The Titration of the Cardiac Glycoside Binding Site of the
(Na\textsuperscript{+} + K\textsuperscript{+})-Adenosine Triphosphatase*  

(Received for publication, May 26, 1972)  

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SUMMARY  

N-(4'-Amino-n-butyl)-3-aminoacetylstrophanthidin is a potent inhibitor of the (Na\textsuperscript{+} + K\textsuperscript{+})-adenosine triphosphatase, with a dissociation constant two orders of magnitude smaller than that of the parent compound, strophanthidin. The kinetics of inhibition of this new compound is qualitatively similar to that of strophanthidin. The difference in free energy of binding of the new inhibitor to the form of the enzyme present at saturating concentrations of sodium and MgATP and no potassium and the form present at saturating concentrations of potassium and MgATP and no sodium has been calculated. A kinetic titration of the cardiac glycoside binding sites of several preparations of the enzyme demonstrates that there is one cardiac glycoside binding site for each molecule of the polypeptide chain which is phosphorylated during turnover of the enzyme.

The cardiac glycosides and aglycones, such as ouabain, digoxin, and strophanthidin, are very specific inhibitors of the (Na\textsuperscript{+} + K\textsuperscript{+})-adenosine triphosphatase, that enzyme responsible for the active transport of sodium and potassium across the cell membrane. The interaction of this enzyme with these inhibitors has been studied in both kinetic (1-5) and direct binding (6, 8-9) experiments. It has been shown that, in the presence of MgATP, sodium enhances the specific inhibition (1, 5) while potassium decreases it (1, 3, 4, 8). Matsui and Schwartz pointed out that these effects paralleled the effects of ions on the phosphorylation state of the enzyme and proposed that the phosphorylated form\textsuperscript{1} of the enzyme bound inhibitor more tightly than the dephosphorylated form (6). This hypothesis has been further substantiated (7, 10, 11), and it has been shown that the energy difference in the binding of ouabain to the two forms of the enzyme can be linked to a phosphorylation of the protein by inorganic phosphate (10-13).

This paper describes the properties of a synthetic derivative of strophanthidin which releases from the (Na\textsuperscript{+} + K\textsuperscript{+})-adenosine triphosphatase with a dissociation constant two orders of magnitude smaller than that of strophanthidin. The inhibition kinetics of this compound was determined in an attempt to understand the reason for this increase in affinity. The kinetics is qualitatively the same as that of strophanthidin and the other inhibitors of this class. The tight and rapid binding of this inhibitor made it an ideal reagent for a kinetic titration (14) of the cardiac glycoside binding sites of the (Na\textsuperscript{+} + K\textsuperscript{+})-adenosine triphosphatase. Therefore, the concentration of these sites in a purified preparation of this enzyme (15) was determined. It was found that there is 1 mole of site present per mole of the polypeptide chain which specifically phosphorylates (16).

EXPERIMENTAL PROCEDURES  

Materials  

Chemicals—Strophanthidin was obtained from Sigma. It was recrystallized from 35\% methanol (m.p. 295-301° dec) and the water of hydration removed at 110°, 20 mm Hg over P\textsubscript{2}O\textsubscript{5}. 1,4-Diaminobutane was obtained from Aldrich. XAD4 resin (Rohm & Haas) was the generous gift of Dr. Ishaiahu Shechter of Harvard University.

Enzyme—Salt-detergent-extracted microsomes and supernatant enzyme have been previously described (15). XAD4 enzyme was prepared from supernatant enzyme by passing it over a column (of a volume one to two times that of the sample) of XAD4 which had been activated by washing with methanol and then equilibrated with 0.25 m sucrose, 1 mm EDTA, 0.03 m histidine chloride, pH 6.8. This resin removed greater than 95\% of the deoxycholate from the sample, based on acetic anhydride assay, while the organic phosphate to protein ratio was unaffected. The XAD4 enzyme, dialyzed into 0.25 m sucrose, 1 mm EDTA, 10 mm 2-mercaptoethanol, 0.03 m histidine chloride, pH 6.8, lost less than 5\% of its activity over 1 week at 4°.

