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Structure-Activity Relationships of Cardiotonic Steroids for the Inhibition of Sodium- and Potassium-Dependent Adenosine Triphosphatase

II. Association Rate Constants of Various Enzyme–Cardiac Glycoside Complexes

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SUMMARY

YODA, ATSUNOBU, YODA, SHIZUKO, AND SARRIF, AWNI, M.: Structure-activity relationships of cardiotonic steroids for the inhibition of sodium- and potassium-dependent adenosine triphosphatase. II. Association rate constants of various enzyme–cardiac glycoside complexes. *Mol. Pharmacol.* **9**, 766–773 (1973).

The association rate constants (k_a) of cardiac glycoside–($Na^+ + K^+$)-ATPase (EC 3.6.1.3) complexes at 25° in presence of magnesium and phosphate and of sodium, magnesium, and ATP were determined by enzymatic assay after dilution. Among the various cardiac monoglycosides, k_a was dependent on the nature of the steroid moiety (aglycone) but not on the sugar moiety in both ligand systems, in contrast to the dissociation rate constants of the complexes formed in the system containing magnesium and phosphate. The order of k_a values was the same as the order of I_{50} values for different steroids in the assay system. Digitoxigenin competed with ouabain in both ligand systems. These results suggest that association reverses the sequence of the dissociation reaction, as follows: association of the steroid moiety of the cardiac glycoside with the steroid-specific site of the enzyme, conformational change of the sugar-specific site, and association of the sugar moiety with the sugar-specific site. In the case of cardiac glycosides, the number of sugars in the oligosaccharide moiety produced a corresponding bulk effect on the association rate constants in both ligand systems. For a gie the association rate constants decreasven aglyconed in the following order: monodigitoxide > bisdigitoxide > tridigitoxide > tetradigitoxide.

INTRODUCTION

Many studies have been carried out on the inhibition of ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) by cardiac steroids. From experiments using radioactive ouabain and digoxin (1–7) it has been recognized that a cardiac glycoside–enzyme complex is formed in the presence of certain ligands. However, as described in an earlier paper (8), the inhibition of ($Na^+ + K^+$)-ATPase by cardiac glycosides is far from an equilibrium or steady-state condition, and the structure-activity relationships of cardiac glycoside in-

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hibition should be re-examined by estimating the association and dissociation rate constants in the presence of suitable ligands.

The association rate of the ouabain–$(Na^+ + K^+)$-ATPase complex has been studied by kinetic analysis of the time course of ATP hydrolysis by $(Na^+ + K^+)$-ATPase (9), or by measurement of bound radioactive ouabain (10). However, the rate has not been reported for other cardiac glycosides.

In the previous paper (8), concerned with structure-activity relationship studies of cardiac glycosides, the dissociation rate constants of various cardiac glycoside–$(Na^+ + K^+)$-ATPase complexes were reported, and conclusions concerning the influence of certain functional groups on dissociation were suggested. This paper presents the association rate constants. Binding was initiated in the presence of Mg^{++} and P_i or of Na^+, Mg^{++}, and ATP, and the association rate was determined by assay of enzymatic activity after termination of the binding reaction by 10- or 20-fold dilution.

MATERIALS AND METHODS

Digitoxigenin bisdigitoxide, digoxigenin bisdigitoxide, digoxin, and digoxigenin tetradigitoxide were purchased from Boehringer/Mannheim Corporation. All other cardiotonic steroids and enzyme preparation and assay procedures used in this study were the same as those used previously (8).

