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Association and Dissociation Rate Constants of the Complexes between Various Cardiac Aglycones and Sodium- and Potassium-Dependent Adenosine Triphosphatase Formed in the Presence of Magnesium and Phosphate

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SUMMARY

YODA, ATSUNOBU & YODA, SHIZUKO (1977) Association and dissociation rate constants of the complexes between various cardiac aglycones and sodium- and potassium-dependent adenosine triphosphatase formed in the presence of magnesium and phosphate. *Mol. Pharmacol.*, 13, 352–361.

Association and dissociation rates of cardiac aglycone–$(Na^+ + K^+)$-ATPase complexes formed in the presence of magnesium and inorganic phosphate were examined by assay of the phosphorylated protein formed in the presence of [γ-^{32}P]ATP, which is influenced by the amount of bound aglycone. Association and dissociation followed pseudo-first-order and first-order rate kinetics, respectively. The dissociation rate constants of four cardiac aglycones – digitoxigenin, digoxigenin, strophanthidin, and ouabagenin – were all the same (0.28 min^{-1} at 25° and 0.63 min^{-1} at 30°), but their pseudo-first-order association rate constants (k_a') varied. Both Mg^{2+} and P_i gave linear relationships against k_a' in double-reciprocal plots over a wide range of concentrations, and the effects of both ligands were identical. The four cardiac aglycones showed the same maximum association rates with increasing drug concentration. These results suggest that the binding of ligands (magnesium and phosphate) results in activation of the enzyme to bind the cardiac aglycone, but that dissociation of ligands from the cardiac aglycone–enzyme complex precedes the release of cardiac aglycone.

INTRODUCTION

The sodium- and potassium-dependent adenosine triphosphatase (ATP phosphohydrase, EC 3.6.1.3) is believed to be integral to the active transport of sodium and potassium ions and is specifically inhibited by cardiac glycosides. Experiments using the radioactive cardiac glycosides ouabain and digoxin (1–4) have established that a cardiac glycoside–enzyme complex is formed in the presence of certain ligands. The two most effective systems are Mg^{2+}-P_i and Na^+-Mg^{2+}-ATP (2). Our rate kinetic studies on cardiac glycoside–$(Na^+ + K^+)$-ATPase complexes (5–7) indicated that cardiac glycosides were bound to the enzyme at two sites, one specific for the steroid moiety and the other for the sugar, and that binding of the steroid moiety to the enzyme results in activation of the sugar binding site, with consequent binding of the glycoside portion. The role of the sugar moiety in binding to $(Na^+ + K^+)$-ATPase is to increase the stability of the complex through interaction of the 3'-hy-

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droxyl and other functional groups on the sugar moiety with the sugar-specific site(s) on the enzyme.

In order to eliminate the effect of the sugar moiety of cardiac glycosides on binding, the binding of [^{3}H]digoxigenin to (Na^+ + K^+)-ATPase was studied at equilibrium, and the following results were obtained (8). (a) Digoxigenin bound to its site on the enzyme with high affinity in the presence of ligands (Mg^{2+} + P_i or Na^+ + Mg^{2+} + ATP), as in the case of glycoside binding. (b) Scatchard plots indicated that all binding was identical and noninteracting. (c) The binding constant of digoxigenin showed little change upon dilution of ligand, except at very low concentrations. However, the number of binding sites on the enzyme was strongly dependent on the ligand concentration. These results are similar to cation effects on dihydromorphine binding (9), and showed that the binding of digoxigenin to (Na^+ + K^+)-ATPase did not follow the usual equations representing a reversible reaction. More detailed information on the elementary steps in binding of the cardiac aglycones seems to be necessary for an understanding of the above results. The rate constants for association and dissociation of cardiac aglycones and (Na^+ + K^+)-ATPase have never been reported, because these reactions are much faster than those of cardiac glycosides. In this study we used a more rapid assay, the determination of protein phosphorylated by ATP, which is influenced by the amount of bound aglycone, and a special apparatus to improve the accuracy of the assay. The association and dissociation rate constants of the (Na^+ + K^+)-ATPase complexes with four cardiac aglycones formed in the presence of Mg^{2+} and P_i were examined.

MATERIALS AND METHODS

The (Na^+ + K^+)-ATPase preparation was NaI-treated microsomes prepared from beef brain by the method of Nakao *et al.* (10) according to the modification of Hegyvary and Post (11). Digoxigenin and strophanthidin were purchased from Boehringer/Mannheim. Digitoxigenin was prepared by acid hydrolysis of digitoxin, and ouabagenin was obtained from ouabain by the method of Mannich and Siewert (12). The homogeneity of each cardiac aglycone was determined by thin-layer chromatography using silica gel G. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappel (13), and used as the Tris salt. The specific radioactivity of the preparation obtained was more than 10^9 cpm/μmole and was diluted to about 200–400 cpm/pmole with nonradioactive ATP in each experiment.