Methods  

Amine was quantitated by ninhydrin (17) and cardenolide was quantitated by acetic anhydride-sulfuric acid (18) following hydrolysis of the ester in 2 m NH\textsubscript{4}. Thin layer plates were de-
The amino derivative of strophanthinid was further purified by using Sephadex as an adsorption chromatographic medium with an aqueous buffer as the solvent. It was applied to a Sephadex G-15 column (2.5 cm x 120 cm) equilibrated with 0.03 M sodium acetate, pH 5.0. The amino cardenolide travels as a sharp peak resolved from the inclusion volume of the column. The ratio of acetic anhydride A135 to ninhydrin A570 was constant through the peak. Fractions 49 to 60 were pooled.

The pooled material was analyzed by thin layer chromatography on silica gel. The plates were developed for both amine and cardenolide. Only one major component was observed in three solvent systems and it was positive for both amine and cardenolide. The solvent systems and mobilities were: RF = 0.06, methylene chloride-methanol-formamide, 80:19:1; RF = 0.45, 0.03 M pyridine-acetate, pH 4.5; RF = 0.58, 0.1 M N-ethylmorpholine acetate, pH 8.0. Several times a minor component positive for ninhydrin was observed. It comprised approximately 5% of the ninhydrin color. However, when the plate was scraped and eluted, only the major component inhibited the (Na+ + K+) adenine triphosphatase.

Fig. 1. Elution profile of amino cardenolide chromatographed on a Sephadex G-15 column (2.5 cm x 120 cm) equilibrated with 0.03 M sodium acetate, pH 5.0. A135 (X-X), acetic anhydride-sulfuric acid assay; A570 (A-A), ninhydrin assay; and the ratio of these two quantities (O-O) are displayed as a function of elution volume.

Hydrolysis of strophanthidin was performed by adding 2-bromoacetic acid (0.2 mmole) in dioxane. After 2 hours, the dioxane was removed by evaporation and the residue taken up in water. A sample of this material was hydrolyzed and added to the amino acid analyzer. The only ninhydrin-positive peak which was observed had an elution time 1.33 that of lysine on the short column, buffered at pH 5.25. 1,4-Diaminobutane was completely retarded in this system. It was assumed that the peak observed was N-carboxymethyl-1,4-diaminobutane, an isomer of lysine.

Verification of Structure—The purified preparation of amino cardenolide was assayed for several molecular structures. The results are presented in Table I, and they are consistent with the hypothesis that the amino cardenolide has the structure shown in Fig. 2. The studies described below, however, indicate that the purified inhibitor contains two components, one with the lactone ring open and the other with the ring closed, present in a ratio of 0.63 to 0.37 and that only the closed ring form is active as an inhibitor.

A portion of the purified product was brought to pH 7.0 and passed over a Dowex 2-X8 column in the Cl- form to remove the acetate. Greater than 90% of the material added was recovered, and this material still strongly inhibited the (Na+ + K+) adenine triphosphatase. A portion of this material was saponified either in 0.1 M NH3 for 8 hours or 12.5 mM Na2CO3 in 95% methanol for 48 hours at 20°C (greater than 98% liberation of N-carboxymethyl-1,4-diaminobutane). The products inhibited the (Na+ + K+) adenine triphosphatase less than 10% as effectively as the starting material. They were solvent extracted between CHCl3 and water. The CHCl3 phase contained only strophanthinid (RF = 0.60, ethyl acetate; RF = 0.77, 10% acetone + 5% methanol in methylene chloride). The aqueous phase also contained cardenolide material which behaved as a single component (RF = 0.26, ethyl acetate; RF = 0.48, 0.1 M N-ethylmorpholine acetate, pH 8.0). When recrystallized strophanthinid was saponified under identical conditions the same two components were produced. It is assumed that the aqueous phase material is strophanthinid whose lactone ring has opened.

