MOLECULAR PHARMACOLOGY, 12, 101-114

The Complexes of Ouabain with Sodium- and Potassium-Activated Adenosine Triphosphatase Formed with Various Ligands: Relationship to the Complex Formed in the Beating Heart

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(Received July 1, 1974)

SUMMARY

AKERA, TAI, KU, DAVID, TOBIN, THOMAS & BRODY, THEODORE M. (1976) The complexes of ouabain with sodium- and potassium-activated adenosine triphosphatase formed with various ligands: relationship to the complex formed in the beating heart. *Mol. Pharmacol.*, 12, 101-114.

The characteristics of complexes of [^{3}H]ouabain with (Na^+ + K^+)-ATPase [Mg^{2+}-dependent, (Na^+, K^+)-activated ATP phosphohydrolase, EC 3.6.1.3.] formed *in vitro* in the presence of various ligands were compared using partially purified rat brain and dog heart enzyme preparations and dog heart homogenates. The differences in characteristics of the ouabain-enzyme complexes were detected by monitoring dissociation rates in media of low ionic strength. It appeared that there are at least three different forms of the ouabain-enzyme complex prepared *in vitro* with rat brain enzyme. The ouabain-enzyme complex formed with Mg^{2+} and P_i was stable and unaffected by K^+ added to the dissociation mixture. The ouabain-enzyme complex formed in the presence of Mg^{2+} and ATP had an intermediary dissociation rate in the absence of K^+ and was not stabilized by K^+. The addition of Na^+ to the binding mixture containing Mg^{2+} and ATP increased the ouabain binding and resulted in the formation of an unstable complex. The addition of K^+ to the mixture after the termination of the binding reaction stabilized the complex. In contrast, the presence of K^+ in the binding mixture with Na^+, Mg^{2+}, and ATP resulted in a K^+-insensitive, intermediary stable complex which was similar to that formed in the presence of Mg^{2+} and ATP. With dog heart enzyme, the ouabain-enzyme complex formed in the presence of Na^+, K^+, Mg^{2+}, and ATP had characteristics similar to that formed in the presence of Na^+, Mg^{2+}, and ATP. Both complexes were stabilized by K^+ added to the dissociation mixture. The [^{3}H]ouabain bound to ventricular tissue during the Langendorff perfusion of isolated puppy hearts and dissociated *in vitro* was stabilized by the addition of K^+, like the complex formed *in vitro* in the presence of either Na^+, Mg^{2+}, and ATP or Na^+, K^+, Mg^{2+}, and ATP, but unlike the complex formed in the presence of Mg^{2+} and P_i, which was not stabilized by K^+. It was concluded that ouabain binds to different forms of the phosphoenzyme under various ligand conditions, resulting in different forms of the ouabain-enzyme complex, and that the ouabain-enzyme complex formed in the presence of Mg^{2+} and P_i is not a suitable model for the drug-enzyme interaction occurring *in vivo*. Additionally, the dissociation characteristics of the ouabain-enzyme complex formed *in vitro* were not influenced by the concentration of ouabain or by the duration of the drug-enzyme interaction.

This work was supported by United States Public Health Service Grant HL 16052 and by the Michigan Heart Association. A part of this study was presented at the 58th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, April 1974.

INTRODUCTION

Cardiac ($Na^+ + K^+$)-ATPase [Mg^{2+}-dependent, (Na^+,K^+)-activated ATP phosphohydrolase, EC 3.6.1.3] has been postulated to be a positive inotropic and/or toxic receptor for cardiac glycosides (1–6; see also ref. 7). Such a hypothesis is based upon the observations that ($Na^+ + K^+$)-ATPase is uniquely sensitive to cardiac glycosides (see ref. 7), and that the inhibition of cardiac ($Na^+ + K^+$)-ATPase is related to the positive inotropic response to cardiac glycosides in dog and guinea pig hearts (4, 8–10). Additionally, the characteristics of interactions between cardiac glycosides and isolated ($Na^+ + K^+$)-ATPase are similar to those between cardiac glycosides and the inotropic receptor (4, 6, 11–15). More recently, we have demonstrated that the inhibition of cardiac ($Na^+ + K^+$)-ATPase by RbCl is associated with a positive inotropic response in guinea pig atrial preparations (16). These data indicate that cardiac ($Na^+ + K^+$)-ATPase is intimately related to the inotropic receptor for cardiac glycosides. Thus the interaction of cardiac glycosides with ($Na^+ + K^+$)-ATPase *in vitro* may be used as a model for the glycoside-receptor interaction occurring *in vivo*.

Cardiac glycosides, such as ouabain, preferentially bind to ($Na^+ + K^+$)-ATPase in the presence of specific combinations of ligands, namely, in the presence of Mg^{2+} and inorganic phosphate at low ionic concentrations or in the presence of Na^+, Mg^{2+}, and ATP (17). The ouabain–($Na^+ + K^+$)-ATPase complexes formed *in vitro* seem to have different properties, depending upon the ligand conditions which prevail during the binding reaction. Such differences may be detected by monitoring the dissociation reaction of the ouabain-enzyme complex in a mixture different from the binding mixture but having a low ionic strength (18). Under these conditions the complex prepared in the presence of Mg^{2+} and P_i is stable and its dissociation rate constant is unaffected by potassium. The ouabain-enzyme complex prepared in the presence of Na^+, Mg^{2+}, and ATP is relatively unstable and is stabilized by the addition of K^+ to the mixture in which dissociation is monitored (18, 19). Whether these differences represent different binding sites for ouabain on the ($Na^+ + K^+$)-ATPase or different conformational states of the enzyme protein is unknown, although several investigators have proposed that binding sites on the enzyme are the same for the complex prepared with Mg^{2+} and P_i and that prepared with Na^+, Mg^{2+}, and ATP and treated with K^+ (20).

Since Na^+, K^+, Mg^{2+}, and ATP are apparently available to ($Na^+ + K^+$)-ATPase in intact cells, the ouabain-enzyme complex formed *in vitro* in the presence of Na^+, K^+, Mg^{2+}, and ATP may represent the complex formed *in vivo* or in the isolated beating heart. However, little is known regarding the characteristics of the ouabain-enzyme complex formed *in vitro* in the presence of Na^+, K^+, Mg^{2+}, and ATP. In the presence of Mg^{2+} and ATP, [^{3}H]ouabain binding to ($Na^+ + K^+$)-ATPase is stimulated by Na^+ and inhibited by K^+ (17). Such effects of Na^+ and K^+ have been suggested to result from their action to increase or decrease the concentrations of phosphoenzyme, to which ouabain binds preferentially (21). If this hypothesis is correct, the bulk of the ouabain-enzyme complex formed *in vitro* in the presence of Na^+, K^+, Mg^{2+}, and ATP should be similar to that formed *in vitro* in the presence of Na^+, Mg^{2+}, and ATP, and may be representative of the binding of ouabain to ($Na^+ + K^+$)-ATPase *in vivo*.