The semirapid mixing apparatus described by Kanazawa *et al.* (14) was employed for measuring the association and dissociation rates. This apparatus consisted of a reaction tube and three pipettes. Each solution of the enzyme or of a reagent in the pipette was injected into the reaction tube by means of a solenoid-controlled syringe. Each solenoid was controlled by a timer (0.5–60 sec) or by a manual switch. The reaction mixture was vigorously stirred with a magnetic stirrer and was kept at constant temperature by circulating water. Each pipette was used after calibration. In the present study the reaction tube contained 1.9 or 2.0 ml of reaction mixture. Two of the three Folin-type pipettes were the 0.1-ml type. One of them contained the phosphorylating solution, consisting of NaCl, $MgCl_2$, and [γ-^{32}P]ATP, and the other was used for the enzyme suspension in the dissociation experiments, or for the cardiac aglycone solution in the association experiments. The third pipette was the 0.6-ml type, which was used for the quenching solution, containing 40% TCA,[1] 1 mM P_i, and 0.1 mM unlabeled ATP. By measuring alkaline hydrolysis of 2,4-dinitrophenylacetate (15), it was shown that the apparatus could be used at intervals of 0.5 sec.

Dissociation of cardiac aglycone-(Na^+ + K^+)-ATPase complex. The cardiac aglycone-(Na^+ + K^+)-ATPase complex was prepared by incubating a mixture containing enzyme (150–200 μg/ml), 4 mM Mg^{2+}, 2 mM P_i, 25 mM imidazole HCl buffer (pH

[1] The abbreviation used is: TCA, trichloroacetic acid.

7.35), and cardiac aglycone[2] for 30–50 min, which was sufficient time to reach equilibrium (9). As shown in the flow sheet (Fig. 1), dissociation was started by the injection of 0.1 ml of the above complex mixture into a reaction tube containing 2.0 ml of 1 mM EDTA solution in 25 mM imidazole HCl buffer (pH 7.35). At various times after starting dissociation, phosphorylation was initiated by injection of the phosphorylating solution, containing 1 M NaCl, 50 mM $MgCl_2$, and 0.2–0.3 mM [γ-^{32}P]ATP. After 3 sec phosphorylation was stopped by addition of 0.6 ml of 40% TCA solution containing 1 mM P_i and 0.1 mM unlabeled ATP. The amount of phosphorylated protein (*E*P) was determined as described below.

In order to calculate inhibition of phosphorylation by the aglycones, control experiments were carried out under the same conditions without any cardiac aglycone. No significant change in *E*P was observed during the incubation after dilution (less than 2.5 min) in every experiment. At various time intervals *E*P formation was assayed in triplicate. From such inhibition data at five or six intervals, a stability curve as shown in Fig. 4 was obtained, and the slope of this curve was the dissociation rate constant (k_d), as explained for cardiac glycosides (5).

Association of cardiac aglycones with (Na^+ + K^+)-ATPase. A mixture (1.9 ml) containing 40–50 μg of enzyme, 4.0 μmoles of Mg^{2+}, 4.0 μmoles of P_i, and 50 μmoles of imidazole HCl (pH 7.35) was stirred in a reaction tube at 15°, and the cardiac aglycone association reaction was started by the injection of 0.1 ml of cardiac steroid solution, as shown in the flow sheet (Fig. 2). At various times after starting the association, phosphorylation was performed as described above. *E*P formation was assayed in quadruplicate at five or six intervals, and from these data an inhibition curve as shown in Fig. 5 was obtained. The slope of this curve was the pseudo-first-order rate constant (k_a'), as explained for cardiac glycosides (6).

[2] The concentration of aglycone was selected to inhibit the formation of phosphorylated enzyme by 70–80% during preliminary incubation.

