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

V. Dissociation Rate Constants of Digitoxin Acetates

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SUMMARY

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The changes in dissociation rate constants (k_d) of (Na^+ + K^+)-ATPase complexes with digitoxin and digitoxigenin bisdigitoxide brought about by acetylation of their sugar moieties were examined in the hope of elucidating the role of each sugar hydroxyl group in binding to the enzyme. With every acetyl derivative the k_d value of the drug-enzyme complex formed in the presence of Na^+, Mg^{2+}, and ATP (type I complex) exceeded that of the corresponding complex formed in the presence of Mg^{2+} and P_i (type II complex). A remarkable increase in k_d values for both types of complexes was produced by acetylation of the 3′-hydroxyl group in the first sugar moiety. Binding by the 3′-hydroxyl group of the first sugar dominates binding by the second and third digitoxose moieties in both types and occurs via hydrogen bonding, as is the case for the monoglycoside. The axial 3‴-hydroxyl group of the third digitoxose moiety may also bind to the enzyme by hydrogen bonding in both types of complexes, but the equatorial 4‴-hydroxyl group is not involved. The second digitoxose group does not bind to the enzyme in the same manner as the others. In the type II complex the 3″-hydroxyl group is not involved in binding, but the hydrophobic component of the sugar opposite to the 3″-hydroxyl group or the pyranoside oxygen may bind to the extended area of the first sugar binding site. On the other hand, in the type I complex, the 3″-hydroxyl group may bind to another specific site of the enzyme, opposite to the first sugar binding site.

INTRODUCTION

It has been recognized that the interaction between the sugar moiety of a cardiac glycoside and (Na^+ + K^+)-ATPase increases the stability of this drug-enzyme complex, although the aglycone moiety is responsible for the physiological effect (1–4). Previously we suggested that the 3′-α- and β-hydroxyl and the 5′-α-methyl groups of the sugar moiety in the cardiac monoglycoside are involved in the binding to (Na^+ + K^+)-ATPase in the presence of

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Mg^{2+} and P_i (2), whereas the 2′-α-hydroxyl group is also involved in binding in the presence of Na^+, Mg^{2+}, and ATP (3). In the preceding paper (5) we showed that not only the first but the second and third sugar moieties of the cardiac oligosaccharides digitoxigenin and digoxigenin digitoxides can bind to the enzyme, lack of suitable cardiac oligosaccharides prevented determination of the functional group(s) responsible for binding. Satoh and Morita (6) synthesized various digitoxin acetates and showed by NMR analysis of their acetyl compounds and NMR data for other acetylated cardiac glycosides (7, 8) that the conformations of the digitoxose moieties of digitoxin were all of the C-1 (D) form.

In this paper we examine the k_d values of (Na^+ + K^+)-ATPase complexes with 10 different acetyl derivatives of digitoxin or digitoxigenin bisdigitoxide to evaluate the contribution of each hydroxyl group on the sugar moiety of digitoxin. For convenience, we refer to the cardiac glycoside–(Na^+ + K^+)-ATPase complex formed in the presence of Na^+, Mg^{2+}, and ATP as a type I complex, and to that formed in the presence of Mg^{2+} and P_i, as a type II complex, as previously noted (5). Acetylated compounds are referred to with the Roman numerals I, II, and III for the first, second, and third digitoxose moieties, respectively, with the subscripts *a* and *e* denoting 3-acetyl (axial) and 4-acetyl (equatorial) positions.

MATERIALS AND METHODS

The silicic acid for column chromatography was Mallinckrodt analytical reagent grade washed with methanol, and silica gel G was used for thin-layer chromatography. Thin-layer chromatograms were developed with chloroform–acetone (1:1 or 2:1, v/v) and visualized by spraying with 3% ceric sulfate in 2 N H_2SO_4 and heating.

3′-Acetyldigitoxin (I_a-II-III), 3″-acetyldigitoxin (I-II_a-II), and 3′,3″-diacetyldigitoxin (I_a-II_a-III) were kind gifts of Dr. D. Satoh (Shionogi and Company, Osaka) (6). 3‴,4‴-Diacetyldigitoxin (I-II-III_{ae}) and 3″,3‴,4‴-triacetyldigitoxin (I-II_a-III_{ae}) were prepared by Satoh's method (6). For 4‴-acetyldigitoxin (I-II-III_e), Satoh's method was slightly modified as follows: 0.2 ml of acetic anhydride was added to a solution containing 500 mg of digitoxin, 5 ml of tetrahydrofuran, and 2 ml of pyridine. After standing overnight at room temperature, the mixture was added to ice water. From the precipitate that had formed, pure I-II-III_e (about 200 mg) was obtained by recrystallization in aqueous methanol.