Determination of association rate constant. When magnesium and phosphate were used as promoting ligands, the enzyme preparation was first incubated with $MgCl_2$, Tris-phosphate, and imidazole HCl buffer (pH 7.3). Association was initiated by the addition of cardiac glycoside. The final concentrations of the components of the association reaction were the following, unless otherwise indicated: about 0.2 mg/ml of enzyme preparation, 1 mM Tris-phosphate, 1 mM $MgCl_2$, and 20 mM imidazole HCl buffer (pH 7.3). The final volume was 0.5 ml. After suitable intervals, the interaction between glycoside and enzyme was stopped by dilution with 5.0 ml of 1 mM Tris-EDTA (pH 7.3), and 0.5 ml of the diluted solution was assayed immediately by the linked pyruvate kinase–lactate dehydrogenase spectrophotometric method (8). In each set of experiments, total incubation time (initial incubation and association reaction) was kept constant (usually 5 min unless the association was too slow). For the zero-time incubation, the cardiac glycoside solution was added after dilution with Tris-EDTA solution. All procedures were carried out at 25° ± 0.2°.

In the Na^+-Mg^{++}-ATP system the final volume of the reaction mixture was 0.25 ml, and the final concentrations of the components were: about 0.6 mg/ml of enzyme, 58 mM NaCl, 2 mM $MgCl_2$, 4 mM ATP, and 20 mM imidazole HCl buffer (pH 7.3). The reaction was started by the addition of the ATP–cardiac glycoside mixture to minimize the production of phosphate during the initial incubation. Other procedures were the same as those for the Mg^{++}-P_i system.

RESULTS

Kinetics of association of cardiac glycoside–$(Na^+ + K^+)$-ATPase complex. The over-all reaction can be shown as follows, where k_a and k_d are association and dissociation rate constants, respectively, and E, I, and EI are active enzyme, inhibitor, and the enzyme-inhibitor complex, respectively.

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

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

As described in the previous paper (8), 10-fold dilution of the reaction system reduces the association rate to less than 1%, and in the case of cardiac glycosides the dissociation reaction is slow. Therefore, after dilution the change in enzymatic activity during the assay is negligible, and only the stable complex of enzyme and cardiac glycoside remains as the inactive form of the enzyme. When the complex formed is relatively stable after dilution compared with the time required for assay, this dilution method is useful to determine the association or dissociation rate of that complex.

The inhibition by cardiac aglycones, which are reversible inhibitors (11), reached a new equilibrium corresponding to the diluted concentration of drugs after dilution by addition of Tris-EDTA (Fig. 1). The inhibi-

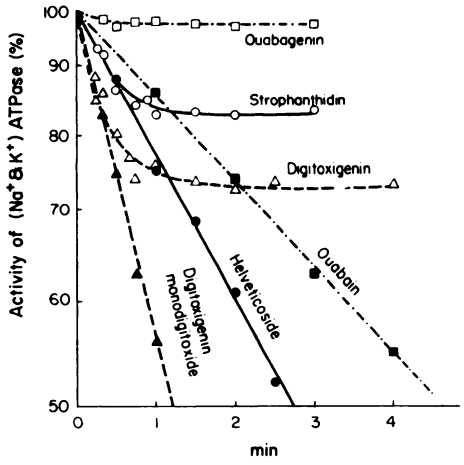

FIG. 1. *Time course of* $(Na^+ + K^+)$*-ATPase inhibition by cardiotonic steroids*

Magnesium and phosphate were used as the promoting ligands. Inhibition was studied with 0.5 μM cardiac steroid (□, ouabagenin; ○, strophanthidin; △, digitoxigenin) or 0.2 μM cardiac monoglycoside (■, ouabain; ●, helveticoside; ▲, digitoxigenin monodigitoxide). Other experimental details were the same as in the text.

tion by cardiac glycosides was not altered, and followed pseudo-first-order kinetics. Slope × 2.3 of this inhibition curve is the pseudo-first-order rate constant.