In the present study the characteristics of the ouabain–($Na^+ + K^+$)-ATPase complexes prepared *in vitro* in the presence of various ligands have been examined, using a partially purified rat brain enzyme preparation. Subsequently the dissociation *in vitro* of ouabain from the cardiac tissue following a dog heart Langendorff perfusion was compared with that of the ouabain–cardiac ($Na^+ + K^+$)-ATPase complexes formed *in vitro* in the presence of various ligands.

MATERIALS AND METHODS

Rat brain ($Na^+ + K^+$)-ATPase preparations. Sprague-Dawley rats, weighing 200–300 g, were killed by decapitation, and the brains were rapidly removed.

After the meninges had been wiped away, brains were weighed and homogenized in a Dounce ball-type homogenizer with 5 volumes of ice-cold solution containing 0.25 M sucrose, 5 mM histidine, 5 mM disodium EDTA, 0.15% sodium deoxycholate, and 0.01 mM dithiothreitol (pH adjusted to 6.8 with Tris base). Deoxycholic acid and dithiothreitol were added immediately before use. Thorough homogenization with a tight pestle is essential for a good yield and high enzyme activity. The homogenate was centrifuged at 10,000 × *g* for 15 min, and the resulting sediment was resuspended using a Dounce homogenizer in a solution (6 ml/brain) similar to the above homogenizing solution but containing 0.1% sodium deoxycholate, and centrifuged again as above. The supernatants from the two centrifugations were combined and centrifuged at 100,000 × *g* for 30 min. The resulting sediment was resuspended using a Potter-Elvehjelm Teflon homogenizer in a solution containing 0.25 M sucrose, 5 mM histidine, 1 mM disodium EDTA, and 0.01 mM dithiothreitol (pH 7.0) to approximately the same volume as that prior to centrifugation, and centrifuged at 20,000 × *g* for 20 min. The resulting supernatant was further centrifuged at 100,000 × *g* for 30 min, and the sediment was resuspended in a solution containing 2.0 M NaI, 2.5 mM disodium EDTA, 3.0 mM $MgCl_2$, 5 mM histidine, 2.0 mM disodium ATP, and 0.01 mM dithiothreitol (pH adjusted to 7.3 with Tris base). The mixture was gently mixed for 30 min at 0° and then diluted with 2.5 volumes of 1 mM disodium EDTA and centrifuged at 100,000 × *g* for 30 min. The resulting sediment was washed twice by suspending it in 10 mM Tris-HCl buffer (pH 7.5) containing 1.0 mM disodium EDTA and centrifuging it at 100,000 × *g* for 30 min. The final sediment was resuspended in a solution containing 0.25 M sucrose, 1.0 mM EDTA (acid, dissolved in a Tris base solution), and 5 mM histidine (pH adjusted to 7.0 with Tris base) and stored frozen at −20° until use. All preparative procedures were performed at 0–5°.

The present enzyme preparations had specific ATPase activities of approximately 350 μmoles of inorganic phosphate released from ATP per milligram of protein per hour in the presence of 100 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, 5 mM Tris-ATP, and 50 mM Tris-HCl buffer (pH 7.5) at 37°. Mg^{2+}-ATPase activity, assayed in the absence of added NaCl and KCl, was less than 5% of the total ATPase activity (for details of ATPase assay, see ref. 22).

Dog heart homogenates and $(Na^+ + K^+)$-ATPase preparations. Cardiac homogenates were prepared from mongrel puppies of either sex weighing 500–1000 g. Ventricular muscle was minced and homogenized in 9 volumes of a solution containing 0.25 M sucrose, 1.0 mM Tris-EDTA, and 5 mM histidine (pH 7.0), using a Dounce ball-type homogenizer.

Cardiac $(Na^+ + K^+)$-ATPase preparations were obtained from frozen dog hearts (Pel-Freez Biologicals) by the method of Pitts *et al.* (23). $(Na^+ + K^+)$-ATPase activity of cardiac enzyme preparations was approximately 100 μmoles of inorganic phosphate released from ATP per milligram of protein per hour. Mg^{2+}-ATPase activity was less than 10% of the total activity.

[^{3}H]Ouabain binding and release studies. The [^{3}H]ouabain-enzyme complex was prepared by incubating the enzyme preparation or homogenate (0.2 mg of protein per milliliter) and 0.01 μM [^{3}H]ouabain (New England Nuclear Corporation; specific activity, 13.2 Ci/mmole) in the presence of various ligands at 37° for 10 min unless otherwise indicated (for details, see ref. 19). The mixture was then centrifuged at 100,000 × *g* for 30 min at 0°, and the sediment was suspended in 10 mM Tris-HCl buffer (pH 7.5). The suspension was kept in an ice bucket and the dissociation reaction was studied immediately. In some experiments the binding of the labeled ouabain was terminated by the addition of nonradioactive ouabain (final concentration, 0.1 mM) with a simultaneous 10-fold dilution of the mixture.

The release of bound [^{3}H]ouabain from $(Na^+ + K^+)$-ATPase observed with these two different methods may theoretically represent two separate phenomena, i.e., dissociation of the ouabain-enzyme complex and turnover of the bound ouabain. These two methods, however, yielded similar rates of decline of the bound

[^{3}H]ouabain in a mixture containing the same cation composition if dissociation studies were performed under conditions in which rebinding of released [^{3}H]ouabain was negligible as a result of the absence of Mg^{2+} or by dilution of the released [^{3}H]ouabain with excess nonlabeled ouabain (19, 24, 25).[1] Thus it would appear that nonradioactive ouabain does not actively displace bound [^{3}H]ouabain from its binding site and that values obtained with either method represent the dissociation reaction of the [^{3}H]ouabain-enzyme complex.

The dissociation of the [^{3}H]ouabain-enzyme complex was monitored at 37°. Aliquots were taken at appropriate time intervals, and the amount of bound [^{3}H]ouabain remaining undissociated was estimated using a Millipore filter system to separate the unbound ouabain and liquid scintillation counting to assay radioactivity (19). Millipore filters were dissolved in ethylene glycol monomethyl ether before counting. Counting efficiency (approximately 30%) was monitored by the external standard channel ratio, which was occasionally calibrated with internal standards. The levels of phosphate ligand-independent binding were estimated from the results of [^{3}H]ouabain binding and dissociation experiments which were performed concurrently, excluding ATP or Tris-phosphate from the [^{3}H]ouabain binding mixture. Although such ATP-independent (or P_i-independent) binding represented less than 2% of the total binding when the assay was performed with low concentrations (0.01 μM) of [^{3}H]ouabain, the radioactivity in the absence of nucleotide or inorganic phosphate was routinely subtracted from that observed in its presence to calculate phosphate ligand-dependent (specific) [^{3}H]ouabain binding. It has been previously reported that ATP-dependent binding of [^{3}H]ouabain is the stoichiometric binding to (Na^+ + K^+)-ATPase (26). All values reported in this paper are those due to the ATP- or P_i-dependent portion of the binding, except for those in Fig. 5.