3′,3″,3‴,4‴-Tetraacetyldigitoxin (I_a-II_a-III_{ae}) was prepared by 90-min refluxing of a mixture of 500 mg of digitoxin, 12 ml of acetic anhydride, and 250 mg of sodium acetate. After evaporation of excess acetic anhydride under vacuum, the residue was treated with water, and 150 mg of pure tetraacetyldigitoxin were obtained by silicic acid chromatography.

Digitoxigenin bisdigitoxide was obtained from digitoxin via periodate oxidation of the terminal digitoxose by the method of Satoh and Aoyama (9). The yield of this compound was 93%.

The acetylation of digitoxigenin bisdigitoxide was based on the method used for digitoxin acetates (6). A mixture of 100 mg of digitoxigenin bisdigitoxide, 1 ml of tetrahydrofuran, 0.4 ml of pyridine, and 0.05 ml of acetic anhydride was allowed to stand at room temperature for 15 hr. After treatment with ice water, the resulting precipitate contained mainly 4″-acetyldigitoxigenin bisdigitoxide (I-II_e) and slight amounts of the 3″,4″-diacetyl compound (I-II_{ae}). When a mixture of 500 mg of digitoxigenin bisdigitoxide, 2.5 ml of pyridine, and 0.5 ml of acetyl anhydride was treated in the same manner, a mixture of I-II_{ae} and small amounts of I-II_e and 3′,3″,4″-triacetyldigitoxigenin bisdigitoxide (I_a-II_{ae}) was obtained. From these mixtures pure I-II_e, I-II_{ae}, and I_a-II_{ae} were separated by silicic acid chromatography.

The isomerization of 4″-I-II_e to the 3″-acetyldigitoxigenin bisdigitoxide (I-II_a) was carried out by the same method as that for 4‴-acetyldigitoxin (6). A solution of I-II_e (200 mg) in 50 ml of 0.1% $KHCO_3$ in 80% acetone was allowed to stand overnight at room temperature and then neutralized with 0.1 N HCl, and the solvent was removed under vacuum and extracted

with chloroform. I-II_a was separated from the extract, which contained unreacted I-II_e, by silicic acid chromatography.

The physical properties of the acetyl compounds are shown in Table 1. Infrared spectra of these compounds were also taken in KBr pellets, and the presence of acetyl, hydroxyl, ester, and butenolide groups was identified in each compound. The NMR spectra, shown in Table 2, were measured in $CDCl_3$ with a Bruker HX90E NMR spectrometer.[1] The NMR spectra of the digitoxin derivatives were identical with those reported by others (6, 7). Except for the 3''-acetyl compound, the acetates of digitoxigenin bisdigitoxide have not been reported, but the configuration of these acetates can be determined from their NMR spectra, which are coincidental with those of digitoxin derivatives. From these data it was also confirmed that the order of acetylation in digitoxigenin bisdigitoxide is 4'' (equatorial) → 3'' (axial) → 3' (axial), which is similar to the acetylation of digitoxin (6, 11).

The methods and calculations for determining the dissociation rate constants were the same as previously reported (2).

RESULTS

K_d values of digitoxin derivatives acetylated on the first digitoxose moiety. If the 3'-hydroxyl group on the first digitoxose moiety was acetylated, the stability of both type I and II $(Na^+ + K^+)$-ATPase complexes was remarkably reduced (Table 3). Although the k_d values obtained are somewhat inaccurate because of the high dissociation rates, the type I complex appears to be less stable than the type II complex, and as more acetyl groups are introduced on the second or third sugar moiety, the drug-enzyme complexes become less stable. In both complex types the stability was reduced as follows: digitoxin >> 3'-acetyldigitoxin (I_a-II-III) > 3',3''-diacetyldigitoxin (I_a-II_a-III) > 3',3'',3''', 4'''-tetraacetyldigitoxin (I_a-II_a-III_{ae}).

k_d values of enzyme complexes with acetyl derivatives of the third digitoxose moiety of digitoxin. If only the equatorial 4'''-hydroxyl group of the third digitoxose moiety was acetylated, the k_d value of the type I drug-enzyme complex increased only slightly and that of the type II complex was unchanged (Fig. 1).