The velocity of the association of cardiac glycosides with the $(Na^+ + K^+)$-ATPase was directly proportional to their concentrations, as shown below (Figs. 4 and 5). Therefore the kinetics of association can be given by Eq. 1, where v_a is the association rate, k_a is the second-order rate constant for association, $[E_a]$ is the concentration of unbound (active) enzyme, $[I]$ is the concentration of cardiac glycoside, and $k_a[I]$ is the pseudo-first-order rate constant when the concentration of inhibitor is $[I]$.

$$v_a = k_a[I][E_a] \qquad (1)$$

Effect of ligands on association rate. The association rate was dependent on the concentration of each ligand (Figs. 2 and 3). These ligands probably exert their effect on the enzyme rather than on the glycoside, because there was no significant difference in the relative rates of association of ouabain and digitoxigenin monodigitoxide. In agreement with our earlier qualitative results (11), Na^+ and ATP or Mg^{++} and ATP promoted the association of cardiac glycosides with the enzyme, but Mg^{++} alone or together with Na^+ did not. By contrast, others (3, 12) have found the binding of radioactive ouabain to be stimulated by Mg^{++} alone but not by Na^+ and ATP alone. The reason for these differences is obscure at this time.

Association rates of various cardiac monoglycosides with $(Na^+ + K^+)$*-ATPase.* The pseudo-first-order rate constants $(K_a[I])$ of different cardiac monoglycosides in the Mg^{++}-P_i system at 25°, as a function of the concentration curves of each compound, are shown in Fig. 4. They are straight lines and intersect the zero point, as predicted by second-order kinetics. The slope of each curve gives k_a. The variation of k_a between glycosides with the same sugar moiety was much larger than that between glycosides with the same steroid moiety [see Table 1 of the previous paper (8)]. Therefore it can be concluded that k_a is exclusively dependent on the steroid portion of cardiac monoglycosides and independent of the sugar portion. The order of k_a of each glycoside parallels the I_{50} value of each corresponding aglycone [see Table 2 of the previous paper (8)].

In the Na^+-Mg^{++}-ATP system the over-all $K_a[I]$ value of each cardiac glycoside with $(Na^+ + K^+)$-ATPase was qualitatively the same as in the Mg^{++}-P_i system (Fig. 5); that is, k_a was dependent on the nature of the steroid portion of the cardiac glycosides. However, the k_a value of each cardiac monoglycoside in the Na^+-Mg^{++}-ATP system was lower than the corresponding value in the Mg^{++}-P_i system (Table 1).

Interaction between digitoxigenin and ouabain. Since cardiac aglycones are reversible inhibitors of $(Na^+ + K^+)$-ATPase (11), and their inhibition during assay can be minimized by dilution as shown in Fig. 1, interaction between cardiac glycosides and cardiac aglycones might be predicted if binding of the steroid moiety is the essential reaction in the cardiac glycoside binding sequence. The interaction between ouabain, the least active glycoside used here (Table 1), and digitoxigenin, the most active aglycone [Table 2 of the previous paper (8)], is plotted in

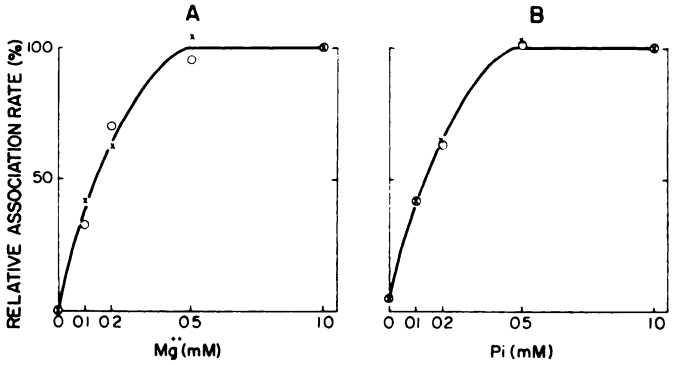

FIG. 2. *Effects of magnesium (A) and phosphate (B) on association rate of ouabain or digitoxigenin monodigitoxide with $(Na^+ + K^+)$-ATPase in the Mg^{++}-P_i system*

Phosphate (1 mM) and various concentrations of magnesium (A) or 1 mM magnesium and various concentrations of phosphate (B) were used as ligands. Inhibitors were 0.4 μM ouabain (○) or 0.1 μM digitoxigenin monodigitoxide (×). Other experimental conditions were the same as in Fig. 1. Percentages of relative association rate were normalized by taking the association rate of each inhibitor at the saturated ligand concentration (1 mM magnesium and 1 mM phosphate) as 100%. In absolute terms 100% corresponds to 0.31 min^{-1} for 0.4 μM ouabain and to 0.28 min^{-1} for 0.1 μM digoxigenin monodigitoxide.

