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

I. Dissociation Rate Constants of Various Enzyme–Cardiac Glycoside Complexes Formed in the Presence of Magnesium and Phosphate

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SUMMARY

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The dissociation rate constants (k_d) of cardiac monoglycoside–($Na^+ + K^+$)-ATPase complexes in the presence of magnesium and inorganic phosphate were determined by enzymatic assay after dilution. Among various cardiac monoglycosides, k_d was dependent on the nature of the sugar and the temperature but not on the steroid. The 3′-hydroxyl and the 5′-α-methyl group of the sugar markedly influenced the stability of the complex. The order of stability of cardiac monoglycoside–($Na^+ + K^+$)-ATPase complexes is the following: L-rhamnoside > D-6-deoxyguloside ≒ D-digitoxide > D-fucoside ≒ D-6-deoxyglucoside. Methylation or acetylation of the 3′-hydroxyl group decreased this stability. These data indicate a sugar site on the enzyme, and they suggest that the 3′-hydroxyl is bound to two groups at this site, a proton-donating group and a proton-accepting group. The 3′-α-hydroxyl binds to either group, but the 3′-β-hydroxyl and the 3′-α-methoxyl bind only a proton-accepting group and a proton-donating group, respectively. The activation energy of this dissociation was rather constant (20 kcal/mole) with various cardiac monoglycosides. The rate-determining step of the dissociation might be a conformational change of the enzyme, and it is suggested that the reaction order is the following: dissociation of the sugar portion of the cardiac glycoside from the sugar site of enzyme, conformational change of the sugar site, and dissociation of the steroid portion from the steroid site.

INTRODUCTION

The cardioactive steroids exert a specific and powerful cardiotonic action on heart muscle and have been successfully used in the treatment of heart failure. These compounds are also specific inhibitors of sodium and potassium transport and the membrane-bound ($Na^+ + K^+$)-dependent adenosine triphosphatase (EC 3.6.1.3), which is believed to be an integral part of this transport

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process (1). This inhibition has attracted considerable attention not only because of its therapeutic implications (2) but also because it serves as an excellent model for the study of drug-receptor interaction at the molecular level. Experiments using radioactive digoxin and ouabain (2–8) have shown that the enzyme–cardiac glycoside complex is formed in the presence of certain ligands. It has been suggested that the phosphoenzyme is one form to which cardiac glycosides will bind, but recently evidence has been presented that the nonphosphorylated enzyme can bind cardiac glycosides under appropriate conditions (4, 8).

There has been some controversy concerning the stability of the enzyme–cardiotonic steroid complex. Recent studies indicate that the stability of the ouabain-enzyme complex is dependent on temperature (9, 10), associated ligands (8, 10), and species of the enzyme (2, 11, 12). In a previous paper (13) we showed that the binding of all cardenolide aglycones to $(Na^+ + K^+)$-ATPase was reversible, but the binding of their glycosides was irreversible at 0°, based on recovery of enzymatic activity after dilution.

Extensive studies of structure-activity relationships of cardiotonic steroids have been carried out by toxicity assay (14–18). Similar studies of the inhibition of $(Na^+ + K^+)$-ATPase have been carried out and have revealed that in many cases the ability to inhibit the enzyme activity qualitatively paralleled cardiotonic activity and toxicity (19–21). It has been recognized that the 14-β-hydroxyl and the 17-β-unsaturated lactone were the essential groups of the cardiac steroid for inhibition of $(Na^+ + K^+)$-ATPase. The effects of other functional groups on the steroid and sugar portions have been studied, and it has been claimed on the basis of toxicity that the monoglycoside is more potent than the aglycone or di- or triglycosides. Wilson *et al.* (21) presented a model for the sugar-binding site of $(Na^+ + K^+)$-ATPase as well as the steroid site.

Structure-activity studies on the inhibition of $(Na^+ + K^+)$-ATPase by cardioactive steroids has been based on estimation of K_i or I_{50} values. However, as shown in our previous paper (13), the inhibition of $(Na^+ + K^+)$-ATPase by glycosides might be irreversible under the usual assay conditions and might be far from the *equilibrium* or *steady state*. Therefore the structure-activity relationships of the glycosides should be re-examined by estimation of the association rate constant and the dissociation rate constant instead of the apparent K_i or I_{50}.

