ethanol and addition of TBS 37. A 10-mM trypan blue (Sigma Chemical Co.) solution, pH 6.0, was prepared and stored at room temperature.

Stock solutions of ADP, grade II ATP, Ap\textsubscript{5}A, type VI CTP, and type III UTP (all from Sigma Chemical Co.), and AMP-PNP (ICN Nutritional Biochemicals, Cleveland, OH) were prepared in hemolysing solution, and of vanadium-free ATP (Boehringer Mannheim Biochemicals, Indianapolis, IN) and \(\gamma\textsuperscript{32P}\)ATP (triethylamonium salt; New England Nuclear) in deionized water and stored at \(-90^\circ\text{C}\).

Stock solutions of creatine phosphate (CP) and creatine kinase (CK) in hemolysing solution and of hexokinase (all from Sigma Chemical Co.) in CHB were made just before the respective ghost preparations.
Preparation of Erythrocyte Ghosts

Human blood was collected by venipuncture using heparin or Alsevier's solution (Bukantz et al., 1946) as anticoagulants and used immediately or stored at 0-4°C for no longer than 20 h. The methods of ghost preparation were modifications of those originally described by Passow's laboratory (Bodemann and Passow, 1972; Lepke and Passow, 1972; Schwoch and Passow, 1973; Wood and Passow, 1981). Three types of ghosts were defined on the basis of dilution and the appearance of the packed pellet of ghosts due to their residual hemoglobin concentration.

Red Ghosts

Red ghosts were prepared by diluting erythrocytes 27-fold (Funder and Wieth, 1976). A 5-min lysis period in HS-IA was followed by addition of the restoring solution, a 15-min incubation at 0.5°C, and a 1-h resealing period at 37°C. The resealed high Na⁺ ghosts were collected after 45 min on a sucrose cushion (43% sucrose; 50 mM NaCl; 25 mM TrisCl; pH 7.2, at 0°C) spun at 27,000 g.

Pink Ghosts

Pink ghosts were prepared by an initial 170-fold dilution of red cells in hemolysing solution (HS-IB), and the suspension was left for 5 min in the ghosting chamber at pH 6.0. The ghosts were concentrated for 6 min at 30,000 g using a GSA head in a Sorvall RC5-B centrifuge (Dupont Instruments, Newton, CT) with no brake at 2-0°C, resuspended in ~30 ml of supernatant, and returned to the ghosting chamber. Compounds to be incorporated into the ghosts were added, and the suspension (final volume = 33 ml) was stirred for 5 min, followed by the addition of 3 ml of restoring solution and stirring for 15 min. Subsequent steps were identical to the preparation of red resealed ghosts with the exception that the sucrose cushion was eliminated after experiments revealed that all pink ghosts were sucrose impermeable.

White Ghosts

White ghosts were lysed analogously to pink ghosts initially but restored after 5 min to isotonicity and pH 7.2 with 3 ml of restoring solution and equilibrated for 15 min. Ghosts were concentrated and the packed ghosts (in 3-ml volumes) were added directly to HS-II. The volume of this second hemolysis solution ranged from 0.030 to 1.0 liter, depending upon the desired dilution of intracellular constituents. After 5 min at pH 6.0, isotonicity and pH 7.2 were restored.

Column Ghosts

Column ghosts were prepared by the method of Wood (1975; Wood and Passow, 1981) with a modified composition and order of addition of column buffers. A K100/45 column (Pharmacia Fine Chemicals, Piscataway, NJ) was equilibrated with CHB, and subsequently a volume of HBS (equal to 10% of the bed volume) was applied, followed by a 15% suspension of red blood cells washed thrice in HBS (5% of the bed volume). White ghosts were collected directly from the column into an ice-cold container, concentrated, restored to isotonicity, and resealed with the desired internal constituents.