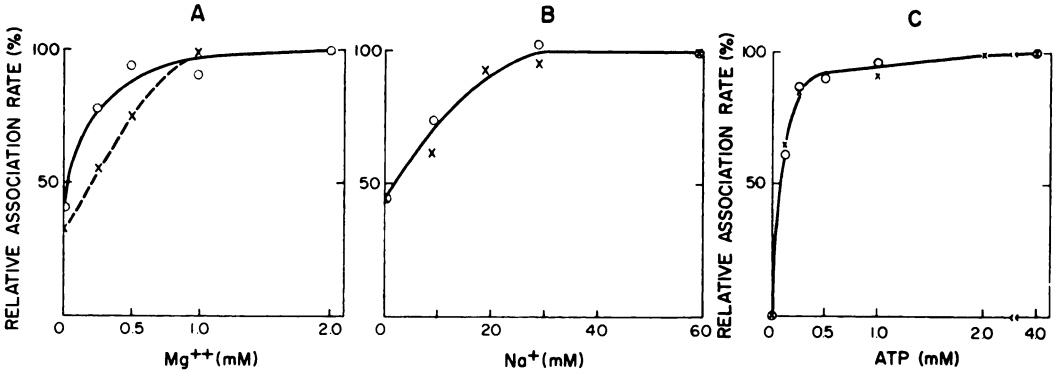

FIG. 3. *Effects of magnesium (A), sodium (B), and ATP (C) on association rate of ouabain or digitoxigenin monodigitoxide with $(Na^+ + K^+)$-ATPase in the $Na^+ - Mg^{++}$-ATP system*

Unless otherwise shown, 58 mM sodium, 2 mM magnesium, and 4 mM ATP were used in each experiment. As the inhibitor, 0.5 μM ouabain (○) or 0.2 μM digitoxigenin monodigitoxide (×) was added to the reaction mixture. Other experimental conditions were the same as in Fig. 2. In absolute terms 100% relative association rate corresponds to 0.33 min^{-1} for 0.5 μM ouabain and to 0.28 min^{-1} for 0.2 μM digitoxigenin monodigitoxide.

Figs. 6 and 7. In both the Mg^{++}-P_i and Na^+-Mg^{++}-ATP systems digitoxigenin antagonized the inhibition by ouabain.

Association rates of cardiac oligosaccharides. In the two series of cardiac digitoxides containing the same aglycones, the $K_a[I]$ value decreased in the order mono-, di-, and tridigitoxides (Figs. 8 and 9). The magnitude of the changes in each series of digitoxides varied with the nature of the aglycone or with the ligand system, but the order of the association rates in each series was consistent.

DISCUSSION

In the previous paper (8) it was shown that the dissociation rate constant of the cardiac monoglycoside–$(Na^+ + K^+)$-ATPase complex formed in the presence of Mg^{++} and phosphate was dependent on the nature of the sugar moiety. However, our present study of the association rate of nine cardiac