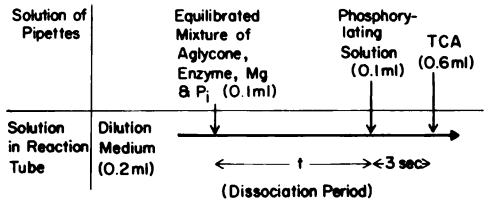


FIG. 1. *Flow sheet of semirapid mixing procedure for determination of dissociation rate constants*

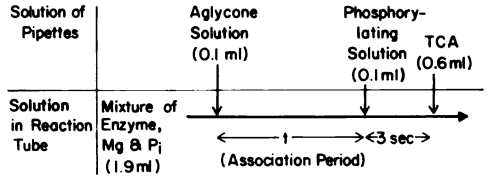


FIG. 2. *Flow sheet of semirapid mixing procedure for determination of pseudo-first-order association rate constants*

Determination of phosphorylated protein (EP). After phosphorylation was stopped, the reaction mixture was placed in ice for 30–40 min; 1 ml of the mixture was charged on a Millipore filter of 0.45-μm pore size, formed into a spot less than 7 mm in diameter, washed by dripping on it about 70 ml of ice-cold 5% TCA solution containing 1 mM P_i and 0.1 mM unlabeled ATP, and finally washed with 10 ml of ice-cold water. The washed precipitate on the filter was solubilized with 1.5 ml of 2-methoxyethanol in a counting vial, and radioactivity was measured with a Packard Tri-Carb liquid scintillation counter, using 5 ml of scintillation medium (5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene in 1 liter of toluene and 500 ml of Triton X-100).

In all experiments shown here, the amount of *E*P formed was calculated by subtraction of nonspecific phosphoprotein, which was determined by measuring phosphorylation in the presence of additional 25 mM KCl.

RESULTS

Inhibition of enzyme phosphorylation by cardiotonic steroids. As shown in Table 1, (Na^+ + K^+)-dependent ATPase activity, (Na^+ + Mg^{2+})-dependent phosphorylation by ATP (*E*P formation), and K^+-dependent umbelliferone phosphatase activity,

TABLE 1

Ouabain inhibition of (Na^+ + K^+)-ATPase, EP formation, and K^+-dependent phosphatase

The enzyme (80 μg/ml) was incubated for 10 min at 30° as described below, diluted 10-fold with 1 mM Tris-EDTA, and precipitated by centrifugation at 100,000 × *g* for 15 min. The precipitate was homogenized with 1 mM Tris-EDTA, and aliquots were assayed. (Na^+ + K^+)-ATPase activity was measured by the linked pyruvate kinase-lactate dehydrogenase spectrophotometric method (5), and K^+-dependent umbelliferone phosphatase activity, by the method of Pitts and Askari (16). *E*P formation was measured as described in the text. The values are percentage inhibition relative to control experiments, in which the enzyme was incubated under the same conditions but without ouabain, which was added later after dilution. In absolute terms, 100% activity corresponds to 0.86 μmole of ADP per minute per milligram at 30° for (Na^+ + K^+)-ATPase, to 215 pmoles/mg for *E*P formation, and to 0.12 μmole of umbelliferone per minute per milligram at 30° for umbelliferone phosphatase.

Conditions	Inhibition		
	(Na^+ + K^+)-ATPase	*E*P formation	Umbelliferone phosphatase
	%	%	%
0.2 μM ouabain + 2 mM Mg^{2+} + 2 mM P_i	23	21	25
0.3 μM ouabain + 2 mM Mg^{2+} + 2 mM P_i	56	55	52
0.3 μM ouabain + 150 mM Na^+ + 2 mM Mg^{2+} + 1 mM ATP	66	65	61

which is equivalent to K^+-dependent *p*-nitrophenyl phosphatase activity (16), were inhibited to the same extent by ouabain treatment, when assayed after the enzyme had been removed from the inhibition medium. The presence of Mg^{2+} and P_i partially inhibited *E*P formation, by forming a different phosphorylated protein $(E\langle^{Mg}_{P})$ (Fig. 3, line *A*), as Post *et al.* reported (17). This reduction in *E*P by Mg^{2+} and P_i was rapid, reaching a steady-state level within 0.5 sec at 15°. Phosphorylation of the digoxigenin-treated enzyme in the presence of Mg^{2+} and P_i decreased with time of exposure of the enzyme to the aglycone (Fig. 3, lines *B* and *C*). The presence of P_i or P_i and digoxigenin did not affect *E*P formation in the absence of Mg^{2+}.