Another drawback of the use of K_i is that the ligands used for binding are restricted to those required for assay, although, as has been shown in the binding studies of radioactive ouabain, other ligands or combinations of ligands different from those used for assay also are very effective in promoting binding of the cardiac glycosides to the enzyme (3, 4, 7, 9, 22). With these latter ligands the dilution method, as described here, has proved to be very useful. This is based on the fact that the enzyme–cardiac glycoside complex remains rather stable after dilution and during assay.

In this study the stability of the binding between $(Na^+ + K^+)$-ATPase and cardiac monoglycosides has been studied by following the change of enzyme activity at various temperatures after dilution of the enzyme which had been incubated with various cardiac monoglycosides. For technical reasons binding was promoted only in the presence of magnesium and inorganic phosphate as ligands, because other systems are more complicated.

MATERIALS AND METHODS

Cardiotonic steroids. The homogeneity of all cardiotonic steroids used in this study was examined by silica gel thin-layer chromatography. The infrared and NMR[1] spectra were determined on all prepared compounds, and the results coincided with the predicted spectra.

Digitoxigenin 6-deoxy-D-glucoside, digoxigenin, digoxigenin monodigitoxide, and helveticoside were purchased from Boehringer Mannheim Corporation. Convallatoxin and ouabain were obtained from Aldrich Chemical Company and Nutritional Biochemicals, respectively. Digitoxin was the kind gift of Eli Lilly and Company and Wyeth Labora-

[1] NMR spectra were obtained by Mr. Jim Munson, School of Pharmacy, University of Wisconsin. The author thanks him.

tories. Deglucocheirotoxin was the kind gift of Dr. T. Reichstein (Institute of Organic Chemistry, University of Basel, Switzerland). Odoroside H, digiproside, and triacetyldigiproside were kindly provided by Dr. D. Sato and Dr. Y. Nozaki (Shionogi and Company, Osaka, Japan).

Digitoxigenin was prepared by acid hydrolysis of digitoxin (23). Cymarin was prepared by the enzymatic hydrolysis of strophanthidin (24) with Clarase (Miles Laboratories). The above two products were identical with the authentic samples based on melting points, thin-layer chromatography, and ultraviolet and infrared spectra.

Digitoxigenin monodigitoxide was prepared by the partial hydrolysis of digitoxin (25) and purified by silicic acid column chromatography: m.p. 182°; λ_{max} (methanol) = 217 nm; $\log \epsilon = 4.22$ [reported m.p. 181–184° (25), 197–200°; (26); λ_{max} (ethanol) = 218 nm; $\log \epsilon = 4.20$ (25, 26)].

$C_{29}H_{44}O_7$
Calculated: C 69.02, H 8.79
Found: C 69.41, H 9.06

Acetylation of cymarin, convallatoxin, and digitoxigenin monodigitoxide was done with pyridine and acetic anhydride, and the products were purified by recrystallization. Digitoxigenin monodigitoxide diacetate: m.p. 209–210°; λ_{max} (ethanol) = 218 nm; $\log \epsilon = 4.20$.

$C_{33}H_{48}O_9$
Calculated: C 67.33, H 8.22
Found: C 66.87, H 8.08

Acetylcymarin: m.p. 173–174°/203° [reported m.p. 170–177° (27)].

$C_{32}H_{46}O_{10} \cdot H_2O$
Calculated: C 63.14, H 7.94
Found: C 63.52, H 8.03

Triacetylconvallatoxin: m.p. 228° [reported m.p. 231–234° (28)]; λ_{max} (methanol) = 217 nm: $\log \epsilon = 4.22$.

$C_{35}H_{48}O_{13}$
Calculated: C 62.12, H 7.15
Found: C 61.93, H 6.92

Digitoxigenin 3-tretrahydropyranyl ether was prepared from digitoxigenin and dihydropyran by a slight modification of the method for strophanthidin 3-tetrahydropyranyl ether (29) and purified by alumina column chromatography. The melting point was 154–155°; λ_{max} (methanol) = 216 nm; $\log \epsilon = 4.18$.

$C_{28}H_{42}O_5$
Calculated: C 73.31, H 9.23
Found: C 72.92, H 8.99

From the NMR spectrum ($CDCl_3$) it appeared that this preparation contained only the isomer which has the ether bond equatorial to the tetrahydropyranyl ring. The chemical relations of the cardiac monoglycosides used here and the structures of each aglycone and sugar are shown in Tables 1 and 2 and Fig. 1.