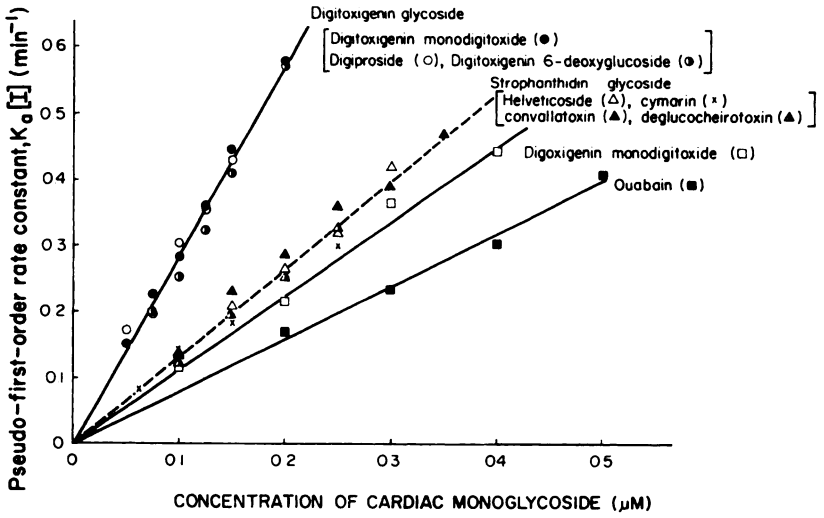

FIG. 4. *Pseudo-first-order association rate constants of various cardiac monoglycosides with* $(Na^+ + K^+)$-*ATPase in the* Mg^{++}-P_i *system*

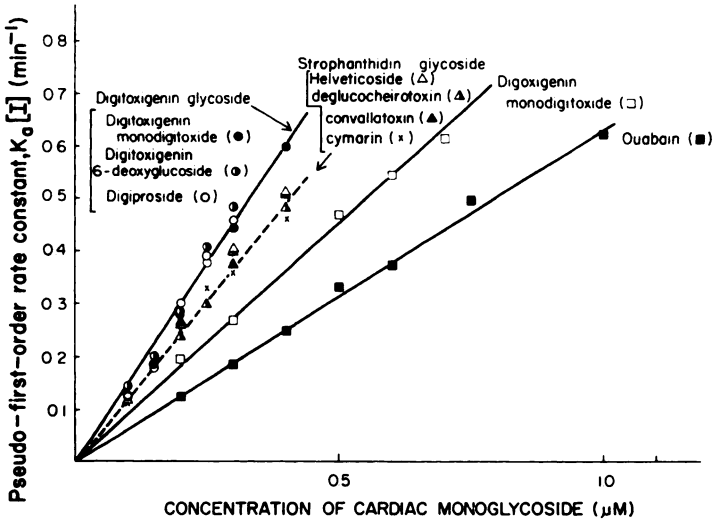

FIG. 5. *Pseudo-first-order association rate constants of various cardiac monoglycosides with* $(Na^+ + K^+)$-*ATPase in the* Na^+-Mg^{++}-*ATP system*

monoglycosides with the enzyme in two ligand systems (Mg^{++} and P_i ; Na^+, Mg^{++}, and ATP) shows that association follows second-order kinetics and that the association rate constant is dependent on the nature of the steroid moiety, in contrast to dissociation. This is the salient difference between association and dissociation reactions.

The association rate constants for a series of cardiac monoglycosides bore the same relationships with each other whether association was carried out in the Mg^{++}-P_i system or in the Na^+-Mg^{++}-ATP system. However, the absolute values of the association rate constants obtained in the Mg^{++}-P_i system were larger than those obtained in the Na^+-Mg^{++}-ATP system. This finding contradicts the result for ouabain obtained by Van Winkel *et al.* (13) but is in accord with the report of Taniguchi and Iida (14). The dis-

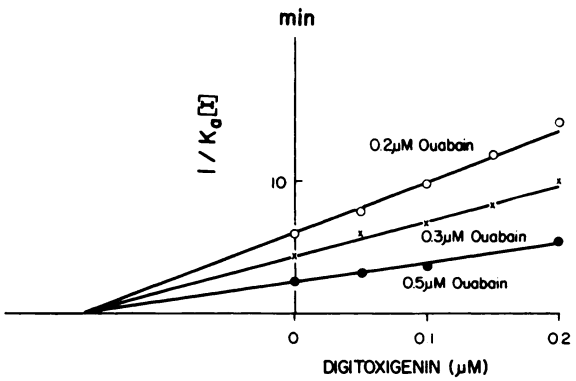

FIG. 6. *Interaction of ouabain inhibition with digitoxigenin in the Mg^{++}-P_i system*

Association was carried out in the presence of 20 mM imidazole HCl (pH 7.3), ouabain and digitoxigenin as indicated, 1 mM $MgCl_2$, and 1 mM Tris-phosphate. After termination by 20-fold dilution with 1 mM Tris-EDTA, the enzymatic activity was assayed. The abscissa shows the reciprocal value of the pseudo-first-order rate constant of ouabain with the enzyme.