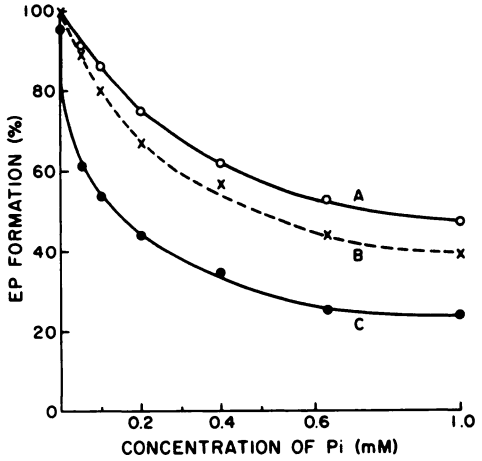


FIG. 3. *Effect of Mg^{2+}, P_i, and digoxigenin on phosphorylation of (Na^+ + K^+)-ATPase by ATP*

After incubation with 2 mM Mg^{2+} and various concentrations of P_i in the presence of 25 mM imidazole HCl buffer (pH 7.35) for 4 min, the enzyme (54 μg/ml) was treated with 1 μM digoxigenin (or left untreated) as indicated and phosphorylated as described in the text. All experiments were carried out at 15°. Line *A*, without digoxigenin, 20 sec (the results of zero-time treatment with 1 μM digoxigenin overlap line *A*); line *B*, with 1 μM digoxigenin, 20 sec; line *C*, with 1 μM digoxigenin, 60 sec.

Post *et al.* observed that Na^+ reduced the Mg^{2+}-P_i effects on *E*P formation (17, 18), and it also was found to decrease the steady-state binding of digoxigenin in the presence of Mg^{2+} and P_i.[3] Since the phosphorylating solution contained 1 M Na^+, it was necessary to determine whether this affected the amount of *E*P formed. When 1/20 the volume of phosphorylation solution was added to enzyme suspensions that had been incubated for 5 min at 25° with 2 mM Mg^{2+} and 2 mM P_i in the absence and presence of 0.5 μM digoxigenin, the amount of *E*P formed between 1 and 6 sec was the same in both cases. Therefore it appears that the Na^+ effect is not fast enough to change the amount of enzyme complexed with Mg^{2+} and P_i during phosphorylation by [γ-^{32}P]ATP.

[3] A. Yoda and S. Yoda, unpublished observations.

The reduction in EP by digoxigenin binding in the presence of Mg^{2+} and P_i was examined further. After the enzyme had been equilibrated with [^{3}H]digoxigenin in the presence of ligands, part of the mixture was centrifuged to separate the supernatant, and the amount of unbound [^{3}H]-digoxigenin was measured (8). Another sample was used for phosphorylation by [^{32}P]ATP. The results (Table 2) indicate that the relative amount of EP coincides with the amount of unbound enzyme. These results indicate that the reaction conditions employed for phosphorylation did not alter the steady state of the enzyme, and that the EP value corresponds to the free form of the enzyme. The reduction in amount of EP was plotted against the reaction time between enzyme and digoxigenin in the presence of Mg^{2+} and P_i, and a first-order plot was obtained (see Fig. 5). The slopes of these lines are the pseudo-first-order rate constants for the association of (Na^+ + K^+)-ATPase and digoxigenin.

Dissociation rate constants (k_d). When an equilibrated mixture of enzyme, cardiac aglycone, and ligands was diluted with 20 volumes of the ligand mixture, the fractional inhibition (i.e., the fraction of cardiac aglycone associated with the enzyme) was decreased and reached a new equilibrium (Fig. 4, line A). The initial rate of this change is the dissociation rate

TABLE 2

EP formation from enzyme-digoxigenin mixture

The enzyme preparation (0.96 mg/ml) was treated with different concentrations of [^{3}H]digoxigenin (specific activity, 284 cpm/pmole) in the presence of 2 mM Mg^{2+}, 2 mM P_i, and 25 mM imidazole HCl buffer (pH 7.35) at 25° for 30 min. The binding of digoxigenin was measured as reported previously (8). Aliquots of each suspension were centrifuged at 25°, and the unbound digoxigenin was determined from the radioactivity in the supernatant. The total number of binding sites (0.22 μM) was estimated by Scatchard plots from the digoxigenin binding data at six different concentrations (0.05, 0.10, 0.20, 0.30, 0.40, and 0.50 μM). This value coincided with the total number of ouabain binding sites, which was determined by the same method as for digoxigenin binding, except that the incubation time was 60 min. Other aliquots were phosphorylated with 1/20 the volume of phosphorylating solution as described in the text. In absolute terms, 100% EP formation corresponds to 115 ± 5 pmoles/mg and was obtained in the presence of 2 mM Mg^{2+} and 2 mM P_i. The values are the averages of three sets of experiments. In each experiment, measurements of bound digoxigenin were made in duplicate, and those of EP, in quadruplicate.