Ghost Parameters

Hemoglobin concentrations were determined at 415 nm in the hemolysate supernatants obtained after the ghost resealing period using an extinction coefficient of 8.132
(gHb/liter)$^{-1}$ at 415 nm. Ghost counting was performed with a model F Coulter Counter (Coulter Electronics Inc., Hialeah, FL). Calculation of mean ghost volumes of red ghosts was based on the number of ghosts per liter, cytocrit, and percent trapped extracellular volume on the same population. Estimates of the mean ghost volume of pink and white resealed ghosts were made from a series of diameter measurements of negatively stained samples prepared for electron microscopic examination as previously described (Ting-Beall et al., 1981).

ATP was measured by the coupled fluorometric assay of Lowry et al. (1964) for concentrations of ATP in the range of 0.1–2.0 mM, and for ATP concentrations below 0.1 mM, a slightly modified version of the luciferin-luciferase assay of Kimmich et al. (1975) was used. Extracts were prepared either by acid elution with 0.51 M perchloric acid and subsequent neutralization with 5.62 M K$_2$CO$_3$ or by boiling the sample for 2 min. Intracellular ATP concentrations were calculated from the total ATP in the sample using the mean ghost volume, the ghost fractional water content as determined by the method of Cook (1967), and the number of ghosts per sample.

$[^3]$H]Ouabain Binding

At zero time, preequilibrated ghosts were suspended to 25% cytocrit in temperature-equilibrated TBS 37 containing $[^3]$H ouabain with concentrations ranging from $10^{-7}$ to $10^{-8}$ M. Lower concentrations of $[^3]$H ouabain gave anomalous binding kinetics which were interpreted as resulting from the deterioration of the ghost preparation over the time required to reach saturation binding (6 h). At various times, triplicate samples of the incubation suspension were squirted into 50 vol of ice-cold TBS 0. During the course of the incubation, duplicate samples were also taken for determination of the number of ghosts per milliliter of incubation suspension. The ghosts were washed five times in TBS 0 to reduce the tritium counts in the membrane pellet to a stable value. Ghosts were then hemolysed by the addition of 10 TCI and washed an additional three to five times to obtain the lowest level of hemoglobin possible. The ghost pellet was digested for 15 min at room temperature in 0.5 ml of 0.1 N NaOH, neutralized with 0.2 ml of 0.5 N HCl and counted in 10 ml Scintillant. Vials were counted for tritium in a model LS-250 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA) with an efficiency of 25–30%.

The number of ouabain binding sites per ghost was calculated using the disintegrations per minute (dpm) per sample, the measured specific activity of $[^3]$H ouabain (previous studies determined the stated specific activity of $[^3]$H ouabain [New England Nuclear] to be inaccurate [Joiner and Lauf, 1978a]), and the number of ghosts per sample. Nonspecific binding was determined in the presence of a 1,000-fold excess of cold ouabain and never exceeded 2%.

Protein Determination

Membrane protein concentrations were measured by the method of Lowry et al. (1951) with the addition of 1% sodium dodecyl sulfate to all samples and standards.

$Na^+\text{-}K^+\text{-}ATPase Assay$

The method of Blöstein (1968) was used. The assay media consisted of the following (mM): 10 KCl, 75 NaCl, 0.525 MgCl$_2$, 0.025 EGTA, 10 Tris-Cl, 0.5 ATP, 0.1 ouabain (when present), pH 7.6, in a volume of 0.5 ml. Membranes were added in TCE to give a final concentration of 200 $\mu$g/ml in the presence of 0.3 $\mu$Ci/ml $[^3]$P ATP. Incubations were carried out routinely for 10 min at 37°C.
**Binding vs. Inhibition Studies**

Two sets of samples were taken at various times, one for determination of the number of [³H]ouabain binding sites, and the other for the fractional activity of the Na⁺,K⁺-ATPase. Ghosts used for determination of Na⁺,K⁺-ATPase activity were taken from the [³H]ouabain binding incubation and squirted into ice-cold TCE. The membranes were pelleted at 27,000 g for 15 min at 0-4°C and residual hemoglobin was removed by one wash in TBS0 and three washes in TCE.