TABLE 1

Second-order association rate constants of cardiac monoglycosides at 25°

In the Mg^{++}-P_i system both components were 1 mM; in the three-ligand system the concentrations were: Na^+, 58 mM; Mg^{++}, 2 mM; ATP, 4 mM.

Cardiac glycoside	Mg^{++}-P_i system	Na^+-Mg^{++}-ATP system
	$\mu M^{-1}\ min^{-1}$	$\mu M^{-1}\ min^{-1}$
Digitoxigenin monodigitoxide	2.9 ± 0.1	1.4 ± 0.2
Digitoxigenin 6-deoxyglucoside	2.6 ± 0.3	1.5 ± 0.2
Digiproside	2.8 ± 0.2	1.5 ± 0.2
Helveticoside	1.3 ± 0.1	1.3 ± 0.1
Convallatoxin	1.4 ± 0.1	1.2 ± 0.1
Cymarin	1.3 ± 0.3	1.2 ± 0.1
Deglucocheirotoxin	1.3 ± 0.1	1.2 ± 0.1
Digoxigenin monodigitoxide	1.2 ± 0.1	0.90 ± 0.08
Ouabain	0.80 ± 0.06	0.64 ± 0.02

crepancy might be the result of different experimental conditions, especially the reaction temperature, and/or the concentrations of ligands.

The large differences in association rates of cardiac monoglycosides and oligosaccharides might be explained by the large difference in molecular weight between them, i.e., the bulk effect of the sugar moiety. As the molecular weight varied little among the monoglycosides used here, the bulk effect of each sugar moiety might be negligible, and the effect of the aglycone moiety would be predominant.

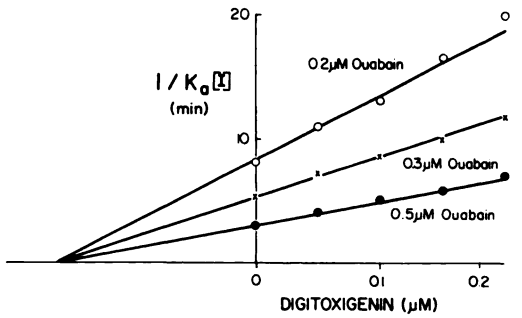

FIG. 7. *Interaction of ouabain inhibition with digitoxigenin in the Na^+-Mg^{++}-ATP system*

All experimental conditions were the same as in Fig. 6, except that 58 mM sodium, 2 mM magnesium, and 4 mM ATP were used as ligands instead of 1 mM magnesium and 1 mM phosphate.

In the previous paper (8) the following reaction sequence was suggested for the dissociation of the cardiac monoglycoside–$(Na^+ + K^+)$-ATPase complex: dissociation of the sugar portion of the cardiac glycoside from the sugar site of the enzyme, a conformational change of the sugar site from an active to an inactive form, and dissociation