Total digoxigenin	Bound digoxigenin	Ratio of bound digoxigenin to total binding sites	Relative EP value
μM	μM	%	%
0			100
0.10	0.07 ± 0.01	32	70 ± 4
0.20	0.12 ± 0.02	55	44 ± 3
0.40	0.16 ± 0.02	73	25 ± 1

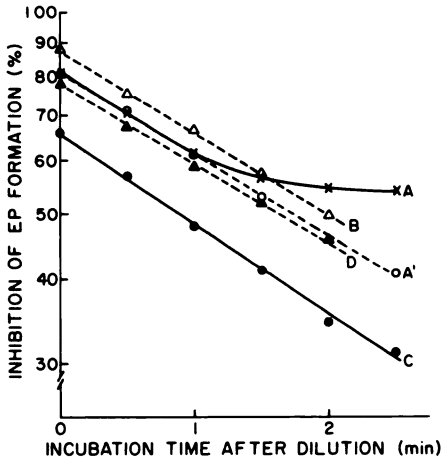


FIG. 4. *Stability of cardiac aglycone-(Na^+ + K^+)-ATPase complexes at 25° after dilution*

A mixture of the enzyme (0.8–1.0 mg/ml), 2 mM Mg^{2+}, 2 mM P_i, and cardiac aglycone was incubated for 30–45 min. The equilibrated mixture (0.1 ml) was diluted with 2 ml of 1 mM EDTA in 25 mM imidazole HCl buffer (pH 7.35), and enzyme inhibition was determined at 30-sec intervals by assaying the formation of EP. See the text for experimental details. Line A, the enzyme was treated with 0.6 μM digoxigenin and diluted with a solution containing 2 mM Mg^{2+}, 2 mM P_i, and 25 mM imidazole HCl buffer instead of 1 mM EDTA. Line A', the same as A, except that the dilution buffer contained 1 mM EDTA. Line B, the same as A', except that the enzyme was treated with 0.8 μM digoxigenin. Line C, the same as A', except that the enzyme was treated with 0.2 μM digoxigenin. Line D, the same as A', except that the enzyme was treated with 0.2 μM digitoxigenin.

of the complex. If the ligands in the dilution medium were omitted, or replaced with 1 mM Tris-EDTA, the shift in equilibrium was increased by the dilution, and the initial change in inhibition followed first-order kinetics over a wider range (Fig. 4, line A'), but the k_d value was not changed. In all other experiments a mixture of 1 mM EDTA and 25 mM imidazole HCl buffer (pH 7.35) was used as the dilution medium. As expected from first-order kinetics, the initial percentage of inhibition by digoxigenin did not influence the dissociation rates (Fig. 4, lines A', B, and C).

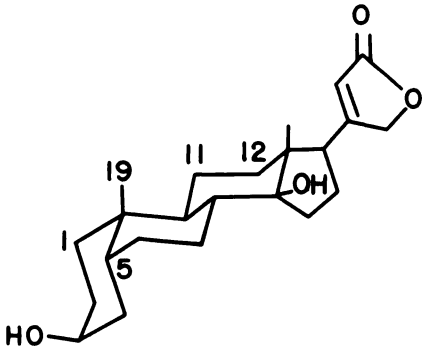
As shown in Table 3, the four cardiac aglycones gave identical k_d values (0.28 ± 0.01 min^{-1} at 25° and 0.63 ± 0.02 min^{-1} at 30°). The k_d value of the cardiac aglycones at 25° is more than 10 times that of odoroside H, which had the highest k_d of the cardiac monoglycosides studied previously (5).

Association rate constants of cardiac aglycones with (Na^+ + K^+)-ATPase. Since dissociation of the cardiac aglycone-enzyme complex was relatively slow ($t_{1/2}$ about 2.5 min at 25°), the amount of aglycone-enzyme complex dissociated during the EP assay should be negligible. If suitable concentrations of enzyme and aglycone are used, EP formation can be determined without significant change in the amount of active enzyme. As the EP value appears to parallel the amount of active enzyme, the time course of EP formation after addition of the cardiac aglycone can give the time course of association between cardiac aglycone and (Na^+ + K^+)-ATPase.

As shown in Fig. 5, the logarithm of EP decreased linearly with time. That is, association of the cardiac aglycone with (Na^+

TABLE 3

Structures and dissociation rate constants of cardiac aglycones used in this study



Compound	Substituents	Dissociation rate constant[a]	
		25°	30°
		min^{-1}	
Cardiac aglycones			
Digitoxigenin	19-CH_3	0.27	0.65
Digoxigenin	19-CH_3, 12β-OH	0.29	0.64
Strophanthidin	19-CHO, 5β-OH	0.27	0.62
Ouabagenin	19-CH_2OH, 1β-OH, 5β-OH, 11α-OH	0.28	0.61
Cardiac glycosides[b]			
Ouabain		0.0037	0.0075
Digoxigenin monodigitoxide		0.0075	0.013
Odoroside H (digitoxigenin 3'-methoxyfucoside)		0.021	

[a] The error was about ±0.03 min^{-1}.