Bound ouabain dissociates during the enzyme assay. Hence, the Na⁺,K⁺-ATPase activity reactivated during the course of the enzyme assay incubation was monitored by the appearance of activity from a ouabain-saturated enzyme. The fractional amount of Na⁺,K⁺-ATPase reactivated during a 10-min assay was 7.6% and was subtracted from the observed activities to obtain the correct values. The degree of inhibition was calculated from the Na⁺,K⁺-ATPase activity in membranes exposed to [³H]ouabain and control membranes in the absence of [³H]ouabain.

**Substrate Support of [³H]Oubain Binding**

Substrates were added to either the primary or secondary hemolysis media, depending upon acid lability, molecular weight, and expense. The resealing period was shortened to 15 min in experiments using the CP-CK ATP-regenerating system to avoid depletion of the system. [³H]Oubain incubation times were also varied to obtain binding curves delineating the initial rate of binding.

Two enzymes known to be present in membrane preparations of human red blood cell ghosts are adenylate kinase (Cavieres and Glynn, 1976, 1979) and nucleoside diphosphokinase (Mourad and Parks, 1966; Kaplan and Hollis, 1980). Adenylate kinase catalyzes the reaction ATP + AMP ⇄ 2ADP, whereas nucleoside diphosphokinase facilitates the reaction X₁TP + X₂DP ⇄ X₁DP + X₂TP where X₁ and X₂ can be any of a variety of purine or pyrimidine ribo- or deoxyribonucleosides. Ap₅A was shown by Lienhard and Secemski (1973) to be a potent inhibitor of the adenylate kinase from rabbit muscle and also effective in human red blood cell ghosts (Cavieres and Glynn, 1976, 1979; Kennedy et al., 1980). Trypan blue inhibits nucleoside diphosphokinase in resealed human red cell ghosts prepared at very high dilutions of internal constituents (Kaplan and Hollis, 1980).

For the ATP support of ouabain binding, a constant concentration of nucleotides over the incubation time was necessary. The CP-CK ATP-regenerating system first used by Garrahan and Glynn (1967a) inside resealed ghosts was chosen. Because of the high molecular weight of CK (82,600; Watts, 1973) and the acid lability of CP (Fiske and Subbarow, 1929), the system was incorporated into the secondary hemolysis media in the preparation of white ghosts. A system comprised of 5 mM CP and 5 U/ml CK was capable of maintaining constant ATP values throughout a 20-min incubation provided the resealing period during the ghosting process was shortened from 60 to 15 min.

**RESULTS**

Resealed red ghosts were prepared with ~2.0 mM ATP sealed inside initially. Fig. 1 shows that ATP was hydrolysed while the ghosts were prepared; hence, ATP was added in excess to saturate the Na⁺,K⁺ pump (the condition existing in intact cells) during the ouabain binding step. As only a high-affinity site for ATP ($K_m = 1 \mu M$) is available under the existing experimental conditions...
(Moczydlowski and Fortes, 1980, 1981), the 500 μM residual ATP was considered sufficient. This assumption was also supported by the fact that internal ATP higher than 2 mM did not increase further the initial rate of [³H]ouabain binding. Finally, incorporation of 5 mM CP and 5 U/ml CK together with 2 mM ATP did not change the rate of ouabain binding (not shown), which indicates a lack of support by the elevated ADP levels resulting from ATP hydrolysis.

There was a considerable difference in the ghost volume of pink and white resealed ghosts. The mean corpuscular volume of red ghosts was 84.9 ± 5.8 μm³, whereas pink and white ghosts were smaller and heterogeneous, ranging in volume from 40 to 60 μm³.