Enzyme preparation and assay. Sodium iodide-treated microsomes were prepared from beef brain by the method of Nakao *et al.* (31). The specific activity of the $(Na^+ + K^+)$-ATPase was 50–60 μmoles of P_i liberated per milligram of protein per hour, and more than 95% of the ATPase activity was ouabain-sensitive.

Disodium ATP was purchased from P-L Biochemicals. Tricyclohexylammonium phosphoenolpyruvate, NADH, pyruvate kinase, and rabbit muscle lactate dehydrogenase were obtained from Sigma Chemical Company. Lactate dehydrogenase and pyruvate kinase suspensions were centrifuged to remove most of the ammonium sulfate and were dissolved in 0.5 M imidazole-HCl buffer (pH 7.3). They were mixed together at a final concentration of 1 mg/ml each.

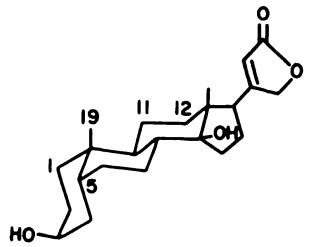
The activity of $(Na^+ + K^+)$-ATPase was assayed by the linked pyruvate kinase–lactate dehydrogenase spectrophotometric method (5, 7, 32). This procedure measures formation of ADP by the hydrolysis of ATP (33). The final concentrations in the assay medium were 100 mM NaCl, 10 mM KCl, 2.5 mM $MgCl_2$, 1 mM ATP, 1 mM phosphoenolpyruvate, about 0.15 mM NADH, about 50 μg/ml of lactate dehydrogenase, about 50 μg/ml of pyruvate kinase, and 30 mM imidazole-HCl buffer (pH 7.3). The hydrolysis of ATP by $(Na^+ + K^+)$-ATPase was followed by measuring the decrease in optical density at 340 nm. A Cary 14 spectrophotom-

TABLE 1

Structures of cardiac monoglycosides used in this study

Compound	Aglycone	Sugar	Glycoside bond
Ouabain	Ouabagenin	L-Rhamnose	α
Convallatoxin	Strophanthidin	L-Rhamnose	α
Helveticoside	Strophanthidin	D-Digitoxose	β
Digitoxigenin monodigitoxide	Digitoxigenin	D-Digitoxose	β
Digoxigenin monodigitoxide	Digoxigenin	D-Digitoxose	β
Deglucocheirotoxin	Strophanthidin	D-6-Deoxygulose	β
Digiproside	Digitoxigenin	D-Fucose	β
Digitoxigenin 6-deoxyglucoside	Digitoxigenin	D-6-Deoxyglucose	β
Cymarin	Strophanthidin	D-Cymarose (3'-methoxy-D-digitoxose)	β
Odoroside H	Digitoxigenin	D-Digitalose (3'-methoxy-D-fucose)	β

TABLE 2



Structures and I_{50} values of aglycones which form steroid moieties of glycosides used in this study

Compound	Group	Substitution	I_{50}[a]
			μM
Digitoxigenin	19-CH_3		0.15
Digoxigenin	19-CH_3	12-β-OH	0.40
Strophanthidin	19-CHO	5-β-OH	0.30
Ouabagenin	19-CH_2OH	1-β-OH, 5-β-OH, 11-β-OH	2.3

[a] These data are taken from a previous paper (13), except in the case of digoxigenin.

eter with a slide wire range of 0–0.1 absorbance unit was used. The temperature of the assay medium was controlled by circulating water at constant temperature. The slope of the optical density change was proportional to the amount of ATPase added. The rapidity of this assay procedure, compared to the usual inorganic phosphate assay method, is of considerable advantage.

Determination of dissociation rate constant. Between 0.1 and 0.6 mg of enzyme preparation was incubated in a final volume of 0.5 ml containing 0.2–1.0 μM cardiac glycoside, 1 mM $MgCl_2$, 1 mM Tris-phosphate, and 10 mM imidazole-HCl buffer (pH 7.3). After 2–10 min of incubation the interaction between glycoside and enzyme was stopped by dilution with 5 ml of 1 mM Tris-EDTA (pH 7.3), and the incubation was continued in order to measure dissociation.[2] During this period 0.5-ml aliquots were taken at suitable intervals and enzymatic activities were assayed by the above method. All procedures were carried out at the same temperature (±0.2°), using a Haake model FK constant-temperature circulator, and all solutions were first heated to the same temperature. As a control, dilution was made before the addition of inhibitor. The reduction of the enzyme activity in the control experiment was less than 10% below 25° and about 15% at 40°.