[b] These data were obtained previously (5).

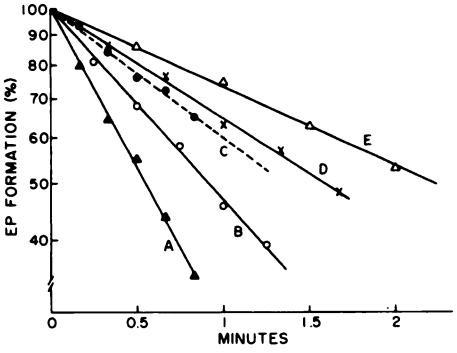


FIG. 5. *Time course of (Na⁺ + K⁺)-ATPase inhibition by cardiac aglycones*

The enzyme (40–60 μg/ml) was inhibited with various concentrations of cardiac aglycones, as indicated, in the presence of 2 mM Mg^{2+}, 2 mM P_i, and 25 mM imidazole HCl buffer (pH 7.35) at 15° (——) or 25° (- - -). At the times shown, phosphorylation was started as described in the text. Line *A*, 0.4 μM digitoxigenin; line *B*, 0.5 μM digoxigenin; line *C*, 0.2 μM digoxigenin at 25°; line *D*, 0.5 μM ouabagenin; line *E*, 0.5 μM digoxigenin, but the concentration of P_i was 0.25 mM instead of 2 mM.

+ K^+)-ATPase followed pseudo-first-order kinetics. The pseudo-first-order association rate constants (k_a') are the slopes of the time courses × 2.3. The k_a' values were affected by temperature and the concentrations of Mg^{2+}, P_i, and cardiac aglycones (Fig. 5).

Since the plots of k_a' vs. concentration of cardiac aglycone were not linear (Fig. 6), the association of cardiac aglycone does not follow a second-order rate equation. The effects of Mg^{2+} and P_i are shown as double-reciprocal plots in Fig. 7. Both Mg^{2+} and P_i gave linear relationships over a wide range of concentrations, and the effects of both ligands were identical.

As shown in Fig. 8, the k_a' values of the four cardiac aglycones are linear functions of their concentration in double-reciprocal plots, and each of the lines crosses at the same point on the *y*-axis. This shows that the maximum association rates of the four cardiac aglycones are the same.

DISCUSSION

In this study the association and dissociation rates for cardiac aglycone–enzyme complexes were obtained from assay of the protein phosphorylated by [γ-^{32}P]ATP, and only the Mg^{2+}-P_i system was used as the ligand mixture to promote the binding of cardiac aglycones.

Although no data have been published concerning the association and dissociation rates of cardiac aglycones and (Na^+ + K^+)-ATPase, it has been suggested that both rates, especially the dissociation rates, are higher than those of cardiac glycosides, because the binding of aglycones is reversible but that of glycosides is not (19). The present results not only confirm that suggestion, but also point out the characteristic differences between cardiac aglycones and glycosides.

The k_d values (dissociation rate constants) of all four cardiac aglycones examined were the same at each temperature, whereas those of the glycosides depend exclusively on the nature of the sugar moiety (6). As shown in Table 3, the k_d values of the cardiac aglycones were 10–90 times greater than those of glycosides at 25°.

In contrast, the k_a' values (pseudo-first-order association rate constants) of the cardiac aglycones were all different; they followed the same order as those of the corre-

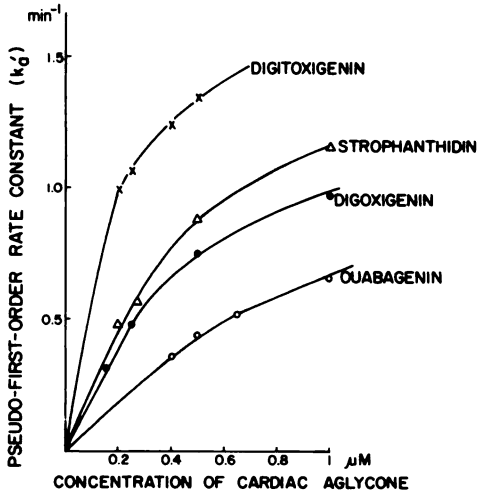


FIG. 6. *Pseudo-first-order association rate constants of various cardiac aglycones with (Na⁺ + K⁺)-ATPase at 15°*

Each value was obtained from the time course of *E*P formation as shown in Fig. 5. The reaction medium contained 2 mM Mg^{2+}, 2 mM P_i, 25 mM imidazole HCl buffer (pH 7.35), various concentrations of cardiac aglycones, and the enzyme (40–60 μg/ml).