[³H]Ouabain Binding to ATP-replete Ghosts

The rate of [³H]ouabain binding to ATP-replete red, pink, and white resealed ghosts (Fig. 2) revealed identical binding kinetics and the same maximum number of ouabain binding sites per ghost (468 ± 14; n = 5) on a limited sample size (n = 5). Study of a greater sample of individuals over a prolonged time period (3 yr) yielded a range of 400–600 ouabain binding sites/ghost.
The same binding maximum was achieved at each ouabain concentration. Because the nucleotide concentrations within the ghosts could not be controlled for prolonged periods of time, a $K_1$ of the enzyme-ouabain interaction was not obtained. Therefore, the initial rate of ouabain binding was the qualitative tool used to illustrate modulation of the binding process.

$[^3H]$Ouabain Binding vs. Inhibition of Na$^+$,K$^+$-ATPase

We decided to relate ouabain binding to the inhibition of the Na$^+$,K$^+$-ATPase activity and not to K$^+$ pump influx (Dunham and Hoffman, 1971; Joiner and Lauf, 1978a) because the passive cation permeabilities in resealed ghosts are known to be approximately four times that of intact red blood cells (Bodemann and Hoffman, 1976a). Furthermore, the state of the pump substrates once incorporated into resealed ghosts is uncertain. The relationship between the number of ouabain binding sites occupied and the fractional inhibition of the Na$^+$,K$^+$-ATPase is shown in Fig. 3: each molecule of bound ouabain inhibited one Na$^+$,K$^+$-ATPase complex. The extrapolation to >500 ouabain binding sites/ghost may reflect some dissociation of ouabain during the membrane preparation for Na,K-ATPase assay.

Substrate Support of Ouabain Binding

Before determination of the ATP dependence of ouabain binding, the effect of the reaction products of the Na$^+$,K$^+$-ATPase reaction, ADP and Pi, were tested.
Mg and Mg with $P_i$

Fig. 4 illustrates $[^3]$H]ouabain binding to high Na$^+$ resealed ghosts containing MgCl$_2$ and MgCl$_2$ with $P_i$. Ghosts containing $P_i$ were assayed in the presence of $P_i$ in the external medium to prevent loss from the ghosts through the anion exchange pathway. It is known that external $P_i$ does not affect the rate of ouabain binding to resealed ghosts (Lishko et al., 1972; Bodemann and Hoffman, 1976b). The ouabain binding rate was negligible in comparison with ghosts replete with ATP, but still exceeded the nonspecific binding measured in the presence of $10^3$-fold excess of unlabeled ouabain. Results similar to those shown in Fig. 4 were obtained in two other experiments with pink ghosts. The rate of Mg$^{2+}$-supported ouabain binding to white ghosts was consistently 50% of that obtained for pink ghosts (not shown), which was due to lower levels of endogenous ATP in white ghosts.

It was found that dilution of intracellular constituents in excess of 20,000-fold in the preparation of white ghosts did not succeed in lowering the ATP concentration within the ghosts to $<3 \times 10^{-7}$ M. Also, column ghosts, which should theoretically contain immeasurable amounts of nucleotide, were routinely found to have ATP concentrations in the range of $3-7 \times 10^{-7}$ M. We propose that this may be a manifestation of the compartmentalized ATP...
found by other studies of the human erythrocyte membrane (Proverbio and Hoffman, 1977; Mercer and Dunham, 1981).

**ADP**

Ouabain binding has previously been shown to be supported by ADP in both human red blood cell membranes (Hoffman, 1969) and purified Na,K-ATPase from calf heart (Schwartz et al., 1968). To exclude the possibility that ADP was supporting ouabain binding by conversion to ATP through the adenylate kinase reaction, the inhibitor Ap5A was also incorporated into the ghosts along with Mg and ADP. It was first necessary to determine what effect, if any, the compound Ap5A had on the rate of ouabain binding. Fig. 5 shows the effect of three different concentrations of Ap5A on the rate of ouabain binding. This significant support of ouabain binding was determined to be due to contaminating ATP in the Ap5A.