Perfusion of isolated hearts. Langendorff perfusions of isolated puppy hearts were performed as previously described (6). The hearts were perfused with Krebs-Henseleit solution at 30° at a constant flow rate of 8 ml/min and electrically driven at 1.5 Hz with a square pusle of 5-msec duration and a voltage 10% above threshold. Contractile force was monitored with an FT-03 force displacement transducer (Grass Instruments). [^{3}H]Ouabain perfusion was started after a 45-min equilibrium period and terminated 20 min later. At that time the ventricular muscle was excised, homogenized, centrifuged at 100,000 × *g* for 30 min at 0°, and resuspended in 10 mM Tris-HCl buffer (pH 7.5), and the release of [^{3}H]ouabain from the resuspended homogenate was monitored at 37° in the presence and absence of added KCl or NaCl.

Miscellaneous. Protein concentrations were assayed by the method of Lowry *et al.*, using bovine serum albumin as the standard (27). The results were analyzed for statistical significance by Student's *t*-test. Tris-ATP and other reagents were purchased from Sigma Chemical Company.

RESULTS

Complexes of ouabain with partially purified rat brain (Na^+ + K^+)-ATPase formed in the presence of various ligands. Figure 1A shows the rates of release of [^{3}H]ouabain previously bound to rat brain (Na^+ + K^+)-ATPase at 37° under various conditions and dissociated at the same temperature in 10 mM Tris-HCl buffer (pH 7.5) in the absence of K^+. In these experiments [^{3}H]ouabain-enzyme complexes were formed by incubating the enzyme preparation with [^{3}H]ouabain in the presence of various ligands for 10 min at 37°. Subsequently unbound [^{3}H]ouabain was removed by centrifugation, washing, and resuspension of the pellet, and dissociation of the bound ouabain was monitored at 37° in 10 mM Tris-HCl buffer (pH 7.5). The levels of bound [^{3}H]ouabain at the beginning of the dissociation reaction were different, depending upon the ligand conditions which prevailed during the binding reaction: high for those complexes prepared in the presence of Na^+, Mg^{2+}, and

[1] Unpublished observations.

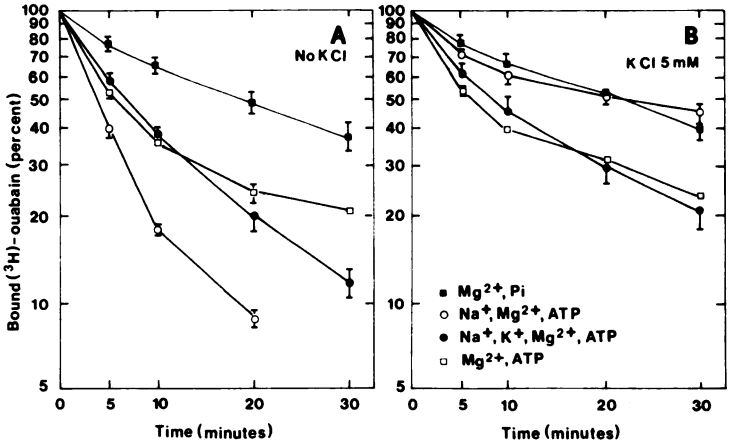

FIG. 1. *Dissociation in the absence (A) or presence of KCl (B) of ouabain-ATPase complexes formed in the presence of various ligands*

Rat brain (Na^+ + K^+)-ATPase preparations were incubated with 0.01 μM [³H]ouabain at 37° for 10 min in the presence of various ligands: ■, 1 mM $MgCl_2$ and 1 mM Tris-phosphate; ○, 20 mM NaCl, 5 mM $MgCl_2$, and 5 mM Tris-ATP; ●, 20 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, and 5 mM Tris-ATP; □, 5 mM $MgCl_2$ and 5 mM Tris-ATP. The mixture was centrifuged at 100,000 × *g* for 30 min at 0°, and the pellet was resuspended in 10 mM Tris-HCl buffer (pH 7.5) containing no (A) or 5 mM KCl (B). Dissociation was monitored at 37°. Bound [³H]ouabain for each preparation at the beginning of the dissociation reaction was set at 100%. Vertical lines indicate the standard errors of four experiments.

ATP or Mg^{2+} and P_i, and lower for those prepared in the presence of Mg^{2+} and ATP or Na^+, K^+, Mg^{2+}, and ATP. These differences in [³H]ouabain binding under various ligand conditions are in good agreement with those reported earlier by Schwartz *et al.* (17). In order to facilitate the comparison of dissociation rate constants, bound [³H]ouabain for each preparation at the beginning of dissociation reaction was set at 100% in Fig. 1. The [³H]ouabain-enzyme complex formed in the presence of Na^+, Mg^{2+}, and ATP dissociated most rapidly, with a half-life of less than 5 min. The ouabain-enzyme complex formed in the presence of Mg^{2+} and P_i dissociated slowly, with a half-life of approximately 30 min. These values are comparable to half-lives reported earlier from our laboratory (18). The complexes formed in the presence of either Mg^{2+} and ATP or Na^+, K^+, Mg^{2+}, and ATP had intermediate stabilities. The addition of 5 mM KCl to the dissociation mixture caused marked stabilization of the complex formed in the presence of Na^+, Mg^{2+}, and ATP; the half-life of the complex in the presence of KCl approached that of the complex prepared with Mg^{2+} and P_i (Fig. 1B). The addition of 5 mM KCl to the dissociation mixture, however, failed to stabilize further the stable ouabain-enzyme complex formed in the presence of Mg^{2+} and P_i (compare Fig. 1A and B). These data are in good agreement with previous reports (18, 28). The ouabain-enzyme complexes prepared in the presence of Mg^{2+} and ATP or Na^+, K^+, Mg^{2+}, and ATP were also minimally stabilized by the addition of 5 mM KCl to the dissociation mixture, although these complexes were still less stable than those prepared with Mg^{2+} and P_i. These results indicate that the ouabain-enzyme complexes prepared in the presence of Mg^{2+} and ATP or Na^+, K^+, Mg^{2+}, and ATP are different from those prepared with Na^+, Mg^{2+}, and ATP or with Mg^{2+} and P_i.