A pH titration of the ion exchanged amino cardenolide was performed between pH 3.0 and pH 9.0. A group (1.0 eq) was present with a pK1 = 7.4. This was assigned to the y secondary amino group of the acetyl group attached by an ester linkage to strophanthinid. If the carboxyl group of the N-carboxymethyl-1,4 dianinobutane were free, the pKa of its amino groups would be approximately 9.0 and 10.5 in analogy with

The abbreviation used is: ABA strophanthinid, N-(4'-amino-n-butyl)-3-aminoacetylstrophanthidin.
lysin. In addition to a group with pK₂ = 7.4, this solution contained a group of pK₁ = 4.87 present in 0.63 eq with respect to the α secondary amino group. This was presumably the carboxyl group of the open lactone ring of strophanthidin in analogy with trans-crotonic acid, pK₁ = 4.69.

When the purified amino cardenolide was submitted to electrophoresis on thin layer silica gel plates in 0.07 M N-ethylmorpholine acetate, pH 6.72, at 700 volts for 1 hour, two ninhydrin-positive components were present (mobility, 3.2 × 10⁻³ and 5.3 × 10⁻³ cm per volt hour). They were eluted separately. Only the factor of the two had the capacity to inhibit the (Na⁺ + K⁺)-adenosine triphosphatase. The ninhydrin color contribution of the X-carboxymethyl-1,4-diaminobutane present in the molecule, and that only the lactone ring of the strophanthidin group is altered. These results are in agreement with the structure shown in Fig. 2, N-(4'-amino-n-butyl)-3-aminoacetylstrophanthidin. A certain portion of the molecules, however, is present in the open lactone form.

The purified ABA strophanthidin was titrated with (Na⁺ + K⁺) adenosine triphosphatase to determine whether it was quantitatively bound by the enzyme. To a series of tubes containing 4.8 nmoles of ABA strophanthidin in 2.50 ml of 100 mM NaCl, 3 mM Na₂ATP, 6 mM MgCl₂, 30 mM imidazole HCl, pH 6.8, were added various amounts of salt-detergent-extracted microsomes (15), all in molar excess over biologically active inhibitor present. After incubation at 37° for 5 min, the particulate enzyme was pelleted and the supernates were collected. They contained less than 10% of the active equivalents of inhibitor present prior to the addition of the enzyme but all still contained more than 40% of the added ABA strophanthidin, presumably in an inactive form. The results obtained with six different enzyme concentrations were extrapolated by least square analysis to zero concentration to correct for the nonspecific binding of the inactive material to the membranes. In this way it was determined that 64% of the ABA strophanthidin molecules were biologically inactive.

All of these observations demonstrate that the purified ABA strophanthidin preparation is a mixture of the open and closed lactone ring forms present in a ratio of 0.63:0.37 that only the closed ring form is biologically active. No other component has ever been observed. At pH 7.8 in unbuffered water, the lactone ring of strophanthidin hydrolyzed less than 1% over a period of 30 min at 20°. Over a period of 2 months, no change in kinetic parameters of the ABA strophanthidin stock solution stored at 4° was observed. Therefore, all of the subsequent results are corrected for this heterogeneity.

**Kinetic Measurements**—The enzyme preparation used for all of the kinetic measurements was prepared as described by Skou (26) from rabbit kidney. The nonspecific, Mg⁺⁺-stimulated adenosine triphosphatase generally accounted for 20% of the total activity. This activity was measured by including two tubes, which did not contain that ion which was being varied in the particular experiment, in each series of assays. All ve-

### Table I

| Molecular property                  | Assay                        | Standard                                         | Concentration determined (mM) |
|-------------------------------------|------------------------------|--------------------------------------------------|-------------------------------|
| Strophanthidin                      | Acetic anhydride: H₂SO₄      | Recrystallized anhydrous strophanthidin          | 0.96                          |
| Amine                               | Ninhydrin                    | Lysineα                                          | 1.05                          |
| Nitrogen                            | Kjeldahl                     | Lysineα                                          | 2.02                          |
| N-carboxymethyl-1,4-diaminobutane   | Amino acid analysisα         | Lysineα                                          | 0.98                          |

* Concentration determined by amino acid analysis.