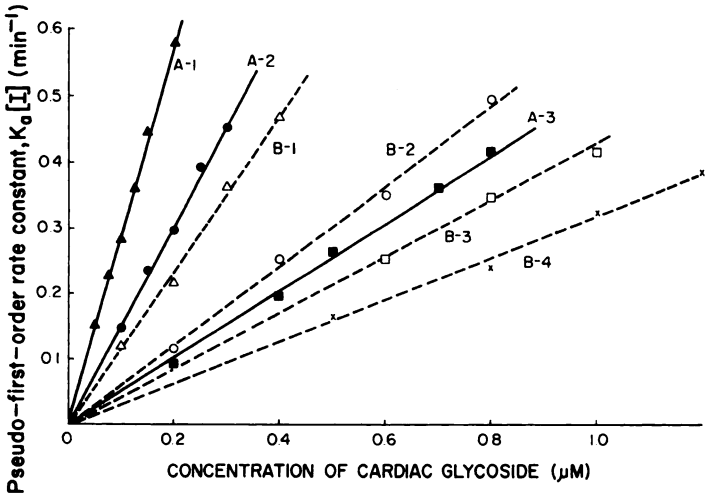

FIG. 8. *Pseudo-first-order rate constants of various digitoxigenin and digoxigenin digitoxides in the Mg^{++}-P_i system*

Curve *A-1*, digitoxigenin monodigitoxide; *A-2*, digitoxigenin bisdigitoxide; *A-3*, digitoxin; *B-1*, digoxigenin monodigitoxide; *B-2*, digoxigenin bisdigitoxide; *B-3*, digoxin; *B-4*, digoxigenin tetradigitoxide.

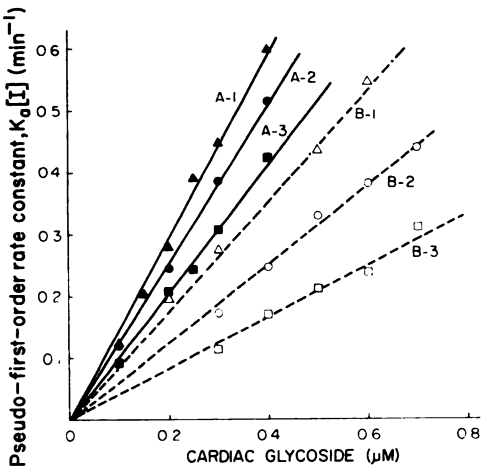

FIG. 9. *Pseudo-first-order rate constants of various digitoxigenin and digoxigenin digitoxides in the Na^+-Mg^{++}-ATP system*

Curve *A-1*, digitoxigen monodigitoxide; *A-2*, digitoxigenin bisdigitoxide; *A-3*, digitoxin; *B-1*, digoxigenin monodigitoxide; *B-2*, digoxigenin bisdigitoxide; *B-3*, digoxin.

of the steroid portion from the steroid site. The results in the present paper indicate that the association rate constant of the enzyme–cardiac glycoside complex is dependent on the nature of the steroid moiety and not on the sugar moiety. The association step between the steroid moiety and the steroid-specific site of the enzyme must precede the rate-determining step or be the rate-determining step itself, and the association step between the sugar moiety and the sugar-specific site of the enzyme must follow the rate-determining step. Although no information was available about the rate-determining step of association or the conformational change of the sugar site during association, it is likely that association reverses the sequence for the dissociation reaction, i.e., (a) reversible binding of the steroid to its receptor site on the (Na^+ + K^+)-ATPase, (b) a conformational change of the sugar site from inactive to active, and (c) binding of the sugar moiety to its receptor site. The kinetic results shown in Figs. 6 and 7 do not permit any conclusion as to whether the interaction of ouabain association with digitoxigenin is competitive or noncompetitive, because the maximum velocity of enzyme inhibition by ouabain is very high. The antagonism of digitoxigenin, however, is consistent with the above association mechanism.

While this manuscript was in preparation, Lindenmayer and Schwartz (10) published second-order association rate constants for ouabain at 30° and 37°, as well as a formu-

lation for the sodium and potassium effects in the Na^+-Mg^{++}-ATP system by following binding of radioactive ouabain to the enzyme. From their data it is possible to calculate the association rate constant of ouabain under conditions used in this paper, assuming that association follows the Arrhenius equation. This calculated value is 0.65 μM^{-1} min^{-1}, and agrees with our value of 0.64 μM^{-1} min^{-1} (Table 1).

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