When both the 3'''- and 4'''-hydroxyl groups of digitoxin were changed to the diacetate, apparent increases in k_d values were observed in both complex types (Fig. 1).

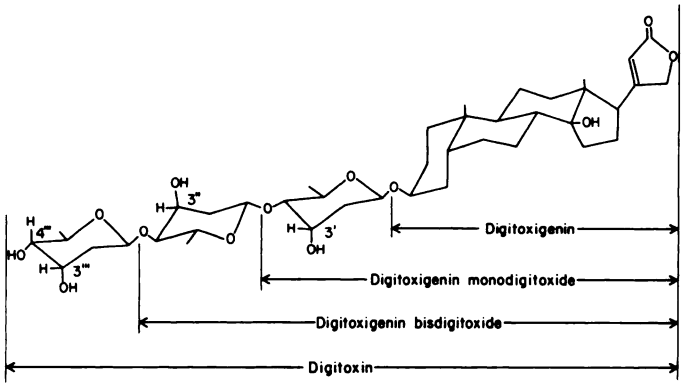
k_d values of enzyme complex with acetyldigitoxigenin bisdigitoxide. As shown in Fig. 2, acetylation of the equatorial 4''-hydroxyl group did not change the k_d value of either type of enzyme complex with digitoxigenin bisdigitoxide. Acetylation of the axial 3''-hydroxyl group affected the k_d values of each complex type differently. As in the case of 4''-acetylation, this 3''-acetylation did not change the k_d value of the type II complex. 3''-Acetylation increased the k_d value of the type I complex at low temperatures (25–20°) but not at high temperatures. Acetylation of both the 3''- and 4''-hydroxyl groups caused an increase in the k_d values of both types of enzyme complexes with digitoxigenin bisdigitoxide.

k_d values of enzyme complex with acetyl derivatives of the second digitoxose moiety of digitoxin. The k_d values of the type II $(Na^+ + K^+)$-ATPase complex with 3''-acetyldigitoxin (I-II_a-III) were the same as those of I-II_a-III_{ae} or I-II-III_{ae} and greater than those of digitoxin or digitoxigenin bisdigitoxide (Fig. 3). The type I complex with I-II_a-III_{ae} was less stable than that with I-II-III_{ae} and was the same as that with digitoxigenin bisdigitoxide. However, based on its k_d values, the type I enzyme complex with I-II_a-III is different in nature from other type I complexes (Fig. 3). At low temperatures (20–23°) the type I complex with I-II_a-III is less stable than that with I-II-III_{ae} or even I-II_a-III_{ae}, but since the temperature change in stability is relatively small in the complex with the 3''-acetyl compound (I-II_a or I-II_a-III), the type I complex with the 3''-acetyl compound becomes more stable at higher temperatures than the complex with I-II_a-III_{ae} or I-II-III_{ae}, and is almost the same as the complex with digitoxin at 35°.

[1] The NMR spectra were obtained by Mr. J. Blackbourn (School of Pharmacy, University of Wisconsin), whom we thank.

TABLE 1

Physical properties of acetyl compounds of digitoxin and digitoxigenin bisdigitoxide prepared for this study



Abbreviation[a]	Position of acetyl group	Formula[b]	Melting points		Recrystallizing solvent
			Found	Reported	
I-II-III$_e$	4‴	$C_{43}H_{60}O_{14}$	265–267°	264–267° (6) 211–227° (10)	CH_3OH–H_2O
I-II-III$_{ae}$	3‴,4‴	$C_{47}H_{68}O_{15}$	245–246°	263–267° (6)	Acetone–ether
I-II$_a$-III$_{ae}$	3″,3‴,4‴	$C_{45}H_{70}O_{16}$	230–233°	232–235° (6)	CH_3OH–H_2O
I$_a$-II$_a$-III$_{ae}$	3′,3″, 3‴,4‴	$C_{49}H_{72}O_{17}$	160–162°	155–162° (7) 154–158° (6)	Acetone–toluene
I-II$_e$	4″	$C_{37}H_{56}O_{11}$	226°		Ether
I-II$_a$	3″	$C_{37}H_{56}O_{11}$	140–145°	143–147° (6)	Ether–isooctane
I-II$_{ae}$	3″,4″	$C_{39}H_{58}O_{12}$	216–218°		Ether–isooctane
I$_a$-II$_{ae}$	3′,3″,4″	$C_{41}H_{60}O_{13}$	186°		Ether

[a] Roman numerals I, II, and III represent the first, second, and third digitoxose moieties in digitoxin and digitoxigenin bisdigitoxide, and the subscripts *a* and *e* denote 3-acetyl (axial) and 4-acetyl (equatorial) groups.