The variations in K^+ sensitivity of the ouabain-enzyme complexes prepared in the presence of Na^+, Mg^{2+}, and ATP and Na^+, K^+, Mg^{2+}, and ATP appear to reflect more than quantitative differences in the affinity of the complexes for K^+, since large differences in K^+ concentration did not appear to alter the dissociation rate constants significantly (Fig. 2). In these

experiments the enzyme preparation was incubated with 0.01 μM [^{3}H]ouabain in the presence of Na^+, Mg^{2+}, and ATP with or without 5 mM KCl at 37° for 10 min. The levels of bound [^{3}H]ouabain in the absence and presence of 5 mM KCl were 18.4 ± 0.5 and 4.04 ± 0.16 pmoles/mg of protein, respectively. The binding of [^{3}H]ouabain was terminated by the addition of nonradioactive ouabain, and the subsequent release of [^{3}H]ouabain was monitored in the presence of 0.5 mM $MgCl_2$, 2 mM NaCl, 0.5 mM Tris-ATP, 10 mM Tris-HCl buffer (pH 7.5), and various concentration of KCl. The half-life of the ouabain-enzyme complex formed in the presence of Na^+, Mg^{2+}, and ATP and dissociated in the absence of KCl (Fig. 2, 0 mM line) was somewhat longer than the corresponding value shown in Fig. 1A. This was due to the presence of 2 mM NaCl in the dissociation mixture rather than to differences in rates of dissociation and turnover (see MATERIALS AND METHODS). The NaCl was originally present in each binding mixture and not washed out but rather diluted 10-fold. A relatively low concentration (1 mM) of NaCl was shown to cause a slight stabilization of the ouabain-enzyme complex formed in the presence of Na^+, Mg^{2+}, and ATP (18). Potassium failed to stabilize the complex prepared in the presence of Na^+, K^+, Mg^{2+}, and ATP, even at high concentrations, whereas it stabilized the complex prepared in the presence of Na^+, Mg^{2+}, and ATP in a concentration-dependent manner. Obviously the effect of "zero" potassium could not be determined in the complex prepared in the presence of 5 mM K^+.

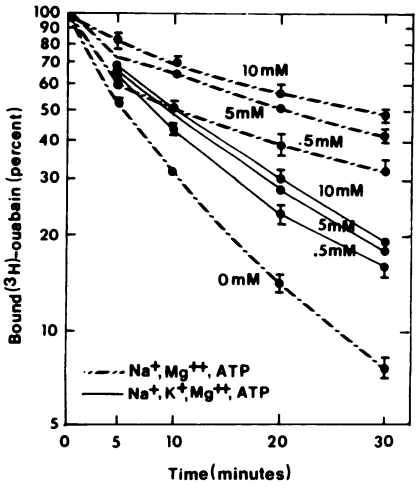

FIG. 2. *Effects of K^+ on dissociation of ouabain-ATPase complex*

Rat brain (Na^+ + K^+)-ATPase preparations were incubated with 0.01 μM [^{3}H]ouabain at 37° in the presence of 20 mM NaCl, 5 mM $MgCl^2$, and 5 mM Tris-ATP, without (-·-·-) or with 5 mM KCl (——) in addition. After a 10-min incubation period, the mixture was diluted 10-fold, nonlabeled ouabain was added (final concentration, 0.1 mM) to terminate the binding of labeled ouabain, and dissociation of the [^{3}H]ouabain-enzyme complex was monitored in the presence of KCl. The final concentration of KCl in the dissociation mixture is indicated along each line. Vertical lines indicate the standard errors of seven experiments.

In Figs. 1 and 2 bound [^{3}H]ouabain remaining after various incubation periods is expressed as a percentage of that at the onset of the dissociation reaction. Although this makes it possible to compare directly the dissociation rate constants of various forms of the ouabain-enzyme complexes, dissociation rate constants may be a function of the magnitude of ouabain-enzyme interactions. Figure 3, however, shows that the differences in initial levels of bound [^{3}H]ouabain or in the duration of the drug-enzyme interaction *per se* did not affect the dissociation of the ouabain-enzyme complex which had been stabilized by K^+. In these experiments the ouabain-enzyme complex was prepared in the presence of Na^+, Mg^{2+}, and ATP by incubating the enzyme preparation with different concentrations of [^{3}H]ouabain, ranging from 0.01 to 1.0 μM, for 10–30 min at 37°, which resulted in different levels of bound [^{3}H]ouabain. The dissociation rates were estimated in the presence of 5 mM KCl after terminating the binding of [^{3}H]ouabain by adding 0.1 mM nonradioactive ouabain to the mixture. Dissociation rate constants of the ouabain-enzyme complex were similar despite more than a 10-fold difference in initial levels of bound [^{3}H]-ouabain (Fig. 3, curves *A*, *B*, and *C*), as indicated by the similar slopes of the semilogarithmic plots. An increase in the incubation period for the binding reaction from 10 to 30 min also failed to affect the dissociation rate constants of this ouabain-enzyme complex (Fig. 3, curve *C* vs. curve

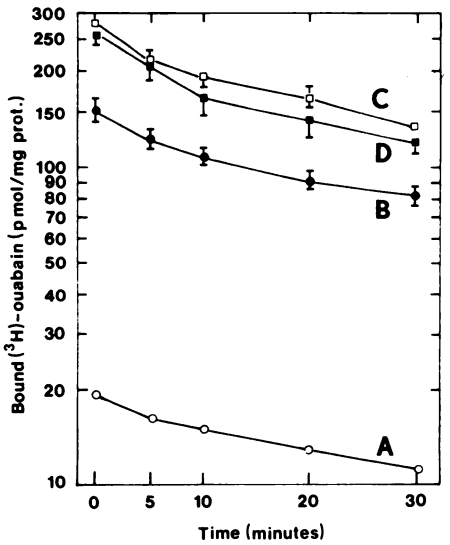

FIG. 3. *Dissociation rates of ouabain-ATPase complex*

Rat brain (Na^+ + K^+)-ATPase preparations (0.2 mg of protein) were incubated at 37° in the presence of 20 mM NaCl, 5 mM $MgCl_2$, and 5 mM Tris-ATP with 0.01 μM ouabain (*A*), 0.1 μM ouabain (*B*), or 1.0 μM ouabain (*C*) for 10 min, or with 1.0 μM ouabain for 30 min (*D*), in a total volume of 1.0 ml. The reaction was terminated at "zero" time by the addition of 9.0 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 0.11 mM nonlabeled ouabain and 5.5 mM KCl. Subsequent dissociation of the [^{3}H]ouabain-enzyme complex was monitored by taking aliquots at the indicated time points. Vertical lines indicate the standard errors of five experiments.

D). Thus the dissociation half-times observed in the presence of 5 mM KCl were independent of the initial levels of bound [^{3}H]ouabain and therefore the lack of K^+-induced stabilization of the ouabain-enzyme complex formed in the presence of Mg^{2+} and ATP or Na^+, K^+, Mg^{2+}, and ATP shown in Figs. 1 and 2 was not due to the lower initial levels of the [^{3}H]ouabain-enzyme complexes associated with these ligand conditions. Regardless of the initial levels of the [^{3}H]ouabain-enzyme complex, KCl was capable of stabilizing the ouabain-enzyme complex formed in the presence of Na^+, Mg^{2+}, and ATP. Furthermore, the dissociation rate constants were independent of the interaction time between ouabain and enzyme.