* When the purified amino cardenolide was added directly to the amino acid analyzer, no ninhydrin-positive material eluted. However, when it was first hydrolyzed in 6 M HCl at 110° for 24 hours, adding lysine as an internal standard, a ninhydrin-positive peak precisely coinciding with that of the N-carboxymethyl-1,4-diaminobutane was observed. The ninhydrin color contributed by the N-carboxymethyl-1,4-diaminobutane present in the amino cardenolide accounts quantitatively for its ninhydrin reaction.

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**Fig. 2. N-(4'-Amino-n-butyl)-3-aminoacetylstrophanthidin.**

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**TABLE I**

**Analysis of a solution of purified amino steroid**

| Molecular property                  | Assay                        | Standard                                         | Concentration determined (mM) |
|-------------------------------------|------------------------------|--------------------------------------------------|-------------------------------|
| Strophanthidin                      | Acetic anhydride: H₂SO₄      | Recrystallized anhydrous strophanthidin          | 0.96                          |
| Amine                               | Ninhydrin                    | Lysineα                                          | 1.05                          |
| Nitrogen                            | Kjeldahl                     | Lysineα                                          | 2.02                          |
| N-carboxymethyl-1,4-diaminobutane   | Amino acid analysisα         | Lysineα                                          | 0.98                          |

* Concentration determined by amino acid analysis.

* When the purified amino cardenolide was added directly to the amino acid analyzer, no ninhydrin-positive material eluted. However, when it was first hydrolyzed in 6 M HCl at 110° for 24 hours, adding lysine as an internal standard, a ninhydrin-positive peak precisely coinciding with that of the N-carboxymethyl-1,4-diaminobutane was observed. The ninhydrin color contributed by the N-carboxymethyl-1,4-diaminobutane present in the amino cardenolide accounts quantitatively for its ninhydrin reaction.
has been described previously (15). The incubation period in all cases was 15.0 min. When sodium was the variable substrate, Tris ATP was used as substrate.

The data were fitted to an equation of the form

$$\frac{1}{v} = \frac{1}{v_{\text{max}}} \left( 1 + \frac{[I]}{K_i} \right)$$

by a weighted least squares analysis (27) where \(1/v\) is the reciprocal of the concentration of specifically-released, inorganic phosphate at the end of the incubation, \([I]\) is the total inhibitor concentration, and \(v_{\text{max}}\) and \(K_i\) are the velocity of the reaction at \([I] = 0\) and the apparent inhibitor dissociation constant at particular ion concentrations of the experiment. The variance associated with each measurement was found to be directly proportional to the square of the velocity (Fig. 3). Therefore, the weighting function \(w_i = v_i^2\) was chosen (27). A FORTRAN program was written to fit the data to the linear form. All of the data gathered in the experiments in which the concentration of K+ was varied, the concentration of Na+ held at saturating levels, and inhibition by ABA strophanthidin was followed (Fig. 4) were normalized with respect to \(K_i\) and \(v_{\text{max}}\), and they are displayed in Fig. 3. It can be seen that they correspond quite closely to the line described by Equation 1. The intersections between all lines in a given data set were then calculated. The tabulated kinetic constants are the means and standard deviations of each set of intersections.

With many preparations of the (Na+ + K+)-adenosine triphosphatase it has been observed that the binding of the cardiac glycoside, ouabain, is time-dependent (4, 5, 8, 29). It has been shown, however, that strophanthidin binds more rapidly than ouabain to the enzyme (10). Moreover, if a linear relationship exists between the reciprocal of the concentration of Pi measured at a given time after initiation of the reaction and the concentration of the inhibitor, then the approach to equilibrium occurs too rapidly to affect the kinetics (Fig. 3). The effect of approach to equilibrium would be to cause the data to deviate below the line at low \([I]\) and above the line at high \([I]\). When apparent inhibition constants of strophanthidin were determined at 20 mM KCl, 100 mM NaCl, values of \(7 \times 10^{-4} \text{ M} \) were obtained with 15-min incubations and \(10 \times 10^{-4} \text{ M} \) with 30-min incubations. If a time-dependent approach to equilibrium were affecting the kinetics, values for \(K_i\) should be significantly less for the second incubation. For these reasons, it is concluded that the binding of strophanthidin and ABA strophanthidin to the (Na+ + K+)-adenosine triphosphatase preparation used for the kinetic studies (26) is rapid and that the measurements reflect inhibitor-enzyme equilibria.