[b] Elemental analysis was done for carbon and hydrogen. All results were within ±0.5% of the theoretical values.

For all acetyl derivatives of digitoxin or digitoxigenin bisdigitoxide, as well as other cardiac glycosides which have previously been examined (3), the type I complex was less stable than the type II complex. The increases in k_d values on acetylation of digitoxin or digitoxigenin bisdigitoxide are summarized as follows.

In type I complexes with digitoxigenin bisdigitoxide (I-II) derivatives:

$$\text{I-II} = \text{I-II}_e < \text{I-II}_a{}^2 < \text{I-II}_{ae}$$

In type II complex with digitoxigenin bisdigitoxide (I-II) derivatives:

$$\text{I-II} = \text{I-II}_e = \text{I-II}_a < \text{I-II}_{ae}$$

In type I complexes with digitoxin (I-II-III) derivatives:

$$\text{I-II-III} \leqq \text{I-II-III}_e < \text{I-II-III}_{ae} < \text{I-II}_a\text{-III}^2 < \text{I-II} = \text{I-II}_a\text{-III}_{ae} << \text{I}_a\text{-II-III}$$

In type II complexes with digitoxin (I-II-III) derivatives:

$$\text{I-II-III} = \text{I-II-III}_e < \text{I-II} < \text{I-II-III}_{ae} = \text{I-II}_a\text{-III} = \text{I-II}_a\text{-III}_{ae} << \text{I}_a\text{-II-III}$$

Arrhenius activation energy of dissociation of acetyl digitoxin and digitoxigenin

[2] The order of 3″-acetyldigitoxin (I-II$_a$-III) or 3″-acetyldigitoxigenin bisdigitoxide (I-II$_a$) in the type I complex is variable with temperature. These orders are correct only in the low temperature range (20–23°).

bisdigitoxide. The Arrhenius activation energy of dissociation of each type I complex with 10 acetyl derivatives of digitoxin or digitoxigenin bisdigitoxide was calculated from Figs. 1–3. All values for type II complexes are around 25 kcal/mole, and those of type I complexes are around 30 kcal/mole except with I-II_a-III and I-II_a. The value for the type I complex with I-II_a-III is 18 kcal/mole, and that for I-II_a is 22 kcal/mole.

Effect of potassium on dissociation of type I complex. As reported previously (12), potassium reduced the k_d value of type I complexes with cardiac oligodigitoxides below the value of the type II complexes, but other ligand effects, e.g., the sodium effect on the type I complex and

TABLE 2

NMR signals of acetyl compounds of digitoxin and digitoxigenin bisdigitoxide

Abbreviation	NMR signals (in $CDCl_3$, 90 MHz)				
	Position of acetyl group	Axial OAc 3′,3″,3‴-positions	Equatorial OAc 4″- or 4‴-positions	Intensity ratio of OAc signals, axial/equatorial	Equatorial H 3′,3″,3‴-positions
Digitoxin (I-II-III) derivatives					
I-II-III_e	4‴		2.12		
I-II-III_{ae}	3‴,4‴	2.11	2.00	1.05	5.43, 5.47[a]
I-II_a-III_{ae}	3″,3‴,4‴	2.11	1.99	1.87	5.41[b]
Digitoxigenin bisdigitoxide derivatives					
I-II_e	4″		2.12		
I-II_a	3″	2.16			5.24, 5.32[a]
I-II_{ae}	3″,4″	2.12	2.01	0.93	5.44, 5.48[a]
I_a-II_{ae}	3′,3″,4″	2.11,[a] 2.09	1.99	1.28, 1.22	5.40[b]

[a] Doublet.
[b] Triplet.

TABLE 3

Dissociation rate constants (k_d) of $(Na^+ + K^+)$-ATPase complexes with 3′-acetates of digitoxin

Average values obtained from duplicate or triplicate experiments are shown. The deviation under each condition was about ± 0.3 hr^{-1}.