In order to determine whether the ouabain-enzyme complexes formed in the presence of Na^+, Mg^{2+}, and ATP and those formed in the presence of Na^+, K^+, Mg^{2+}, and ATP were interconvertible, attempts were made to convert the latter ouabain-enzyme complex from the relatively K^+-insensitive form to the highly K^+-sensitive form. When the [^{3}H]ouabain-enzyme complex was formed in the presence of Na^+, K^+, Mg^{2+}, and ATP, centrifuged, and resuspended in 10 mM Tris-HCl buffer (pH 7.5), the complex dissociated at an intermediate rate and K^+ failed to stabilize it (Fig. 1A). With added KCl, the dissociation of such a complex was significantly faster than that of the complexes formed in the presence of Na^+, Mg^2, and ATP and dissociated in the presence of KCl or those formed in the presence of Mg^{2+} and P_i (Fig. 1B), although the centrifugation and resuspension had been shown to remove K^+ effectively (19). Further attempts to convert the complex by treatment with Na^+ and P_i were only partially successful. (Na^+ + K^+)-ATPase has been shown to be an allosteric enzyme, and it is postulated that Na^+ favors one configuration and that K^+ induces the transition to another configuration (see ref. 18). Ouabain preferentially binds to the Na^+-induced form of the phosphoenzyme (21, 29, 30) in an environment of high ionic strength, and the resulting complex is unstable and highly sensitive to the stabilizing action of K^+ (18, 19, 28). Thus, after the removal of K^+ by centrifugation, washing, and resuspension of the pellet, the ouabain-enzyme complex formed in the presence of Na^+, K^+, Mg^{2+}, and ATP was exposed to relatively high concentrations of NaCl in an attempt to convert it from the K^+-induced form to the Na^+-induced form under conditions which would phosphorylate the ouabain-enzyme complex. Ouabain binds to the phosphoenzyme, forming a ouabain-phosphoenzyme complex from which phosphate dissociates more rapidly than ouabain (29). The addition of P_i, but not ATP, has been shown to rephosphorylate the complex (29). The ouabain-enzyme complex prepared in the presence of either Na^+, K^+, Mg^{2+}, and ATP or Mg^{2+} and P_i was centrifuged at 100,000 × *g* for 30 min at 0°, resuspended in a mixture containing 10 mM Tris-phosphate, 200 mM NaCl, and 10 mM Tris-HCl buffer (pH 7.5),

and incubated at 37° for 5 min. The mixture was then centrifuged twice at 100,000 × *g* at 0°, resuspending each time with 10 mM Tris-HCl buffer (pH 7.5). The dissociation rates of these complexes were observed at 37°. The slow dissociation of the complex prepared with Mg^{2+} and P_i was unchanged by treatment with Na^+ and P_i and further centrifugation and resuspension (open squares, Fig. 4). Additionally, 5 mM KCl failed to alter the stability of the ouabain-enzyme complex prepared with Mg^{2+} and P_i and then treated with Na^+ and P_i (solid squares, Fig. 4). The stability, observed in the absence of KCl, of the complex prepared with Na^+, K^+, Mg^{2+}, and ATP appeared to be only slightly decreased after these treatments (compare with Fig. 1). The addition of 5 mM KCl to the dissociation mixture caused a decrease

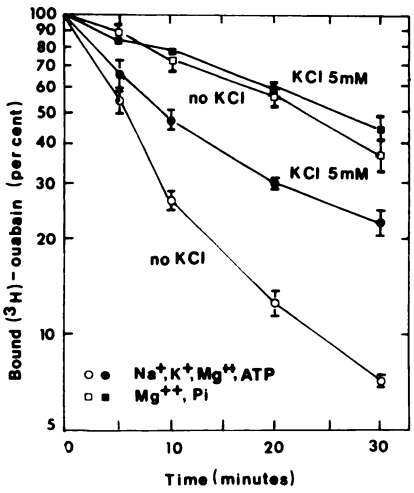

FIG. 4. *Dissociation of ouabain-ATPase complex treated with 10 mM P_i and 200 mM NaCl*

Rat brain (Na^+ + K^+)-ATPase preparations were incubated with 0.05 μM [^{3}H]ouabain at 37° for 10 min in the presence of either 20 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, and 5 mM Tris-ATP or 1 mM $MgCl_2$ and 1 mM Tris-phosphate. The mixture was centrifuged at 100,000 × *g* for 30 min at 0°. The resulting pellet was resuspended in a medium containing 10 mM Tris-phosphate and 200 mM NaCl, incubated at 37° for 5 min, and centrifuged twice at 100,000 × *g* for 30 min at 0°, resuspending each time with 10 mM Tris-HCl buffer (pH 7.5). The final suspension was incubated at 37° and monitored for dissociation of the [^{3}H]ouabain-enzyme complex in the presence and absence of 5 mM KCl. Vertical lines indicate the standard errors of four experiments.

in the dissociation rate constant, but failed to stabilize the complex to the level of that prepared in the presence of Mg^{2+} and P_i (Fig. 4) or that prepared in the presence of Na^+, Mg^{2+}, and ATP and stabilized by KCl (Fig. 1). Thus these treatments failed to significantly convert the ouabain-enzyme complex prepared in the presence of Na^+, K^+, Mg^{2+}, and ATP from the relatively K^+-insensitive form to the highly K^+-sensitive form. Treatment of the complex with either Na^+ or P_i alone was also unsuccessful (data not shown). These treatments also failed to alter the stability of the ouabain-enzyme complex formed in the presence of Mg^{2+} and P_i.

Dissociation in vitro of ouabain from cardiac tissue following dog heart Langendorff perfusion, and dissociation of ouabain–cardiac (Na^+ + K^+)-ATPase complexes formed in vitro in the presence of various ligands. In isolated puppy heart Langendorff preparations, perfusion with 0.6 μM [^{3}H]ouabain for 20 min produced a 29.1±1.9% increase in isometric contractile force (mean ± standard error of four experiments) under the present experimental conditions. At that time the ventricular muscle was excised, homogenized, and centrifuged at 100,000 × *g* for 30 min at 0°, and the pellet was resuspended in 10 mM Tris-HCl buffer (pH 7.5) and assayed for the release of [^{3}H]ouabain at 37°. The release of [^{3}H]ouabain in the absence of added KCl was relatively slow: the half-time was approximately 25 min (Fig. 5). It should be pointed out that values in Fig. 5 represent "total" ouabain binding to the particulate fraction of ventricular tissue. Although it was not possible to distinguish that portion of [^{3}H]ouabain which was specifically bound to (Na^+ + K^+)-ATPase from that nonspecifically bound to the tissue under these experimental conditions, the addition of 5 mM KCl markedly reduced the rate of release of [^{3}H]ouabain. The effects of 5 mM NaCl in reducing the rate of release of [^{3}H]ouabain were significantly smaller (Fig. 5). These differential effects of Na^+ and K^+ on the release of bound ouabain closely resemble those on the dissociation of the ouabain–(Na^+ + K^+)-ATPase complex formed *in vitro* in the pres-

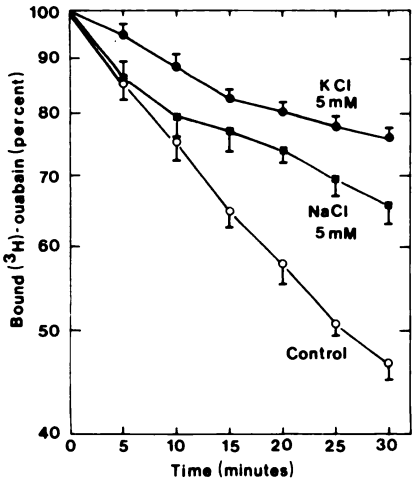

FIG. 5. *Dissociation of [^{3}H]ouabain from ventricular tissue after puppy heart perfusion with the drug*