In all experiments the concentrations of Mg2+ and ATP were held at constant and saturating levels. In this case, the reaction of the (Na+ + K+)-adenosine triphosphatase can be considered a two-substrate reaction. Under these conditions neither of the two substrates of interest, Na+ and K+, is consumed and the reaction velocities remain linear with time even at low substrate concentration. In the experiments presented here, each of the two ions was held at saturating concentration in turn. The other ion’s concentration was varied in the region of its Michaelis constant. Under these circumstances the equations governing inhibition for the two classes of bisubstrate reactions, ping-pong and bi-bi, are formally identical. They yield a family of straight lines intersecting at a point whose projection on the abscissa is the negative of the inhibitor dissociation constant which describes the binding of inhibitor to the form of the enzyme which predominates in the presence of saturating concentrations of the one substrate and zero concentration of the other. In the ping-pong case, these are the unliganded forms of the enzyme, while in the bi-bi case, they are the ion-ligated forms of the enzyme.

These considerations are for the general case in which inhibitor binds to all forms of the enzyme with unique dissociation energies. It is assumed that when any form of the enzyme binds inhibitor it becomes inactive.

### RESULTS

#### Kinetics of Inhibition

It was important to decide whether the inhibition of the (Na+ + K+)-adenosine triphosphatase by ABA strophanthidin was competitive with K+. The results of an experiment in which the concentration of K+ was varied at saturating concentration of Na+ were plotted in the form of \([s]/v\) versus \([s]\) (Fig. 5) (14). It is clear that the inhibition in this case is neither of the purely competitive nor the purely non-competitive type. The assignment is not completely unequivocal, as curvature is present at high and low substrate concentrations due to substrate inhibition and the sigmoidal saturation function (30). This phenomenon, however, seems to be confined to a limited region of the curves.

The results were also plotted in the form \(1/v\) versus \([I]\) (Fig. 4). As can be seen, a family of straight lines is generated which intersect at a point. The projection of this point on the abscissa is the dissociation constant of the inhibitor with that form of the enzyme which predominates at saturating concentrations of Na+ and in the absence of K+. Values for this constant are presented in Table III.

### Table II

| Type of resonance | Chemical shift (6) | Number of protons | Present in strophanthidin | Present in derivative | Assignmenta |
|-------------------|-------------------|-------------------|--------------------------|----------------------|-------------|
| Singlet           | 0.68              | 3                 | +                        | +                    | C18 methyl  |
| Multiplet         | 9.70              | 1                 | +                        | +                    | C17 methine |
| Multiplet         | 3.23              | 2                 | -                        | +                    | Amino acetyl methylene |
| Multiplet         | 4.00              | 1                 | +                        | <0.2 proton          | C9 methine  |
| Multiplet         | 4.90              | ~1                | -                        | +                    | C9 methylene|
| Singlet           | 4.78              | 2                 | +                        | -                    | C11 methylene |
| Singlet           | 4.78              | ~1                | +                        | -                    | C11 methylene |
| Singlet           | 5.08              | ~1                | -                        | +                    | C11 methylene |
| Singlet           | 5.54              | 1                 | +                        | +                    | C22 methine |

*a Numbering in Fig. 2.*
FIG. 3. Normalized kinetic data. The data from all experiments in which K+ was varied while Na+ was held at saturating levels and the inhibition of ABA strophanthidin was measured were normalized by setting [I], the concentration of ABA strophanthidin, which equaled the apparent $K_i$ to a value of 1.00 and the apparent $1/V_{max}$ to a value of 1.00. It can be assumed that

$$E + I \xrightarrow{k_1} EI$$

where $k_1$ is a bimolecular association rate constant and $k_2$ a unimolecular dissociation rate constant. Barnett (4) has shown that the enzyme inhibitor reaction in the forward direction is bimolecular and Tobin and Sen (7) have shown that the reaction in the reverse direction is first order. These results have been confirmed by Hansen (9). In this situation, if [I] is varied in the neighborhood of the apparent $K_i$, then