Complex	k_d values					
	Type I			Type II		
	15°	17°	20°	15°	17°	20°
	hr^{-1}	hr^{-1}	hr^{-1}	hr^{-1}	hr^{-1}	hr^{-1}
Digitoxin (I-II-III)			0.23[a]			0.20[a,b]
Digitoxigenin monodigitoxide			0.74[a]			0.26[a]
Digitoxigenin tetrahydropyranyl ether			1.2[a]			0.70
3′-Monoacetyldigitoxin (I_a-II-III)	2.5	2.8	3.5	1.9	2.3	3.2
3′,3″-Diacetyldigitoxin (I_a-II_a-III)	2.5	3.7	4.4	2.2	2.6	3.8
3′,3″,3‴,4‴-Tetraacetyldigitoxin (I_a-II_a-HI_{ae})	3.4	3.8	5.0	2.2	2.8	4.1

[a] These data were obtained previously (2, 3). The error in these values was less than ± 0.1 hr^{-1}.
[b] At 25°.

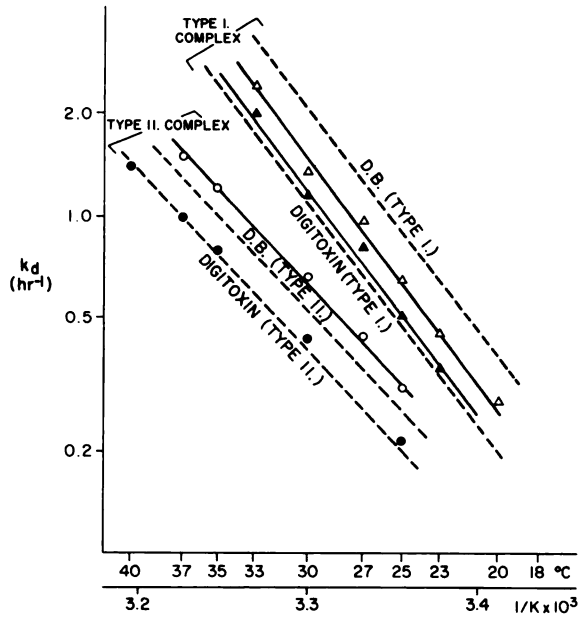


FIG. 1. *Arrhenius plots of dissociation rate constants (k_d) of ($Na^+ + K^+$)-ATPase complexes with 4'''-acetyldigitoxin and 3''',4'''-diacetyldigitoxin*

● and ▲, 4'''-acetyldigitoxin (I-II-III$_e$); ○ and △, 3''',4'''-diacetyldigitoxin (I-II-III$_{ae}$); – – –, digitoxin or digitoxigenin bisdigitoxide (D.B.).

the sodium plus ATP effect on the type II complex, were similar to those on the enzyme complexes with monoglycosides. The effects of potassium on the type I complexes with acetylated derivatives of digitoxin and digitoxigenin bisdigitoxide are shown in Table 4. It appears that the characteristic potassium effect is not observed with compounds with 3''-acetyl derivatives. Here, as in the case of the monoglycoside, potassium reduced the k_d value of the type I complex to the same extent as that of the type II complex.

DISCUSSION

In the preceding paper (5) it was suggested that the second and third digitoxose moieties of the cardiac oligodigitoxides could bind at specific sites on ($Na^+ + K^+$)-ATPase, although binding of the first sugar moiety to the enzyme predominated. In the present study we examined the dissociation rate constants (k_d) of ($Na^+ + K^+$)-ATPase complexes with acetylated digitoxin or digitoxigenin bisdigitoxide. The acetylation of these oligodigitoxides provides more precise evidence to support the above interpretation. When binding between a drug and its receptor depends on hydrogen bonding of the hydroxyl group(s) on the drug, acetylation of that hydroxyl has as its primary effect not only a reduction in hydrogen bonding but also steric hindrance caused by the insertion of the bulky acetyl group. Sometimes steric hindrance is produced by acetylation of a hydroxyl group which is not involved in binding.