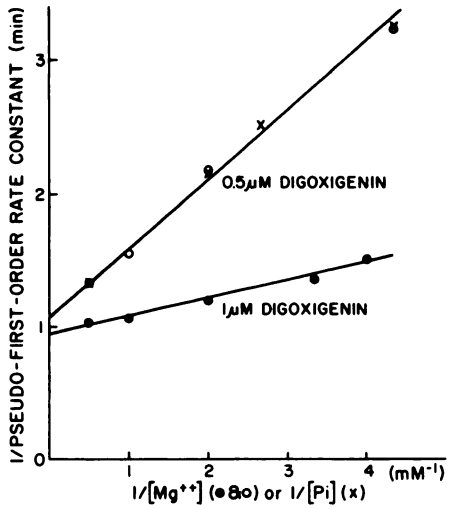


FIG. 7. *Effect of Mg^{2+} or P_i on pseudo-first-order association rate constant of (Na^+ + K^+)-ATPase inhibition by digoxigenin at 15°*

Each value was obtained from the time course of EP formation as shown in Fig. 5. ●, 1 μM digoxigenin, 2 mM P_i, and 0.25–2 mM Mg^{2+}; ○, 0.5 μM digoxigenin, 2 mM P_i, and 0.23–2 mM Mg^{2+}; ×, 0.5 μM digoxigenin, 2 mM Mg^{2+}, and 0.23–2 mM P_i. Other conditions were the same as in Fig. 5.

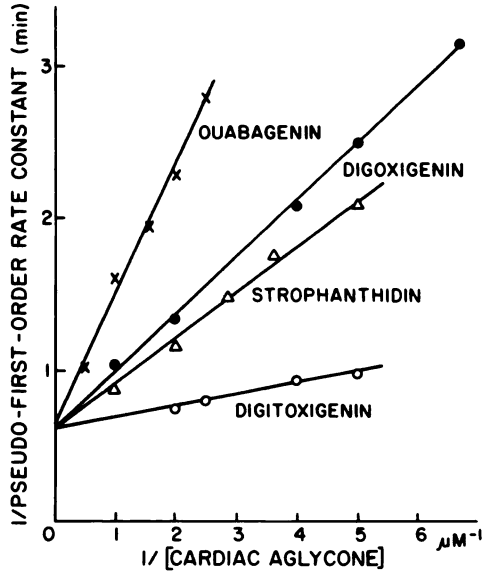


FIG. 8. *Double-reciprocal plots of pseudo-first-order association rate constants vs. concentration of cardiac aglycones*

Each value was calculated from the results shown in Fig. 6.

sponding cardiac glycosides, whose k_a' values are dependent on the nature of the aglycone moiety (7). The k_a' of digoxigenin at 25° was 0.63 min^{-1} (curve C in Fig. 5), which is 3 times the k_a' of digoxigenin monodigitoxide (6). The difference between the k_a' values of cardiac aglycones and glycosides is less than the difference in k_d values, and the increase in inhibitory potency of the cardiac monoglycosides can be attributed mainly to the decrease in k_d values.

As previously reported (6), the association of cardiac glycosides with (Na^+ + K^+)-ATPase followed second-order kinetics, but that of aglycones did not. This discrepancy may be due to the fact that only low concentrations of glycosides were examined. The double-reciprocal plots of k_a' vs. the concentration of each ligand or cardiac aglycone were linear, and the four cardiac aglycones displayed the same maximum association rates with increasing drug concentration. These results suggest that the enzyme binds the ligands to form some active intermediate as the first step, and this intermediate then binds the cardiac aglycone.