$$\frac{[P_i]}{[E]_{tot}} = \frac{1}{v} = \frac{1}{k_{cat}[E]_{tot}}$$

$$\left(1 + \frac{[I]}{K_i}\right)\left(1 + \frac{1}{k_1[I]t}\right)^{-1}$$

where $t$ is any time following initiation; $[P_i]_{tot}$ the concentration of specifically released inorganic phosphate at time $t$; $k_{cat}$ the turnover number of the enzyme under the specific ionic conditions; $[E]_{tot}$ the concentration of active sites; [I], the concentration of inhibitor; and $K_i$, the apparent inhibitor dissociation constant at the specific ionic conditions. It is quite clear that if the approach to equilibrium were significantly affecting the results, $1/v$ would be a nonlinear, exponential function of [I]. The line drawn in the figure is that expected in the case where approach to equilibrium does not affect the data.

When the concentration of K+ was held constant at saturating levels and the concentration of Na+ was varied and the results were plotted in the form $1/v$ versus [I], a family of straight lines whose intersection is in the third quadrant was obtained (Fig. 6). This result demonstrates that sodium actually decreases the apparent dissociation constant of ABA strophanthidin from the enzyme. Dissociation constants calculated from these experiments are presented in Table III. The mean of the distances of the intersections below the abscissa was $-3.6 \pm 1.2$. This
The results obtained when potassium and inhibitor concentrations were varied were qualitatively similar to those obtained with ABA strophanthidin. Potassium was only partially competitive with the inhibitor. The binding constants of strophanthidin to the completely saturated form of the enzyme and to the sodium-saturated form in the absence of potassium are tabulated (Table III). The ratio between these two values is 19. The ratio of the same constants associated with ABA strophanthidin are 1.85 ± 0.37 x 10^-7 M and 3.4 x 10^-7 M, respectively.

The same kinetic analysis was performed with strophanthidin. Concentrations of sodium were varied in the standard assay at different concentrations of ABA strophanthidin. Millimolar concentrations of sodium are inscribed above respective lines. The lines are the weighted least square fits of the data.

As can be seen, the intercept with the abscissa decreases by a factor of 2.47 when protein added to the series of reaction tubes was decreased by a factor of 2.5. This result demonstrates that the inhibitor is binding stoichiometrically to enzyme at the concentrations used in these experiments. This property and the linearity of the data were used as criteria for stoichiometric binding. All results presented are from experiments in which both of these criteria were met.

Several preparations of enzyme were titrated with ABA strophanthidin and the results are presented in Table IV. Also tabulated are the specific activities of each preparation and the calculated turnover numbers. The most interesting result is that the presence of the concentration of deoxycholate necessary to cause the enzyme to remain in the supernate at 100,000 X g significantly depresses its turnover number. This effect can be reversed by removing the deoxycholate with the resin XAD4. However, a decrease in the available binding sites occurs when the detergent is removed.

### Table III

| Inhibitor       | Condition                  | $K_i$       |
|-----------------|----------------------------|-------------|
| I. ABA strophanthidin | Saturating Na$^+$, no K$^+$ | 1.92 ± 0.44 x 10^-9 M |
|                 | Saturating Na$^+$, saturating K$^+$ | 1.86 ± 0.60 x 10^-9 M |
|                 | Saturating K$^+$, no Na$^+$ | 1.85 ± 0.37 x 10^-7 M |

**Fig. 7.** Initial velocities of enzyme to which different amounts of ABA strophanthidin have been bound. Supernatant enzyme (124 μg) was preincubated with inhibitor in the presence of Na$^+$ and MgATP. The reaction was initiated with K$^+$, and samples were taken at various times. The amounts of ABA strophanthidin added to the preincubation are, in descending order, 0 n mole, 0.09 n mole, 0.17 n mole, 0.24 n mole.