RESULTS

Kinetics of dissociation of cardiac glycoside–($Na^+ + K^+$)-ATPase complex. In the enzyme-inhibitor system in which the inhibitor

[2] The concentration of the enzyme was selected to be suitable for assay. The concentration of inhibitor and the incubation time were also selected so that inhibition would be more than 50% and dissociation of the enzyme-inhibitor complex would be favored.

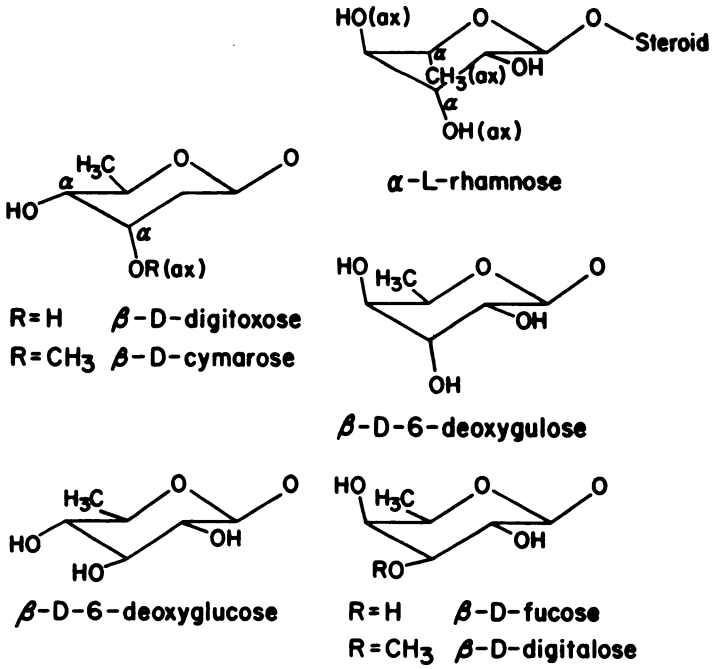


FIG. 1. *Structures of sugars listed in Table 1*
These conformations are those of Klyne (30).

is not saturated, dilution reduces the association rate more than the dissociation rate, and the equilibrium of the system shifts to reduction of inhibition. This is because the association reaction is bimolecular but dissociation is unimolecular. The over-all reaction can be shown as follows, where k_1 and k_2 are association and dissociation rate constants, respectively, and E, I, and EI are active enzyme, inhibitor, and the enzyme-inhibitor complex, respectively.

$$E + I \xrightarrow{k_1} EI \qquad (a)$$

$$EI \xrightarrow{k_2} E + I \qquad (b)$$

Therefore

$$v_{obs} = k_2(EI) - k_1(E)(I) \qquad (1)$$

A 10-fold dilution of the reaction system reduces $k_2(EI)$, the first term of Eq. 1, by 10%, and $k_1(E)(I)$, the second term, by 1%. Therefore dissociation of the enzyme-inhibitor complex is favored after dilution. Also, reaction a is dependent on ligands (Mg^{2+} and P_i), and their dilution will cause even further reduction of the second term of Eq. 1. Inhibition in the control experiment was started after dilution; i.e., (EI) was zero and v_{obs} was almost equal to the second term of Eq. 1. Less than 15% of the inhibition occurred during the total incubation time under these conditions. Since the initial concentration of (EI) is higher than that of (E) upon dilution, the value of $k_1(E)(I)$ might be much less than that observed in the control experiment. Therefore the recovery of enzymatic activity after dilution should follow first-order kinetics derived from reaction b. The kinetic is given by Eq. 2, where k_d is the dissociation rate constant, (E_a) is the concentration of active enzyme, and (E_c) is the concentration of active enzyme in the control experiment.

$$v = k_d\left[1 - \frac{(E_a)}{(E_c)}\right] \qquad (2)$$

As shown in Fig. 2, the recovery of the enzyme activity is first-order and follows Eq. 2. As shown in Table 3, k_d was not dependent on the initial percentage of inhibition, the incubation time, or the concentra-