Fig. 6 shows that the rate of ouabain binding to pink ghosts containing both ADP and Ap5A (upper curve) was caused by the additive effects of ADP (middle curve) and Ap5A (Fig. 5). In white ghosts with ADP and Ap5A at a 2,000-fold dilution of original internal constituents, the rate of ouabain binding fell to baseline levels (Fig. 6, bottom curve). The different results obtained in ouabain binding may be due to different dilution of endogenous adenylate kinase in the two ghost preparations. In pink ghosts, the concentration of Ap5A used was insufficient to inhibit adenylate kinase-supported ATP synthesis from ADP, whereas in the white ghosts, the adenylate kinase was presumably completely inhibited.
To examine whether ATP synthesized via the adenylate kinase was responsible for the ADP support of ouabain binding in pink ghosts, the rate of ouabain binding was followed in white ghosts containing ADP, Ap5A, and exogenous adenylate kinase (rabbit muscle). Adenylate kinase was present at the levels existing in the pink ghost preparation at a 200-fold dilution of original cell constituents (Cavieres and Glynn, 1979). The rate of ouabain binding obtained in this manner was significantly higher (not shown) than baseline levels.

**ATP**

The ATP dependence of ouabain binding was examined using the CP-CK-regenerating system to maintain constant ATP levels. To exclude contaminating ATP, the regenerating system itself was examined for its ability to support ouabain binding. As shown in Fig. 7, the CP-CK-regenerating system supported ouabain binding. However, the individual components of the system failed to do so.

To determine whether ouabain binding was supported by the nonhydrolyzable ATP analogue AMP-PNP, the compound was incorporated into resealed ghosts at a concentration sufficient to saturate both the high- and low-affinity ATP sites of the Na,K pump. Although contaminating ATP at a concentration sufficient to promote ouabain binding may obscure the accurate interpretation (Tobin et al., 1974), it is still evident from Fig. 7 that AMP-PNP did not support ouabain binding to the extent shown by ATP at comparable concentrations. Since it is known that AMP-PNP is capable of supporting K:K
exchange by occupation of the low-affinity ATP site, it was then of interest to examine the rate of ouabain binding supported by concentrations of ATP insufficient to act at the low-affinity site.

We explored whether the ATP-regenerating system was incorporated into column ghosts by measuring ouabain binding and ATP levels at indicated intervals, as shown in the lowest binding curve of Fig. 8. Obviously, submicromolar concentrations of ATP supported ouabain binding significantly, which indicates contamination by endogenous ATP. To test this possibility, exogenous ATP was added to the column ghosts just before resealing, and the ATP concentrations were maintained by the regenerating system. Fig. 8 shows that the rate of ouabain binding was significantly affected by changes at submicromolar levels of ATP. Initial experiments revealed that resealed ghosts containing between 20 and 200 μM ATP exhibited the same rates of ouabain binding as ghosts containing 500 μM ATP.

**UTP and CTP**

The nucleotides UTP and CTP have been shown to effectively support ouabain binding to purified Na⁺,K⁺-ATPase (Tobin et al., 1974) and resealed human erythrocyte ghosts (Hoffman, 1969; Bodemann and Hoffman, 1976b). The nucleoside diphosphokinase inhibitor trypan blue was used to investigate whether the support of ouabain binding was the result of synthesis of ATP from the pyrimidine nucleotides through the nucleoside diphosphokinase or of direct interaction of UTP and CTP with the Na⁺,K⁺ pump.

Initial experiments showed that resealed ghosts containing 2 mM UTP at
the time of resealing supported ouabain binding to an extent almost equivalent to that observed in the presence of ATP. Incorporation of trypan blue did not significantly alter the effect of UTP (Fig. 9), which is consistent with direct support of ouabain binding by UTP or contamination by ATP. To check for ATP contamination, both hexokinase and glucose were included in the resealing mixture, together with UTP and trypan blue. Under these conditions, the rate of ouabain binding was found to be at baseline levels. Ghosts with UTP, glucose, and hexokinase had negligible glucose-6-phosphate content, which excludes UTP as substrate for the hexokinase reaction. Hence, UTP did not directly support ouabain binding to the Na,K pump of human red cell ghosts.