**Fig. 6.** Inhibition of (Na$^+$ + K$^+$)-adenosine triphosphatase as a function of sodium concentration. The data are plotted in the form 1/v versus [I] (28). Concentrations of sodium were varied in the standard assay at different concentrations of ABA strophanthidin. Millimolar concentrations of sodium are inscribed above respective lines. The lines are the weighted least square fits of the data.
ABA strophanthidin is plotted. The initial velocity of the enzyme which has been preincubated with various amounts of supernatant enzyme. The fractional initial velocity of supernatant enzyme at 220 nm, there were 1.7 g of the large polypeptide per g of the enzyme. On the basis of the optical density at 220 nm, there were 1.7 g of the large polypeptide per g of the small one in the supernatant enzyme.

**DISCUSSION**

The effects of the monovalent cations on the dissociation constants of ABA strophanthidin and strophanthidin agree well with results obtained for ouabain inhibition of the (Na\(^+\) + K\(^+\))-adenosine triphosphatase. The kinetics displayed in Fig. 4 is the same as that observed by Matsui and Schwartz with ouabain (2). It has been shown (4, 8) that the addition of K\(^+\) at saturating levels of Na\(^+\) decreases the bimolecular rate of association between enzyme and ouabain by one order of magnitude, while the first order rate of dissociation is unaltered (8). As a consequence, the apparent dissociation constant of ouabain from the enzyme in the presence of K\(^+\) must be 10 fold greater than that in its absence. The level of digoxin binding is also decreased by K\(^+\) (6). These results agree with the differences observed in the present study between the apparent dissociation constants of the Na\(^+\)-saturated enzyme and inhibitor in the absence and presence of K\(^+\), one of 29-fold in the case of ABA strophanthidin and one of 19-fold in the case of strophanthidin (Table III). The apparent K\(_i\) for the release of ABA strophanthidin from the K\(^+\)-saturated enzyme in the presence of Na\(^+\) was 4 fold less than in its absence (Table III). This agrees with earlier observations which demonstrate that Na\(^+\) both accelerates the approach to equilibrium of enzyme and ouabain (4) and also increases the equilibrium level of ouabain binding (5). That ouabain is not purely competitive with K\(^+\) has been clearly demonstrated (3), a result which was also observed during the present experiments, and which indicates that there is a finite dissociation constant of enzyme liganded with K\(^+\) for cardiac glycoside.

Therefore, the dissociation constant of ABA strophanthidin and the form of the enzyme present at saturating concentrations of Na\(^+\) in the absence of K\(^+\) is two orders of magnitude smaller than that of ABA strophanthidin and the form of the enzyme present at saturating concentrations of K\(^+\) in the absence of Na\(^+\). If it is assumed that the phosphorylated enzyme is the former and the dephosphorylated enzyme is the latter (4, 6, 11), then the binding of ABA strophanthidin to the phosphorylated enzyme is stronger by at least 3 Cal per mole than that to the dephosphorylated enzyme. As Scn et al. (11) have pointed out, as a result of thermodynamic linkage (31, 32), this type of energy difference can explain phosphorylation of the enzyme in the presence of Mg\(^{2+}\), Pi, and ouabain (11, 13). The magnitude of this difference, however, has not been measured in the case of ouabain.

One interesting result of these experiments is that ABA strophanthidin, an ester derivative of strophanthidin containing two positive charges, binds to the enzyme with dissociation constants two orders of magnitude smaller than those of the parent compound. As the character of the kinetics observed with variable K\(^+\) concentration is unaffected by the ester substitution, the positive charges of the derivative are probably not interacting with the K\(^+\) sites. However, they may be interacting with surface negative charges on the protein or the phospholipid phosphate groups.

The result of the titration measurements is that 1 mole of ABA strophanthidin binds to 175,000 ± 20,000 g of supernatant enzyme. On the basis of the distribution coefficient of the larger of the two polypeptide chains present in this preparation when it is chromatographed by gel filtration in either 0.2% sodium dodecyl sulfate (33) or guanidine hydrochloride (34), its molecular weight is 135,000 (35). However, on sodium dodecyl sulfate gel electrophoresis it behaves as a polypeptide of molecular weight 90,000 (15, 36). As there are approximately 1.7 g of large chain per g of small chain, the mass of protein per molecule of the larger chain is therefore between 140,000 and 210,000 daltons, and there are between 0.8 and 1.2 binding sites for ABA strophanthidin, per large polypeptide chain. This is the chain which is specifically phosphorylated (16).