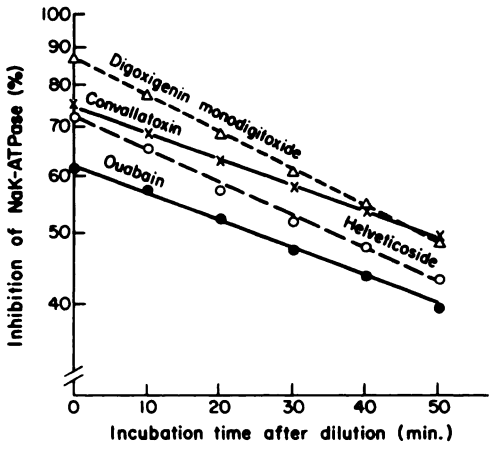


FIG. 2. *Stability of cardiac monoglycoside-($Na^+ + K^+$)-ATPase complexes after dilution*

Enzyme, 1 mM $MgCl_2$, 1 mM Tris-phosphate, and 0.2 μM cardiac monoglycoside were incubated for 3–5 min at 30°. The reaction was stopped by 10-fold dilution with 1 mM Tris-EDTA solution, and enzyme inhibition was determined at 10-min intervals.

TABLE 3

Effect of treatment with ouabain under various conditions on dissociation rate constant (k_d)

Conditions of ouabain treatment[a]		Initial inhibition	k_d
Concentration of ouabain	Incubation time		
μM	*min*	%	hr^{-1}
1.0	5	89	0.43
1.0	2	73	0.42
0.2	10	72	0.42
0.2	5	62	0.46
0.2	2	50	0.49

[a] The temperature was 30°, and other experimental conditions were the same as in Fig. 2.

tion of the inhibitor during the inhibition period, as expected from first-order kinetics.

The initial inhibition by ouabain after dilution was dependent not only on the concentration of the inhibitor but also on the inhibition period (Table 3). This phenomenon indicates that the inhibition by ouabain is far from the usual equilibrium state, as already shown in other systems (2, 5, 33). When the inhibition is reversible, the reaction system reaches the new equilibrium rapidly after dilution. Therefore (E_c) is practically equal to (E_a).

Effects of various aglycone moieties on k_d as a function of temperature. As shown in Fig. 3, the rhamnosides, ouabain and convallatoxin, gave almost identical k_d values at various temperatures, and all monodigitoxides, namely, digitoxigenin monodigitoxide, digoxigenin monodigitoxide, and helveticoside, gave identical k_d values. The glycosides in each of these two groups have a common sugar—L-rhamnose and D-digitoxose, respectively—but the aglycones are different. The activities of the aglycones were not the same, as shown in Table 2, and their inhibition was reversible. Therefore it may be concluded that k_d was dependent exclusively on the sugar portion of cardiac monoglycoside, and independent of the aglycone.

Effects of various sugar moieties on k_d as a function of temperature. Digiproside and digitoxigenin D-6-deoxyglucoside are 4′-epimers, and the k_d values of these glycosides were almost the same (Fig. 4). Not only are the 4′-hydroxyl groups opposite in deglucocheirotoxin and helveticoside, but there is an additional 2′-α-hydroxyl in the former. However, the difference in k_d was small (Fig. 4), and therefore the configurational change in

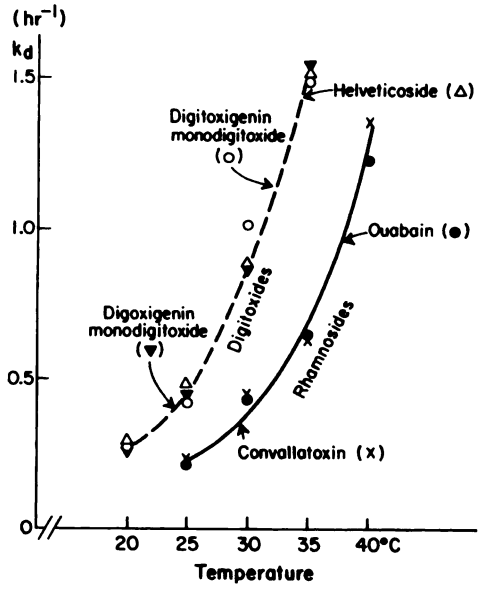


FIG. 3. *Dissociation rate constants (k_d) of digitoxides and rhamnosides at various temperatures*