Isolated puppy hearts were perfused with 0.6 μM [^{3}H]ouabain for 20 min at 30°. After perfusion of the drug into puppy hearts, ventricular muscle was homogenized, centrifuged at 100,000 × *g* for 30 min at 0°, resuspended in 10 mM Tris-HCl buffer (pH 7.5), and assayed for the release of [^{3}H]ouabain at 37° in the absence and presence of either KCl or NaCl. Values are expressed as percentages of bound [^{3}H]ouabain at the start of the dissociation reaction. Vertical lines indicate the standard errors of eight (control and KCl) or four (NaCl) experiments.

ence of Na^+, Mg^{2+}, and ATP (18). While it is possible that K^+ nonspecifically alters the rate of release of bound ouabain which is not associated with (Na^+ + K^+)-ATPase, the differential effects of K^+ and Na^+ observed strongly suggest that the K^+-sensitive portion of bound ouabain is associated with (Na^+ + K^+)-ATPase. The ATP-dependent portion of bound [^{3}H]ouabain formed in the presence of Na^+ and Mg^{2+} has been shown to be associated with (Na^+ + K^+)-ATPase (26), whereas the ATP-independent portion is considered to be nonspecific. While the dissociation of the ATP-dependent portion of bound ouabain is differentially affected by Na^+ and K^+ (18), that of the ATP-independent portion is affected by neither Na^+ nor K^+ (data not shown). Furthermore, the release of bound ouabain during the incubation *in vitro* was accompanied by recovery of the (Na^+ + K^+)-ATPase activity estimated from the initial velocity of [^{3}H]ouabain binding (data not shown; see, however, ref. 10). Thus it appears reasonable to conclude that the [^{3}H]ouabain bound to (Na^+ + K^+)-ATPase during the perfusion of isolated hearts was sensitive to K^+ after a single centrifugation and resuspension. The portion of bound [^{3}H]ouabain sensitive to K^+ constituted at least 30% of the bound ouabain present in the particulate fraction of ventricular tissue (Fig. 5). The relatively slow release of ouabain from the tissue preparations in the absence of added KCl depicted in Fig. 5 may have been due to the presence of nonspecifically bound ouabain, which appears to have a very long dissociation half-time. The "looser" binding is characteristic of specific ouabain binding to (Na^+ + K^+)-ATPase (31). It has been shown that the portion of bound cardiac glycoside associated with the inotropic response is less tightly bound (32) and that the washout of ouabain which is not associated with the inotropic response is slower than the washout of the inotropic response, i.e., slower than the washout of ouabain bound to the inotropic receptor (33).

While the ouabain-enzyme complexes formed with Na^+, Mg^{2+}, and ATP or with Na^+, K^+, Mg^{2+}, and ATP were different in rat brain, these complexes have similar characteristics in dog heart: [^{3}H]ouabain bound to the dog heart homogenate *in vitro* in the presence of Na^+, K^+, Mg^{2+}, and ATP was fully sensitive to K^+ after a single centrifugation and resuspension (Fig. 6), in contrast to that bound to the rat brain enzyme (Fig. 1). In these experiments [^{3}H]ouabain was incubated with dog heart homogenate, centrifuged at 100,000 × *g* for 30 min at 0°, and resuspended in 10 mM Tris-HCl buffer (pH 7.5). The dissociation half-time of the [^{3}H]ouabain-enzyme complex formed in the presence of Na^+, K^+, Mg^{2+}, and ATP was longer than that of the complex formed in the presence of Na^+, Mg^{2+}, and ATP (Fig. 6). However, the addition of 5 mM KCl stabilized both complexes to the same level. Thus the ouabain-enzyme complexes formed with Na^+, Mg^{2+}, and ATP in the presence and absence of K^+ were maximally stabilized by KCl added to the dissociation mixture

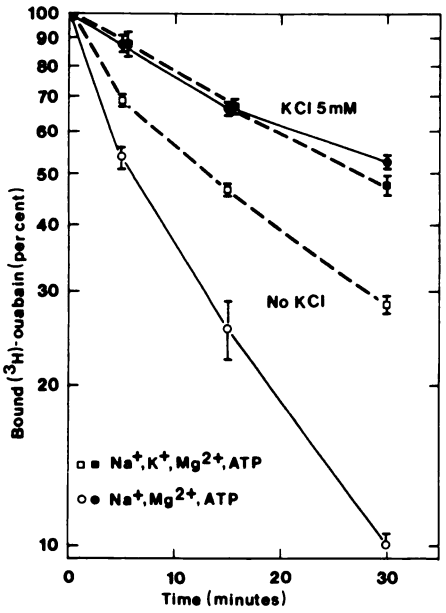

FIG. 6. *Dissociation of [³H]ouabain from dog heart homogenate*

Dog heart homogenate (0.2 mg of protein per milliliter) was incubated with 0.01 μM [³H]ouabain at 37° for 20 min in the presence of either 20 mM NaCl, 5 mM $MgCl_2$, and 5 mM Tris-ATP (○, ●) or 20 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, and 5 mM Tris-ATP (□, ■). The mixture was centrifuged at 100,000 × *g* for 30 min at 0°, resuspended in 10 mM Tris-HCl buffer (pH 7.5), and assayed for the release of [³H]ouabain at 37° in the absence (open symbols) or presence of 5 mM KCl (filled symbols). Values are expressed as percentages of bound [³H]ouabain at the start of the dissociation reaction. Vertical lines indicate the standard errors of four experiments.

when dog heart homogenate was used as the enzyme source.

In order to determine whether these characteristics of the ouabain-enzyme complex prepared with Na^+, K^+, Mg^{2+}, and ATP were due to the source of the enzyme or to the use of the homogenate instead of the partially purified enzyme preparation, dissociation rates of the ouabain-enzyme complex prepared with a partially purified dog heart enzyme were studied. In the absence of KCl, dissociation of the [³H]ouabain-enzyme complex prepared in the presence of Na^+, Mg^{2+}, and ATP was slightly slower (open circles, Fig. 7) than dissociation of a similar complex prepared from rat brain enzymes (Fig. 2). The addition of 5 mM KCl stabilized the complex. Dissociation of the complex prepared with Na^+, K^+, Mg^{2+}, and ATP in the presence of 0.5 mM KCl was somewhat slower than that prepared without K^+ in the binding medium (filled circles, Fig. 7). It should be noted that the [³H]ouabain binding reaction was terminated by the addition of nonradioactive ouabain in these experiments and the dissociation reaction was monitored without centrifugation and resuspension, in contrast to the experiments shown in Fig. 6. Thus, when the binding reaction was performed in the presence of 5 mM KCl and the dissociation was monitored subsequently, the mixture inevitably contained 0.5 mM KCl. When the [³H]ouabain-enzyme complex was prepared in the presence of Na^+, K^+, Mg^{2+},