This finding was consistent with the relatively low affinity of UTP for purified Na,K-ATPase from guinea pig kidney cortex (Hegyvary and Post, 1971). The pyrimidine nucleotide CTP, however, has a significantly higher affinity for the purified enzyme (Hegyvary and Post, 1971), supports uncoupled Na\(^+\) efflux (Karlish and Glynn, 1974), phosphorylates the enzyme (Tobin et al., 1972), and is hydrolyzed appreciably by the purified enzyme (Robinson, 1982). Fig. 9 shows that the ouabain binding rate in the presence of CTP, trypan blue, glucose, and hexokinase was intermediate to that supported by UTP and ATP.
DISCUSSION

The present study indicates ouabain binding to the Na⁺,K⁺ pump of resealed ghosts is supported entirely by ATP interaction at its high-affinity site. A linear slope was obtained in a plot of ouabain molecules per ghost vs. inhibition of the Na⁺,K⁺-ATPase showing that one cardiac glycoside molecule is sufficient to inhibit one enzyme complex. Ouabain binding was not supported by (a) Mg²⁺, (b) Mg²⁺ plus Pᵢ (under the high-Na⁺ conditions), (c) Mg²⁺ plus ADP, or (d) AMP-PNP, which indicated that phosphorylation of the Na⁺,K⁺ pump in resealed ghosts was a prerequisite of the enzyme ouabain interaction.

![Graph showing ouabain binding and inhibition](image_url)
The precipitous decline in the ATP levels of resealed ghosts shown here has been noted previously (Kaplan et al., 1978) and may be due in part to the action of an ouabain-insensitive phosphatase, such as the Mg-ATPase activity of the human erythrocyte membrane (Hoffman, 1980) or to nonspecific phosphatases of the cytosol.

Our verification of the existence of a membrane-associated ATP pool is of relevance in this regard. The previous attempts at quantitation of the amount of ATP associated with this membrane compartment have arrived at values ranging from 100 to 700 molecules of ATP per Na,K pump (Proverbio and Hoffman, 1977; Mercer and Dunham, 1981). Proverbio and Hoffman derived this estimate from a value of 27,000 molecules of ATP/ghost, which is equivalent to a concentration of $5.0 \times 10^{-7}$ M ATP/liter ghost water, assuming a value of $85 \times 10^{-16}$ liter for the mean corpuscular volume. This value corresponds favorably with the ATP associated with the membrane in our preparations of extremely dilute ghosts or column ghosts. In a series of experiments on white ghosts and column ghosts, a range of 0.3–0.7 μM ATP was found to be associated within the human red blood cell ghost when only a fraction of these values should remain as a consequence of the dilution. The mechanism that would explain this finding is one in which ATP is somehow intimately associated with the membrane structure (Mercer and Dunham, 1981).

**Figure 9.** The nucleotide support of ouabain binding to three different preparations of column ghosts. Content: ○, 2 mM ATP, 100 μM trypan blue; ■, 2 mM UTP, 100 μM trypan blue; ▲, 2 mM UTP, 100 μM trypan blue, 6 U/ml hexokinase, 10 mM glucose; ◆, 2 mM CTP, 100 μM trypan blue, 6 U/ml hexokinase, 10 mM glucose. The binding curve obtained with 2 mM CTP and 100 μM trypan blue was superimposable upon the 2 mM UTP and 100 μM trypan blue binding curve. [³H]Ouabain = $10^{-7}$ M.
High Na\(^+\) concentrations are known to inhibit the formation of the Mg\(^{2+}\)- and P\(_{i}\)-supported enzyme-ouabain complex (Skou et al., 1971) and to prevent the formation of a Mg\(^{2+}\)- and P\(_{i}\)-induced, phosphorylated intermediate of purified Na\(^+\), K\(^+\)-ATPase (Post et al., 1975). Despite the rapid decline in ATP and the rise in intracellular ADP and P\(_{i}\), \(^{3}\text{H}\)ouabain must have been bound to the enzyme supported by Na\(^+\), Mg\(^{2+}\), and ATP. This conclusion was reached because neither Mg\(^{2+}\) nor Mg\(^{2+}\) plus P\(_{i}\) nor ADP in the presence of sufficient concentrations of the adenylate kinase inhibitor Ap5A supported significant ouabain binding rates (Figs. 4 and 6).