Acetylation of the axial 3'-hydroxyl group of the first sugar moiety of digitoxin produced a remarkable increase in the k_d values of both types of drug-enzyme complexes. In both complex types the k_d values of the 3'-acetyldigitoxin complex was greater than that of the enzyme complex with digitoxigenin tetrahydropyranyl ether, in which there is no sugar hydroxyl group and no binding group except the pyranoside oxygen. This marked effect of 3'-acetylation can be explained if the first digitoxose group binds with a sugar-specific site on the enzyme in the same manner as the monoglycoside, which is considered to bind by hydrogen bonding between

the 3'-hydroxyl group of the sugar moiety and a sugar-specific site on the enzyme in both types of complexes (2, 3). The abolition of hydrogen bonding and the insertion of a bulky acetyl group between the first sugar moiety and the sugar-specific site of the enzyme appears to be the main reason for the 3'-acetylation effect, and confirms the previous conclusion that the 3'-α-hydroxyl group in the first digitoxose moiety is the dominant functional group in the sugar moiety of digitoxin, which binds with the receptor in both complex types.

As the results in Table 3 show, it appears that the introduction of additional acetyl groups into 3'-acetyldigitoxin (I_a-II-III) increases the k_d values of both complex types. This increase suggests that the second and third digitoxose moieties of I_a-II-III may bind with the enzyme independently of the binding of the first sugar. In the binding of the sugar moiety of the cardiac monoglycoside, however, it is suggested that the sugar binding site of the enzyme is activated by attachment of the cardiac aglycone (13). It is not obvious at present whether binding of the cardiac aglycone is necessary for binding of the second or third sugar moiety to the enzyme.

The effect of 3''-acetylation of the second digitoxose moiety is complicated. The differences in k_d values for the type II enzyme complexes with I-II_a and I-II_e are small, but 3''-acetylation of the digitoxin derivatives produces a greater increase in the k_d value than 3''-acetylation of the bisdigitoxide. The k_d value of I-II_a-III_{ae} is the same as that for I-II-III_{ae} or I-II_a-III and a little greater than that of I-II_a. Therefore it is not likely that the 3''-hydroxyl group of the second digitoxose group is functional in hydrogen bonding with a specific site on the enzyme. On the other hand, the 3''-hydroxyl group may be involved in the binding of the second digitoxose moiety in the type I complex, because the acetylation of the 3''-hydroxyl group increased the k_d value of the drug-enzyme complex in every case examined here (I-II_a, I-II_{ae}, I-II_a-III, and I-II_a-III_{ae}). The type I complexes with 3''-monoacetylated compounds are different from other acetylated or non-acetylated digitoxigenin oligodigitoxides, how-

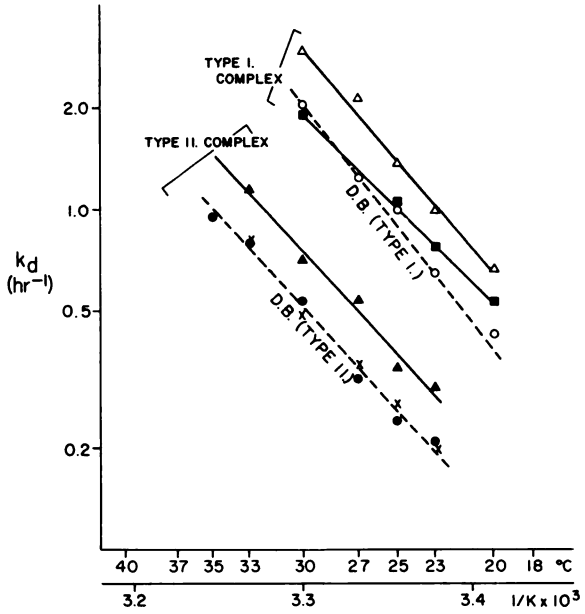


FIG. 2. *Arrhenius plots of dissociation rate constants (k_d) of $(Na^+ + K^+)$-ATPase complexes with acetyldigitoxigenin bisdigitoxides*

● and ○, 4''-acetyldigitoxigenin bisdigitoxide (I-II_e); × and ■, d''-acetyldigitoxigenin bisdigitoxide (I-II_a); ▲ and △, 3'',4''-diacetyldigitoxigenin bisdigitoxide (I-II_{ae}); – – –, digitoxigenin bisdigitoxide (D.B.).