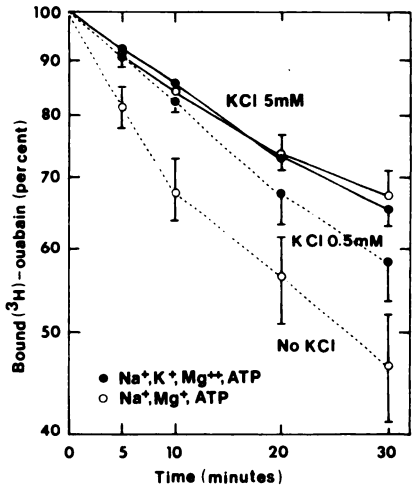

FIG. 7. *Dissociation of [³H]ouabain from dog heart (Na^+ + K^+)-ATPase preparation*

Dog heart (Na^+ + K^+)-ATPase preparations (0.2 mg of protein per milliliter) were incubated with 0.01 μM [³H]ouabain at 37° for 20 min in the presence of either 20 mM NaCl, 5 mM $MgCl_2$, and 5 mM Tris-ATP (○) or 20 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, and 5 mM Tris-ATP (●). The binding of [³H]ouabain was terminated by the addition of nonlabeled ouabain (final concentration, 0.1 mM) and 10-fold dilution of the incubation mixture. Subsequent dissociation of the [³H]ouabain-enzyme complex was monitored at 37° in the presence (——) and absence (----) of added KCl. Values are expressed as percentages of bound [³H]ouabain at the start of the dissociation reaction. Vertical lines indicate the standard errors of four experiments.

and ATP and the dissociation rate was subsequently monitored in the presence of 5 mM KCl, the dissociation rate constants were exactly the same as those of the complexes prepared in the absence of KCl and dissociated in the presence of 5 mM KCl (Fig. 7). Thus the ouabain-enzyme complex prepared from a partially purified dog heart $(Na^+ + K^+)$-ATPase in the presence of Na^+, K^+, Mg^{2+}, and ATP was K^+-sensitive and was fully stabilized by this cation.

Centrifugation and resuspension failed to alter either the stability or the K^+ sensitivity of the [^{3}H]ouabain-enzyme complex prepared with dog heart $(Na^+ + K^+)$-ATPase in the presence of Mg^{2+} and P_i (data not shown). The [^{3}H]ouabain-enzyme complex, prepared from the partially purified dog heart enzyme in the presence of Mg^{2+} and P_i, centrifuged at 100,000 × *g* for 30 min at 0°, and resuspended in 10 mM Tris-HCl buffer (pH 7.5), showed slow dissociation at 37° in the absence of K^+. The half-time for the dissociation was approximately 30 min. The addition of 5 mM KCl to the dissociation mixture failed to influence the stability significantly. Thus centrifugation and resuspension did not convert this ouabain-enzyme complex from a stable, K^+-insensitive form to an unstable, K^+-sensitive form, indicating that the ouabain-enzyme complex formed in the presence of Mg^{2+} and P_i has different characteristics from that prepared in the presence of either Na^+, Mg^{2+}, and ATP or Na^+, K^+, Mg^{2+}, and ATP.

DISCUSSION

It was shown previously that ouabain–$(Na^+ + K^+)$-ATPase complexes formed in the presence of different ligands, such as $(Na^+ + Mg^{2+} + ATP)$, $(Mg^{2+} + P_i)$, Mn^{2+}, or $(Mg^{2+} + ATP)$, dissociate with similar rate constants when the binding of labeled ouabain is terminated by the addition of excess nonlabeled ouabain and the dissociation of the labeled complex is monitored in the presence of binding ligands (34). The ouabain-enzyme complex, however, appears to have different characteristics, depending upon the ligands present during the binding reaction (18). Such differences may be detected by transferring the ouabain-enzyme complex into another environment, e.g., one with low ionic strength, and monitoring the rate of dissociation. Under the latter condition dissociation of ouabain-enzyme complexes formed in the presence of certain ligands are rapid and markedly slowed by the addition of monovalent cations such as K^+, Rb^+, Cs^+, NH_4^+, or Tl^+ to the dissociation mixture (18). The stabilizing effects of Na^+ or Li^+ were much less, indicating that the ouabain-enzyme complex discriminates among monovalent cations in the same manner as does the phosphoenzyme (see ref. 18). Although it is not known which dissociation rate—the slow rate in the presence of binding ligands or the fast rate in the low ionic milieu—is useful in predicting the dissociation rate that determines the level of ouabain-enzyme interaction *in vivo*, the dissociation rate under the latter condition is useful in studying the identity of the ouabain-enzyme complexes formed under various conditions, particularly in the beating mammalian heart.

The present data suggest that there are at least three different forms of the complex of ouabain with rat brain $(Na^+ + K^+)$-ATPase, depending upon the presence or absence of ligands during the binding reaction, namely, the complexes formed in the presence of Mg^{2+} and P_i, in the presence of Na^+, Mg^{2+}, and ATP, and in the presence of Mg^{2+} and ATP. The complex formed in the presence of Na^+, K^+, Mg^{2+}, and ATP appeared to be similar to that formed in the presence of Mg^{2+} and ATP (Table 1).

The ouabain-enzyme complex formed with rat brain enzyme in the presence of Mg^{2+} and ATP dissociated relatively rapidly in the absence and presence of K^+, the complex being relatively unstable and K^+-insensitive. The addition of Na^+ to the binding mixture that contained Mg^{2+} and ATP stimulated ouabain binding, as reported previously (17), and made the resultant complex unstable in the absence of K^+. The addition of K^+ to the dissociation mixture stabilized this complex. Furthermore, addition of K^+ to the binding mixture altered the dissociation characteristics of the resulting complex. These data may sup-

TABLE 1

Dissociation characteristics of ouabain-(Na^+ + K^+)-ATPase complexes formed in the presence of various ligands and dissociated in medium with low ionic strength

Ligands	Stability	Effects of K^+
Mg^{2+}, P_i	Stable	None
Mg^{2+}, ATP	Intermediate	None
Na^+, Mg^{2+}, ATP	Unstable	Marked stabilization
Na^+, K^+, Mg^{2+}, ATP	Intermediate	Minimal stabilization[a] Marked stabilization[b]

[a] Rat brain enzyme.

[b] Dog heart enzyme.

port the hypothesis that ouabain binds to various forms of (Na^+ + K^+)-ATPase, such as an ADP-sensitive phosphoenzyme, a K^+-sensitive phosphoenzyme, and/or a K^+-induced form. Ouabain binding to K^+-sensitive forms of the phosphoenzyme appears to result in an unstable, K^+-sensitive complex, whereas binding to the ADP-sensitive phosphoenzyme and/or the K^+-induced forms of the enzyme (i.e., K^+-insensitive forms) results in a relatively unstable, K^+-insensitive complex. These different dissociation properties of the ouabain-enzyme complex may represent the binding of ouabain to different sites on (Na^+ + K^+)-ATPase, as claimed by Taniguchi and Iida (35). An alternative explanation may be that these different forms represent different conformations of the ouabain-enzyme complex which have different accessibilities to ouabain binding sites. The present data with dog heart (Na^+ + K^+)-ATPase would appear to support the latter contention. Under the present experimental conditions, however, the ouabain-enzyme complex prepared from rat brain enzyme in the presence of Na^+, K^+, Mg^{2+}, and ATP or Mg^{2+} and P_i could not be converted to a highly K^+-sensitive form, which is the characteristic of the complex prepared in the presence of Na^+, Mg^{2+}, and ATP. Thus, while it appears that different forms of the ouabain-enzyme complex represent different conformational states of the enzyme, these different forms are not readily interconvertible.