There is a discrepancy between the present measurements of the cardiac glycoside binding sites and other measurements in the literature. It is usually observed that, under the conditions of saturating MgATP and in the presence of Na\(^+\), the maximum binding of tritiated ouabain is 94 ± 6 pmol per pmole of ATP hydrolyzed per min (4, 6, 9, 29, 37). In similar preparations, the maximum phosphorylation of the enzyme in the presence of Na\(^+\) and MgATP is 90 ± 10 pmol per pmole of ATP hydrolyzed per min (16, 38, 39). These results cannot be com-

![Graph](https://example.com/graph.png)

**Fig. 8.** Titration of the cardiac glycoside binding sites of supernatant enzyme. The fractional initial velocity of supernatant enzyme which has been preincubated with various amounts of ABA strophanthidin is plotted. The initial velocity of the enzyme to which no inhibitor is liganded is assigned a value of 1.00. The micrograms of supernatant enzyme used in each experiment are inscribed above the respective lines.

**Table IV**

| Enzyme preparation | Titratin result | Specific activity | Turnover number |
|--------------------|----------------|------------------|-----------------|
| Salt detergent-extracted microsomes | 285,000 | 12.8 | 3,600 |
| Supernatant enzyme | 175,000 ± 20,000 | 6.7 | 1,100 |
| XAD4 enzyme | 220,000 | 11.1 | 2,900 |

**Ratio of Masses of the Two Chains**—The mass ratio between the larger and the smaller polypeptide chain present in the supernatant enzyme (15, 16) was determined by denaturing a sample of this preparation with sodium dodecyl sulfate and separating the two polypeptides on a Sepharose 4B column (2.5 cm × 65 cm) equilibrated with 0.2% sodium dodecyl sulfate, 0.04 M Tris sulfate, pH 8.0. The two polypeptides were well separated under these conditions. On the basis of the optical density at 220 nm, there were 1.7 g of the large polypeptide per g of the small one in the supernatant enzyme.
pared directly to those of the present study as it is clear that deoxycholate alters the turnover number of the enzyme (Table IV). A crucial disagreement, however, does exist because with the other preparations, the concentration of phosphorylated intermediate, is that the latter are minimum estimates of the true number of cardiac glycoside binding sites. The concentration of specific binding sites will be underestimated if the concentrations of the various substances in solution are not adequate to allow stoichiometric binding. In fact, Tobin and Sen (7) have shown that the concentration of specific binding sites in the presence of MgATP and Na⁺, conditions previously used for maximum binding (4, 6, 9, 29, 37), is actually 1.6 times smaller than the number of sites available in the presence of Mg²⁺, P⁰, and 0.1 mm KCl. These considerations, as well as the discussion of the phosphorylation results presented earlier (15, 16), suggest that neither phosphorylation nor the direct measurements of ouabain binding are adequate estimates of the true concentration of active enzyme.

It has also been observed that the binding of ATP to the enzyme saturates at approximately 150 moles of ATP per mole of ATP hydrolyzed per min (40–42). This figure is significantly larger than that for the phosphorylation of the enzyme, but the difference is not 3-fold. It must be stressed again, however, that this measurement is a lower estimate of the true number of binding sites. In order for the concentration of bound ATP at saturation to be equivalent to the total number of binding sites, all forms of the enzyme must be in rapid equilibrium. This may not be the case when a critical substrate, Mg²⁺, is omitted from the incubation. Therefore, the direct measurements of ouabain binding and ATP binding to the (Na⁺ + K⁺)-adenosine triphosphatase yield lower limits of the true concentration of sites. The kinetic titrations on the other hand are necessarily upper limits of the concentration of specific sites.

As a result of these considerations, I conclude that the preparation of supernatant enzyme contains a uniform collection of enzyme molecules which bind 1 mole of cardiac glycoside per mole of large polypeptide chain and which only phosphorylate under optimal conditions to a level of 0.4 mole per mole.

Acknowledgments I would like to thank Professor Guidotti. Advice from Professor E. J. Corey and Professor Gustav Lienhard was also very useful.

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