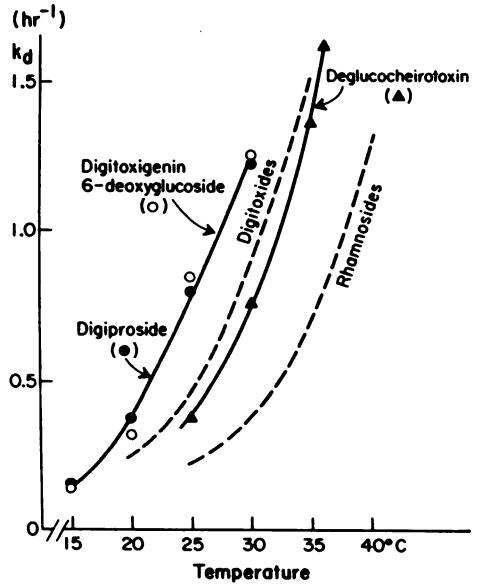


FIG. 4. *Dissociation rate constants (k_d) of deglucocheirotoxin, digiproside, and digitoxigenin 6-deoxyglucoside at various temperatures*

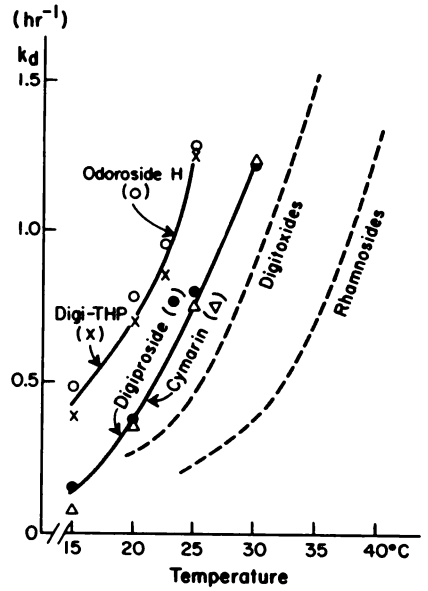


FIG. 5. *Dissociation rate constants (k_d) of methoxyglycosides and digitoxigenin 3-tetrahydropyranyl ether (Digi-THP) at varous temperatures*

in the 4′-hydroxyl and the introduction of a 2′-α-hydroxyl group probably do not affect k_d.

Deglucocheirotoxin is the 5′-epimeride of convallatoxin, but the change in the 5′-methyl group from α (D series) to β (L series) increased the k_d value almost to that of the digitoxides (Fig. 4).

The sugar portions of digiproside and deglucocheirotoxin (D-fucose and D-6-deoxygulose) are 3′-epimers, and there was some difference in k_d (Fig. 4). If the 3′-hydroxyl group of the sugar was changed to a methoxyl group, k_d was increased, as can be seen by comparing cymarin (3′-methoxydigitoxide) and odoroside H (3′-methoxydigiproside) (Fig. 5). Thus k_d was altered in the following order on changing the substituent at the 3′-position: α-OH < α-OCH_3 = β-OH < β-OCH_3.

The 3-tetrahydropyranyl ether of digitoxigenin, which is considered to be the 2,3,4-trideoxypyranoside (no hydroxyl group in the sugar), had almost the same k_d as odoroside H (Fig. 5). Therefore it may be presumed that the 2′-α-hydroxyl, 3′-β-methoxyl, 4′-β-hydroxyl, and 5′-β-methyl groups in the digitalose portion of odoroside H have no effect on the stability of the $(Na^+ + K^+)$-ATPase–cardiac glycoside complex. It would thus appear that only the 3′-hydroxyl group (3′-α-OH and 3′-β-OH), the 3′-methoxyl group (3′-α-OCH_3 only), and the 5′-α-methyl group (L-6′-CH_3) of the sugar portion were able to bind specifically with the ATPase.

Effect of acetylation of sugar hydroxyls on k_d. As shown in Fig. 6, acetylation of sugar hydroxyl groups increased k_d in every case, but the extent of this change was variable. If only the 4′-hydroxyl group of cymarin was acetylated, the increase in k_d was small, but if the 3′-α-hydroxyl of convallatoxin or digitoxigenin monodigitoxide was acetylated, the change was very large, and the inhibition became reversible on dilution. The I_{50} values of the acetylated compounds determined by our previous method (13) were 12 μM and 10 μM, respectively. However, acetylation of the 3′-β-hydroxyl group did not cause so large an increase in k_d as that of the 3′-α-hydroxyl, as exemplified by acetylation of digiproside.