The integrity of nucleotides in resealed ghosts is crucial for the interpretation of our data. Joiner and Lauf (1978c) proposed that a decline in the internal concentration of ATP may have been the cause of the discrepancy in the effect of internal cation concentrations on \(^{3}\text{H}\)ouabain binding in resealed ghosts (Bodemann and Hoffman, 1976a) and nystatin-treated red cells (Joiner and Lauf, 1978c). The resealed ghost preparation of Bodemann and Hoffman differed from our preparation by significantly lower internal Na\(^+\) concentrations, which may allow for ouabain binding by Na\(^+\), Mg\(^{2+}\), and ATP as well as Mg\(^{2+}\) and P\(_{i}\). Joiner and Lauf (1978b, c), on the other hand, measured the ATP concentration after the binding was completed to ascertain maintenance of cellular ATP levels.

Ouabain binding in resealed ghosts resembled that of intact human red cells with regard to the apparent irreversibility of the Na\(^+\), K\(^+\) pump-ouabain interaction (Hoffman, 1966, 1969; Dunham and Hoffman, 1971; Sachs, 1974; Joiner and Lauf, 1975, 1978b). However, as it was difficult to control internal nucleotide concentrations, the experiments were done with a considerable excess of \(^{3}\text{H}\)ouabain, which precluded measurement of a \(K_1\) for the enzyme-ouabain interaction.

Every molecule of \(^{3}\text{H}\)ouabain bound inhibited one Na\(^+\), K\(^+\)-ATPase membrane complex, as indicated by the slope of unity in the binding vs. inhibition plot (Fig. 3), which corroborated previous findings in erythrocytes on the correlation between \(^{3}\text{H}\)ouabain binding and K\(^+\) pump flux inhibition (Dunham and Hoffman, 1971; Joiner and Lauf, 1978a). Hence, also in ghosts, each Na\(^+\), K\(^+\) pump presents only one ouabain binding site to the external medium.

With the ionic conditions chosen, the Na\(^+\), K\(^+\) pump was either dormant relative to the translocation of cations (Garrahan and Glynn, 1967a, b; Glynn and Karlish, 1976) or operating in a reduced Na\(^+\)/Na\(^+\) exchange mode (Garay and Garrahan, 1973). Nevertheless, the maximum rate of \(^{3}\text{H}\)ouabain binding to these resealed ghost membranes was effected. This was of interest in light of previous reports that the rate of ouabain binding was inversely (Bodemann and Hoffman, 1976a) or directly (Joiner and Lauf, 1978b) proportional to the rate of turnover of the Na\(^+\), K\(^+\) pump. Hence, the rate of ouabain binding is primarily dependent not on the turnover rate of the Na\(^+\), K\(^+\) pump through a sequence of conformations, but on the existence of a particular reaction intermediate, most probably \(E_2-P\) (Sen et al., 1969; Post et al., 1969; Tobin et al., 1973).

Previous evidence for nonspecific support of ouabain binding by various Na\(^+\), K\(^+\)-ATPase ligands, substrates, and their analogues has been provided
for purified enzyme preparations (Schwartz et al., 1968; Albers et al., 1968; Tobin et al., 1972) and resealed erythrocyte ghosts (Hoffman, 1969; Bodemann and Hoffman, 1976b). Before concluding that the Na⁺,K⁺ pump-ouabain interaction is related to a particular enzymatic conformation, contaminating activities of adenylate kinase and nucleoside diphosphokinase in resealed ghosts have to be checked. ADP sealed inside high-Na⁺ and -Mg²⁺ ghosts supported an initial ouabain binding rate, as did ATP; however, saturation levels were never obtained (Fig. 6). Incorporation of the adenylate kinase inhibitor Ap5A slightly enhanced the rate of ouabain binding over baseline levels because of hydrolysis of the compound and concomitant ATP production (Fig. 5). However, with ADP plus Ap5A (at concentrations sufficient to completely inhibit the endogenous adenylate kinase), the rate of ouabain binding was close to that found with Ap5A alone. Hence, ADP supports ouabain binding via the synthesis of ATP by the adenylate kinase reaction and not by the molecular interaction of ADP with the Na⁺,K⁺ pump.