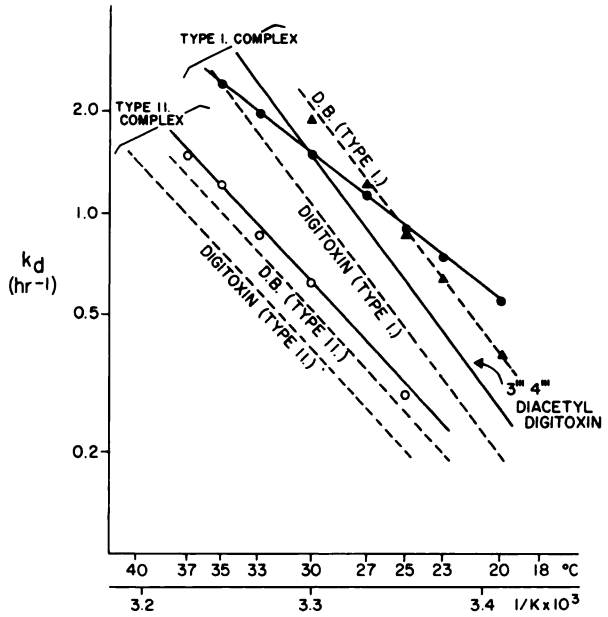


FIG. 3. *Arrhenius plots of dissociation rate constants (K_d) of ($Na^+ + K^+$)-ATPase complexes with 3"-acetyldigitoxin and 3',3''',4'''-triacetyldigitoxin*

○ and ●, 3"-acetyldigitoxin (I-II_a-III); ▲, 3",3''',4'''-triacetyldigitoxin (I-II_a-III_{ae}); – – –, digitoxin or digitoxigenin bisdigitoxide (D.B.).

TABLE 4

Change in dissociation rate constants (k_d) of ($Na^+ + K^+$)-ATPase complexes with digitoxin acetates or digitoxigenin bisdigitoxide acetates by potassium

Compound	Temperature	k_d values		
		Type I complex in 1 mM Tris-EDTA	Type I complex in 2 mM KCl + 1 mM Tris-EDTA	Type II complex in 1 mM Tris-EDTA
		hr^{-1}	hr^{-1}	hr^{-1}
Digitoxin derivatives				
Digitoxin (I-II-III)	30°	1.04	0.25	0.40
4'''-Acetate (I-II-III_e)	30°	1.15	0.20	0.43
3''',4'''-Diacetate (I-II-III_{ae})	30°	1.34	0.30	0.66
3"-Acetate (I-II_a-III)	30°	1.49	0.55	0.62
3",3",4'''-Triacetate (I-II_a-III_{ae})	29°	1.23	0.36	0.40
Digitoxigenin bisdigitoxide derivatives				
Digitoxigenin bisdigitoxide (I-II)	27°	1.30	0.22	0.33
4"-Acetate (I-II_e)	27°	1.25	0.21	0.32
3"-Acetate (I-II_a)	27°	1.28	0.30	0.35
3",4"-Diacetate (I-II_{ae})	27°	2.14	0.46	0.54
Digitoxigenin monodigitoxide	25°	1.90	0.47	0.51

ever: the Arrhenius activation energies of the dissociation of the type I complexes with I-II_a-III and I-II_a are 18 and 22 kcal/mole, respectively, compared with those of the other type I complexes, which are around 30 kcal/mole and rather constant for all the mono- and oligoglycosides examined.

Since the k_d values of I-II-III_e are almost the same as those of I-II-III in both com-

plex types, the 4'''-hydroxyl group in the third sugar moiety is not involved in binding with the enzyme, like the 4'-hydroxyl group of the cardiac monoglycoside (2, 3). In both complex types the difference between k_d values of the I-II-III$_{ae}$ and I-II-III$_e$-complexes is apparently greater than that between the I-II-III$_e$ and I-II-III complexes. Therefore the axial 3'''-α-hydroxyl group may contribute to binding to the enzyme. Moreover, since the k_d value of the type II I-II-III$_{ae}$ complex is greater than that of the type II digitoxigenin bisdigitoxide (I-II), the 3'''-hydroxyl group in digitoxin appears to bind to the enzyme by hydrogen bonds in the type II complex. In the type I complex, however, other functional groups in the third sugar moiety, i.e., pyranoside oxygen, may be involved in binding to the enzyme, because the I-II-III$_{ae}$ complex is more stable than the I-II complex.

In conclusion, we can summarize the contribution of each hydroxyl group in the sugar moieties of digitoxin to binding with (Na^+ + K^+)-ATPase as shown in Table 5. In addition to binding by the hydroxyl group, hydrophobic binding or the binding of pyranoside oxygen is involved in the binding of the second digitoxose moiety in the type II complex and of the third sugar moiety in the type I complex.