It appeared that the hydrolysis of ATP by rat brain (Na^+ + K^+)-ATPase preparations during the ouabain binding reaction was substantial when the binding reaction was performed in the presence of Na^+, K^+, Mg^{2+}, and ATP, conditions optimal for (Na^+ + K^+)-ATPase activity. Ouabain did not inhibit the enzyme activity significantly, because of the low concentration employed to reduce the level of nonspecific binding. Concomitantly, the levels of the ouabain-enzyme complex reached a peak after approximately 10–15 min of incubation and declined slightly thereafter (data not shown). This phenomenon was not observed with other combinations of the ligands. It is not likely, however, that the ouabain-enzyme complex formed in the presence of Na^+, K^+, Mg^{2+}, and ATP is a mixture of those formed with ATP-supported binding and P_i-supported binding. Inorganic phosphate failed to support ouabain binding in the presence of Na^+, K^+, or high ionic strength (36). With other combinations of ligands or with dog heart enzyme or homogenate, the hydrolysis of ATP was less significant.

While in some experiments less than 10% of the enzyme added to the mixture formed a complex with ouabain, it appears that K^+ was capable of stabilizing the ouabain-enzyme complex formed in the presence of Na^+, Mg^{2+}, and ATP independently of the initial level of the interaction. It appears reasonable to assume that the higher concentration of ouabain merely increased the incidence of the ouabain-enzyme interaction but that the characteristics of the resultant complex were unaffected by the drug concentration. A similar result was reported by Lane *et al.* (34). This assumption is further supported by a smooth, symmetrical curve with a midpoint slope of approximately 0.5 on a semi-

logarithmic plot of ouabain concentration vs. $(Na^+ + K^+)$-ATPase inhibition shown in our previous report (Fig. 2, curve *B*, of ref. 24), which tends to rule out a cooperative interaction of ouabain with the enzyme. Barnett (21) reported that inhibition of $(Na^+ + K^+)$-ATPase by ouabain and the binding of [^{3}H]ouabain to the enzyme preparation are first-order in both enzyme and ouabain concentrations. Lindenmayer and Schwartz (25) confirmed that the rates of ouabain binding adhere to pseudo-first-order kinetics.

Although Na^+ stimulates ouabain binding to $(Na^+ + K^+)$-ATPase, this does not necessarily indicate that Na^+ enhances the reaction rates between phosphoenzyme and ouabain. In the presence of Na^+, Mg^{2+}, and ATP, it appears that a large fraction of the enzyme is in the form of the K^+-sensitive phosphoenzyme (29), whereas in the presence of Mg^{2+} and ATP, or Na^+, K^+, Mg^{2+}, and ATP, only a small fraction of the total enzyme may be in a form which optimally binds ouabain. Thus differences in ouabain binding may reflect the concentration of binding forms of the enzyme, as proposed by Barnett (21), rather than differences in the binding reaction rate constant. Present data, however, are incompatible, at least in the brain, with the hypothesis that the K^+-sensitive phosphoenzyme is the only form capable of binding ouabain in a milieu of high ionic strength (21).

In contrast to the ouabain-enzyme complex prepared from rat brain enzyme, the complex of ouabain with dog heart enzyme formed *in vitro* in the presence of Na^+, K^+, Mg^{2+}, and ATP and subjected to centrifugation and resuspension had dissociation characteristics similar to the complex prepared without K^+. These differences may be due to the characteristics of $(Na^+ + K^+)$-ATPase itself, namely, differences in the ease or difficulty of interconversion between the ADP-sensitive, K^+-insensitive form of the phosphoenzyme and the ADP-insensitive, K^+-sensitive form. The existence of such differences among rat brain and guinea pig kidney enzymes has been reported (37).

Since the complexes of ouabain with dog heart $(Na^+ + K^+)$-ATPase formed *in vitro* in the presence of either Na^+, Mg^{2+}, and ATP or Na^+, K^+, Mg^{2+}, and ATP had similar dissociation characteristics, either complex may be used in studies of the drug-enzyme interaction. This however, may not be true for the enzyme obtained from sources such as brain. A significant portion of [^{3}H]ouabain bound to ventricular tissue during Langendorff perfusions of puppy hearts was stabilized by the addition of K^+ to the dissociation mixture. These characteristics are clearly different from those of the complex formed from dog heart $(Na^+ + K^+)$-ATPase in the presence of Mg^{2+} and P_i, which was not K^+-sensitive. It is also well recognized that both the intracellular and extracellular milieus contain in excess of 20 mM Na^+. For these reasons, and because Na^+ at these concentrations completely inhibits the binding of ouabain to the enzyme in the presence of Mg^{2+} and P_i (36), the binding of ouabain to $(Na^+ + K^+)$-ATPase *in vitro* in the presence of Mg^{2+} and P_i is not a suitable model for drug-enzyme interactions occurring in the functioning heart.

ACKNOWLEDGMENTS

The authors thank Mr. Roxy H.-M. So and Mrs. V.-M. K. Cheng for expert technical assistance.

Note.

Since the submission of the original manuscript, three papers pertinent to this study appeared. Schwartz *et al.* (38) reported that the release *in vitro* of [^{3}H]ouabain bound to a cat Langendorff preparation was prevented by 10 mM KCl. Inagaki, Lindenmayer, and Schwartz (39) proposed that cardiac glycosides bind to a particular (conformational) state of the enzyme and presented a scheme in which ouabain binds to this state when the enzyme is bound to either Na^+ or K^+, or is free from monovalent cations. They did not describe the differences of the resultant complexes formed with different ligand-enzyme combinations, however. Yoda and Yoda (40) reported a successful conversion of the complex of beef brain $(Na^+ + K^+)$-ATPase with ouabain or digitoxigenin monodigitoxide, formed in the

presence of Mg^{2+} and P_i, from the slowly dissociating form to a rapidly dissociating one, using an indirect method to estimate the dissociation of the complex. These data, however, are not inconsistent with the present observations, since rapid dissociation was observed only in the presence of Na^+ and ATP, and the removal of Na^+ and ATP resulted in a slow dissociation of the complex as reported here. Since the unstable ouabain-enzyme complex formed in the presence of Na^+, Mg^{2+}, and ATP remains unstable after centrifugation and resuspension (Fig. 1), it appears that (Na^+ + ATP + EDTA) treatment of the complex formed in the presence of Mg^{2+} and P_i does not permanently convert this stable complex to an unstable one.

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