Contaminating adenylate kinase activity in preparations of Na⁺,K⁺-ATPase has been reported previously (Stahl, 1968; Blostein, 1970; Banerjee et al., 1972), and its presence in human red blood cells (Nilsson and Ronquist, 1969; Fajnholc et al., 1972) and resealed ghosts (Cavieres and Glynn, 1976, 1979) is well documented. The observation by Albers et al. (1968) that ouabain binding was supported by ADP can now be explained by the existence of both a contaminating phosphatase and adenylate kinase activities. The studies of Tobin and Sen (1970) as well as Hansen et al. (1971) indicate that ADP inhibits ATP-supported ouabain binding and alone supports the binding to a negligible extent. Therefore, ADP together with Na⁺ and Mg²⁺ does not induce the ouabain binding conformation of the Na⁺,K⁺ pump.

Similarly, support of ouabain binding by the pyrimidine triphosphate UTP can also be discounted. UTP sealed inside resealed ghosts at a level previously shown to support ouabain binding in resealed ghosts (Hoffman, 1969; Bodemann and Hoffman, 1976b) produced binding curves similar to those obtained with ATP. If the nucleoside diphosphokinase inhibitor trypan blue was introduced together with UTP, however, the rate of ouabain binding was only slightly less than that of UTP-supported binding, which indicates that perhaps UTP does support ouabain binding to the pump. Incorporating 10 mM glucose and hexokinase (to deplete ATP stores), together with UTP, produced baseline level of ouabain binding, which indicates that it was contaminating ATP that supported the rate of ouabain binding initially attributed to the presence of UTP. This finding is consistent with the known low affinity of UTP for the Na,K-ATPase (Hegyvary and Post, 1971; Tobin et al., 1972).

In ATP-depleted column ghosts, CTP produced a slow but significant rate of ouabain binding, a finding consistent with the recent report by Robinson (1982) that demonstrated the Na⁺,K⁺-CTPase activity to be only 10% of the Na⁺,K⁺-ATPase activity in a purified enzyme from canine kidney medulla. Tobin et al. (1974), however, found CTP to be as effective as ATP in supporting ouabain binding to a purified Na⁺,K⁺-ATPase from guinea pig kidney.
Having found that in the presence of high concentrations of Na\(^+\) plus Mg\(^+\) ATP effected ouabain binding, we examined whether ATP was acting at the low- and/or high-affinity sites (Robinson, 1976; Glynn and Karlsh, 1976). Initial experiments in which internal ATP concentrations were held constant in the range of 20–200 \(\mu\)M produced maximal rates of ouabain binding. However, when the ATP levels were reduced to submicromolar levels using the column ghosting process, relative rates of ouabain binding became directly proportional to the ATP levels determined. Hence, saturation of the high-affinity ATP binding site in the presence of high Na\(^+\) and Mg\(^{2+}\) was sufficient to induce the ouabain binding conformation of the Na\(^+\),K\(^+\) pump, which is consistent with reports on purified Na,K-ATPase from beef brain (Skou et al., 1971).

The results presented emphasize the specific support of ouabain binding by ATP occupying a high-affinity site in resealed ghosts. In the absence of K\(^+\) and in the presence of Na\(^+\), Mg\(^{2+}\), and ATP, the most likely enzymatic intermediate presenting the ouabain binding site to the external media is the \(E_2-P\) complex, which is not readily dephosphorylated by ADP.

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