Arrhenius activation energy of dissociation of cardiac monoglycoside–$(Na^+ + K^+)$-ATPase complexes. The Arrhenius activation energy of the dissociation of each cardiac glycoside–$(Na^+ + K^+)$-ATPase complex was

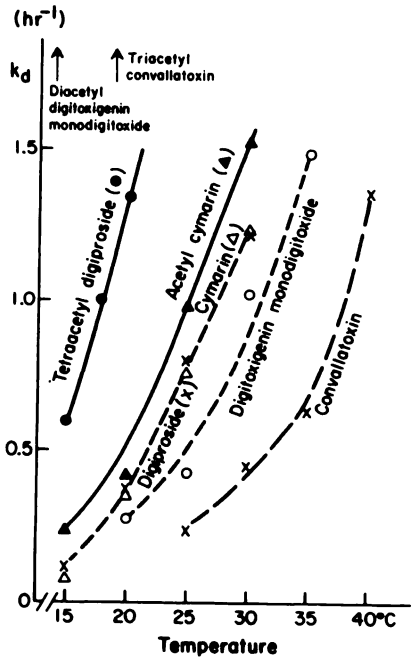


FIG 6. *Effects of acetylation of cardiac glycosides on dissociation rate constants (k_d)*

TABLE 4

Activation energy of dissociation of cardiac monoglycoside-(Na^+ + K^+)-ATPase complexes

Cardiac glycosides	Sugar	Activation energy
		kcal/mole
Ouabain, convallatoxin	Rhamnose	22
Deglucocheirotoxin	6-Deoxygulose	22
Digitoxigenin monodigitoxide, digoxigenin monodigitoxide, helveticoside	Digitoxose	20
Digitoxigenin 6-deoxyglucoside	6-Deoxyglucose	22
Digiproside	Fucose	22
Cymarin	Cymarose	22
Odoroside H	Digitalose	17
Digitoxigenin 3-tetrahydropyranyl ether	Tetrahydropyranyl ether	18
Acetylcymarin	4′-Acetylcymarose	20
Triacetyldigiproside	2′,3′,4′-Triacetylfucose	20

calculated using the data shown in Figs. 2–4. These results are shown in Table 4, and all values are around 20 kcal/mole.

DISCUSSION

In an earlier paper it was suggested that the sugar portion of cardiac glycosides binds to (Na^+ + K^+)-ATPase at a specific site and that dissociation is slow. The effects of temperature on the dissociation rate constants (k_d) of 11 cardiotonic monoglycosides confirmed the presence of the sugar site on (Na^+ + K^+)-ATPase and provided information about the nature of the binding between the sugar portion and the specific site on the enzyme. The k_d was not dependent on the structure of the aglycone portion, but only on that of the sugar portion. A comparison of k_d values for various monoglycosides indicated that no equatorial functional groups except the 3′-β-hydroxyl can bind with the sugar site; the 3′-hydroxyl and 5′-α-methyl can bind with the enzyme, but the 2′-α-hydroxyl, 4′-hydroxyl, and 5′-β-methyl groups cannot. Unfortunately, no information was available about the other functional groups of the sugar portion—the 2′-β-hydroxyl, the pyranoside ring oxygen, and the glycoside bridge oxygen—because suitable compounds were not available. However, the pyranoside ring oxygen might have some affinity for the enzyme because dissociation of the digitoxigenin 3-tetrahydropyranyl ether–enzyme complex was not as fast as that produced by 3-acetylaglycone, which is a reversible inhibitor.

The present results suggest that the 3′-hydroxyl group binds to (Na^+ + K^+)-ATPase by hydrogen bonding, because the substitution of a 3′-methoxyl group for the 3′-hydroxyl caused a reduction in stability of the complex (Fig. 5). The association model shown in Fig. 7 can account for all the changes in k_d related to the 3′-hydroxyl group. Binding occurs only on the lower side of the sugar. The 3′-α-hydroxyl group (axial) can bind with the proton donor group (X) and the acceptor group (Y) of the sugar site on the (Na^+ + K^+)-ATPase, but the 3′-β-hydroxyl (equatorial) can bind only with the acceptor group. The 3′-α-methoxyl can do so only with the donor group, and the 3′-β-hydroxyl cannot bind with either group.

On the other hand, the binding between the 5′-α-methyl group and $(Na^+ + K^+)$-ATPase may involve a hydrophobic interaction, but the evidence for this is weak at the moment.