From NMR data, the C-1 (D) conformation of digitoxose moieties has been demonstrated (6, 8, 9) and the present data confirm this. Since the digitoxose moieties are linked by a (1-4)-β-glycosidic linkage in the digitoxin molecule (14), the preferred conformation is three parallel pyranoside rings, as in cellulose, where the 3''-α-hydroxyl group is present on the side opposite to the 3'- or 3'''-hydroxyl group against the plane of these pyranoside rings (Fig. 4). In the type II complex with cardiac monoglycoside, we suggested previously that binding with specific sites on the enzyme occurred only on the lower side of the pyranoside ring (2), and the same type of binding is also suggested for the type I complex with the monoglycoside, because all functional groups which bind to the enzyme are present on the lower side of the sugar.

The binding model shown in Fig. 4 extends the previous suggestion. This model may account for the complex results obtained in this study. Binding of the first and third sugar groups occurs only on the lower side of the sugars in both types of complexes. In the same manner as the monoglycoside, the 3'-α-hydroxyl group (axial) can bind with a specific site on the enzyme by hydrogen bonding. The difference in stability between type I and type II complexes of monoglycosides suggests that the distance from the 3'-hydroxyl group to the first sugar site is greater in the type I complex than in the type II complex. We suggest that the binding of the third sugar is similar to that of the first sugar, but until now information on the comparative stability at the third sugar site in each type of complex has not been available.

The binding of the second sugar moiety is more complicated. In the type II complex the specific binding site for the second sugar is suggested to be on the extended area of the first sugar site, which binds with the hydrophobic side of the second digitoxose ring,[3] i.e., opposite the 3''-hydroxyl group. In the type I complex, however, the second sugar-specific site may be too far away to bind the second digitoxose, and another specific site of the enzyme may be involved. This second sugar-specific site in the type I complex appears to be on the side opposite to the first or second sugar-specific site in the type II complex. The nature of this binding between 3''-hydroxyl group and the enzyme in the type I complex may be different from that at the first sugar binding site, but not enough data are available to discuss this in detail.

From this model, the specificity of the potassium effect on the type I complex with cardiac oligodigitoxides is explained as follows. Not only may potassium convert the first sugar site of the type I complex to that of the type II complex, but it also may change the extended area of the first sugar site to the same active second

[3] Since the pyranoside oxygen can serve as the proton acceptor in hydrogen bonding, binding between this pyranoside oxygen and proton donor at the specific binding site of the enzyme is also possible.

TABLE 5

Contribution of each hydroxyl group on sugar moiety of digitoxin to binding with ($Na^+ + K^+$)-ATPase

	3'-OH	3''-OH	3'''-OH	4'''-OH
Binding in type I complex	++	+	+	−
Binding in type II complex	++	−	+	−
Potassium effect	+	+	−	−

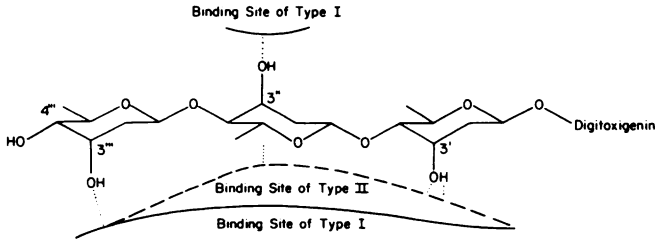


FIG. 4. *Model indicating spatial relationship of each sugar-specific site*

sugar-specific site as in the type II complex. However, the second sugar-specific site in the type I complex, which is present on the side opposite to the first sugar site, is not changed by potassium. Therefore the second sugar moiety in the type I complex can bind with two sites on the enzyme, that is, hydrogen binding by the 3''-hydroxyl group and hydrophobic binding, just as in the type II complex.

The present results demonstrate the complexity of cardiac oligosaccharide binding. The 3'-α-hydroxyl group of the oligodigitoxide binds to the same specific site on ($Na^+ + K^+$)-ATPase as the monoglycoside, and this binding dominates all other binding by the second and third digitoxose moieties in both complex types. The binding of the third digitoxose moiety may also be dependent on a hydrogen bond involving the 3'''-α-hydroxyl group, but that of the second digitoxose moiety may not be involved in the same manner. In the type II complex the 3''-α-hydroxyl group is not involved in binding of the second sugar moiety to the enzyme.

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