Since the intermediate formed in the presence of Mg^{2+} and P_i is not phosphorylated by ATP (17) (see also Fig. 3), the following scheme can be postulated as the association of cardiac aglycone to (Na^+ + K^+)-ATPase:

$$E \underset{k_{-1}}{\overset{k_1[Mg^{2+}][P_i]}{\rightleftharpoons}} E\cdot Mg^{2+}\cdot P_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\begin{matrix}Mg\\P\end{matrix} \xrightarrow{k_3[I]} E\begin{matrix}Mg\\P\\I\end{matrix}$$

where I is the cardiac aglycone, and $E\cdot Mg^{2+}\cdot P_i$ is the intermediate, formed possibly by noncovalent binding between the enzyme, Mg^{2+}, and P_i. In $E\begin{matrix}Mg\\P\end{matrix}$, phosphorus binds covalently, as shown by Post *et al.* (17). E and $E\cdot Mg^{2+}\cdot P_i$ are phosphorylated by [γ-^{32}P]ATP to produce EP, but $E\begin{matrix}Mg\\P\end{matrix}$ and $E\begin{matrix}Mg\\-P\\I\end{matrix}$ are not. From this scheme,

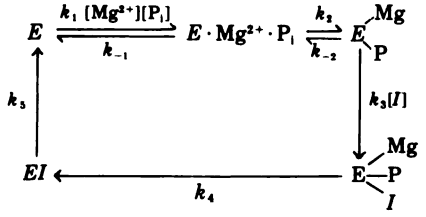
the observed pseudo-first-order rate constant k_a' is

$$\frac{1}{k_a'} = \frac{1}{k_2}\left[1 + \frac{k_{-1}}{k_1[Mg^{2+}][P_i]} + \frac{k_2}{k_3[I]} + \frac{k_{-1}k_{-2}}{k_1[Mg^{2+}][P_i]k_3[I]}\right]$$

This formula accords well with the present results (Figs. 7 and 8). This mechanism also agrees with the report by Post *et al.* (17) that $(Na^+ + K^+)$-ATPase is phosphorylated with Mg^{2+} and P_i and can then bind ouabain.

The notable finding in this study is that the dissociation rate constants of the four cardiac aglycones are the same. This result strongly suggests that the dissociation of cardiac aglycones from the enzyme follows the rate-determining step of dissociation. In such a sequence, the differences among the cardiac aglycones would not influence the dissociation rate, and a more complicated reaction step, such as a conformational change of the enzyme and/or the dissociation of ligands from the enzyme, might precede or be the rate-determining step. Post *et al.* (17) found the dephosphorylation rate of the ouabain-enzyme complex formed in the presence of Mg^{2+} and P_i to be 0.42 min^{-1} at 0°; this value is higher than the k_d of the cardiac aglycones (0.28 min^{-1} at 25°). It is likely that other slow reaction steps occur before the rate-determining step.

The following binding scheme, derived from the scheme above and the dissociation rate constants, can account for the k_a' and k_d values found for the various cardiac aglycones:



This scheme suggests that cardiac aglycone can bind to the active form of the enzyme, which is formed by covalent binding of phosphorus. However, the dissociation of the cardiac aglycones occurs after release of the phosphate. From this scheme, the following formula is obtained at equilibrium:

$$B = \frac{\frac{1}{k_4} + \frac{1}{k_5}}{\frac{1}{k_2} + \frac{1}{k_4} + \frac{1}{k_5} + \frac{1}{k_1[M]}\left(1 + \frac{k_{-1}}{k_2}\right)} N - \frac{\frac{1}{k_3} + \frac{k_{-2}}{k_2k_3} + \frac{k_{-1}k_{-2}}{k_1[M]k_2k_3}}{\frac{1}{k_2} + \frac{1}{k_4} + \frac{1}{k_5} + \frac{1}{k_1[M]}\left(1 + \frac{k_{-1}}{k_2}\right)} \frac{B}{F}$$

where $[M] = [Mg^{2+}][P_i]$, B is the total concentration of cardiac aglycone-enzyme complex ($B = [E\overset{Mg}{\underset{I}{\diagup\!-\!P\diagdown}}] + [EI]$), F is the concentration of the unbound cardiac aglycone ($F = [I]$), and N is the total concentration of the enzyme:

$$N = [E] + [E \cdot Mg^{2+} \cdot P_i] + [E\overset{Mg}{\underset{P}{\diagup\quad\diagdown}}] + [E\overset{Mg}{\underset{I}{\diagup\!-\!P\diagdown}}] + [EI]$$

This equation suggests that Scatchard plots of binding are linear, and the number of binding sites obtained by such plots changes with the concentration of ligands $[M]$. Sufficient data with which to estimate the apparent dissociation constant, which is a coefficient of B/F, are not presently available. If it could be shown that the coefficient of B/F varied little with $[M]$ compared with the coefficient of N, this reaction scheme for cardiac aglycone binding might explain the unusual observation on [^{3}H]digoxigenin binding, reported previously (8), that the apparent number of binding sites changed with the concentration of each ligand but the apparent dissociation constant did not.

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