Acetylation of some of the cardiac monoglycosides provides additional support for the above interpretation. The reduction in stability on acetylation of cymarin was slight compared with that of diacetyldigitoxide and triacetylconvallatoxin. With acetylcymarin, the acetyl group was introduced only on a hydroxyl group which is not involved in binding, and the decrease in tightness of binding probably resulted from a bulk effect of the acetyl group. However, in the cases of digitoxigenin diacetyldigitoxide and triacetylconvallatoxin, the essential group for binding, 3′-α-hydroxyl, was also acetylated. Therefore a reduction in hydrogen bonding of the sugar to the enzyme would be expected. Also, the insertion of a bulky acetyl group between the axial 3′-α-hydroxyl and the enzyme may have produced steric hindrance in the binding between the 5′-α-methyl group and the hydrophobic site on the enzyme. It is also probable that the same type of steric hindrance may have prevented binding between the pyranoside ring oxygen and the enzyme. In the case of digiproside the weak binding by the 3′-β-hydroxyl group was lost on acetylation, and the introduction of three acetyl groups may have produced a large bulk effect. Therefore a rather large reduction in the stability of the complex would be expected. But since all the acetyl groups here are equatorial, steric hindrance of the binding may not be as large as with acetylated convallatoxin and digitoxigenin monodigitoxide.

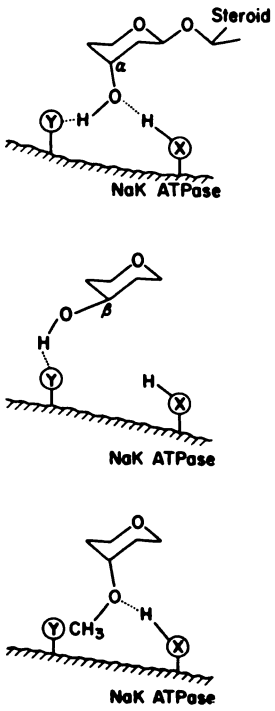


FIG. 7. *Model indicating spatial distribution of functional groups at binding site of sugar moiety*

X is the proton donor group and Y is the proton acceptor group of the $(Na^+ + K^+)$-ATPase.

The activation energy of the dissociation was rather constant in the series of cardiac monoglycosides. As the rate-determining step is independent of the variety of glycosides and will traverse the highest point on the reaction coordinate diagram, this step might be the one in which the enzyme changes its conformational nature. The value for the activation energy, 20 kcal/mole, is in good agreement with that of Tobin and Sen (9), 28.3 kcal/mole, obtained from studies on the radioactive ouabain-$(Na^+ + K^+)$-ATPase complex. This value is much higher than the energy of formation of one or two hydrogen bonds. Therefore this

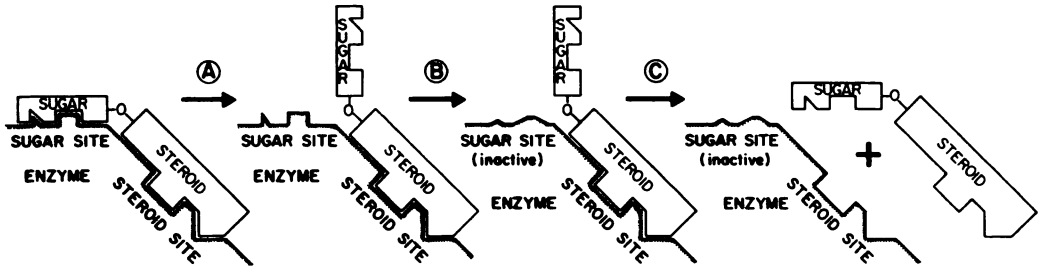


FIG. 8. *Model for dissociation mechanism of cardiac monoglycoside-$(Na^+ + K^+)$-ATPase complex*

activation energy may be related to some conformational change in the enzyme, as suggested by Tobin and Sen (9).

As the dissociation rate of the enzyme–cardiac glycoside complex is dependent on the nature of the sugar portion, dissociation of the bond(s) between the sugar moiety of the cardiac glycoside and the sugar site(s) on the (Na^+ + K^+)-ATPase must precede the rate-determining step. Unlike cardiac glycosides, aglycones bind to the enzyme reversibly (high dissociation rate), and the dissociation rate of the glycosides is not dependent on the nature of the steroid portion. Therefore dissociation of the steroid portion might follow the rate-determining step. If the rate-determining step were assumed to be a conformational change of the sugar site from active to inactive, the model shown in Fig. 8 could explain the mechanism of dissociation of the cardiac monoglycoside–(Na^+ + K^+)-ATPase complex. In reaction A the bond between the sugar moiety and the enzyme is disrupted. Reaction B is the rate-determining step and involves a conformational change in the sugar site on the enzyme, and reaction C is the dissociation of the steroid moiety from the steroid site on the enzyme.

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