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Inhibition of Calf Brain Membranal Sodium- and Potassium-Dependent Adenosine Triphosphatase by Cardioactive Sterols

A Binding Site Model

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SUMMARY

The optimal inhibitory responsiveness of calf brain membranal ($Na^+ + K^+$)-dependent ATPase to cardioactive sterols was determined to be consistent with a model depicting a three-point or area interaction between inhibitor and a complementary binding site on the enzyme. Each functional ATPase molecule could be inhibited reversibly by 1 molecule of sterol at the binding site. The A component of the binding site interacted optimally with the sugar portion of an aglycone monosaccharide rather than that of an aglycone di-, tri-, or tetrasaccharide or the 3β-hydroxyl group of the aglycone. The B component of the binding site yielded optimal enzyme inhibition as a result of interaction with the sterol 14-hydroxyl group. Relative affinities at the C component of the binding site were related to the following order of preference for the lactone ring substituted at position 17β of the sterol: α-pyrone > crotonolactone > γ-butyrolactone. Although the sterol lactone rings possess large dipole moments, the compounds most inhibitory toward ($Na^+ + K^+$)-ATPase favored ring planarity and a relatively extensive π-electron system. Binding of the crotonolactone and α-pyrone substituents to the C component was postulated to result from multiple interactions, one or more of which are independent of the lactone ring group dipole moment, namely, π-π system interaction or π-complex formation.

INTRODUCTION

The ($Na^+ + K^+$)-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) represents a fundamental component of the system that regulates alkali metal ion distribution across cell membranes (1, 2). Several investigators have observed that activation of the membranal ATPase by K^+ is inhibited reversibly by cardioactive sterols (3, 4).

The most significant pharmacological effect of cardiac glycosides is the inotropic response of heart muscle. Recent reports have indicated that there is a significant correlation between the extent of inotropic responsiveness and the degree of inhibition of membranal ($Na^+ + K^+$)-ATPase by cardiac glycosides (5–7). Structural requirements for the relative toxicities of cardioactive aglycones to KB carcinoma cells have also been correlated with the requirements for ($Na^+ + K^+$)-ATPase inhibition (5).

The present study deals with the influence of cardiac glycoside structure on the extent

of reversible inhibition of the membranal ($Na^+ + K^+$)-ATPase system. Within the limits of availability of inhibitory compounds, this study has permitted the determination of an optimal spatial array of inhibitor functional group types that bind or interact in such a manner as to inhibit ($Na^+ + K^+$)-ATPase activity.

Each functional ($Na^+ + K^+$)-ATPase system behaved as if it interacted with only 1 molecule of inhibitor. Correlation of the extents of enzyme inhibition with variations in cardioactive sterol structure provided an indication of the topological characteristics and functional group requirements at the complementary binding site of the enzyme. A model demonstrating several properties of such a binding site has been constructed, and the qualitative aspects of possible sterol-binding interactions at this site are discussed.

METHODS

Chemical preparation and homogeneity. Dihydroouabain was prepared by hydrogenating ouabain (Sigma) according to a modification of the technique of Brown *et al.* (8). The Brown[2] Hydroanalyzer apparatus (obtained from Delmar Scientific Laboratories, Maywood, Ill.) was modified to contain a 100-ml reaction flask assembly inserted between the hydrogen generator and the mercury valve. After 0.3 g of 10% palladium on carbon (Engelhard Chemicals, Newark, N.J.) had been placed in the reaction flask and 5 ml of 1 M $NaBH_4$ solution in the hydrogen generator flask, the system was flushed with dry nitrogen gas. Fifteen milliliters of methanol were added to the reaction flask, rotation of the magnetic stirring bar in each flask was initiated, and 2 ml of 10 N HCl were added to the hydrogen generator to flush the system. A solution of 360 mg of ouabain in 10 ml of methanol was introduced into the reaction flask, following which 2.3 ml of 0.05 M $NaBH_4$ solution were consumed during a 24-hr hydrogenation procedure. The contents of the reaction flask were filtered through a fine fritted glass funnel, and methanol was removed with a jet of dry nitrogen. Dihydroouabain crystallized after standing as a syrup for 1 month; recrystallization was effected using acetone–water.

The melting point range for dihydroouabain was 191–193°, and for ouabain it was 190–193°. The carbonyl stretching vibrations for dihydroouabain were at 1740 cm^{-1}, as compared to 1715 cm^{-1} for ouabain. Dihydroouabain was homogeneous on thin-layer chromatography in three different solvent systems, and was resolved from ouabain in each case (Table 1).

Trimethylsilyl ether and acetate derivatives of several of the sterols were prepared as described previously (10). Acetates were recrystallized from acetone–water.

TABLE 1

Thin-layer chromatographic behavior of cardiotonic compounds with different types of lactone rings

Compound	The compounds were chromatographed on 250-μ layers of adsorbent using a single 15-cm solvent development.					Fluorescence of spots on excitation at 320 nm[a]
	Kieselguhr			Silica gel G-F		
	Toluene–2-propanol (1:2)	Toluene–chloroform (1:1)	Toluene	Toluene–2-propanol (1:2)	Methanol	
	R_F	R_F	R_F	R_F	R_F	
Ouabain	0.58	0.00	0.00	0.09	0.65	Yellow
Dihydroouabain	0.77	0.00	0.00	0.11	0.62	Green
Ouabain trimethylsilyl ether				0.75	0.72	Yellow
Dihydroouabain trimethylsilyl ether				0.75	0.70	Green
Strophanthidin	0.98	0.39	0.07	0.57	0.72	Yellow
Hellebrigenin	0.99	0.66	0.24	0.60	0.70	Brown
Strophanthidin trimethylsilyl ether				0.53	0.73	Tan
Hellebrigenin trimethylsilyl ether				0.71	0.70	Tan

[a] The fluorescent spot represented the sterol reaction product obtained upon spraying the developed plate with a modified (9) Liebermann-Burchard reagent.

Strophanthidin and hellebrigenin were gifts from L. E. Hokin. Strophanthidin was subjected to preparative thin-layer chromatography on silica gel, using toluene–2-propanol (1:2 by volume) as the developing solvent. After desorption in methanol and filtration, strophanthidin was crystallized by adding water.

Other bufodienolide compounds were obtained from Alfred Bader Company, Milwaukee. The melting point ranges of these compounds corresponded to previously reported values.

The remaining cardenolides were obobtained from Boehringer-Mannheim, New York. Many of the cardenolide sterols and glycosides had previously been examined by gas-liquid chromatography, NMR spectrometry, and mass spectrometry, in addition to thin-layer chromatography (10, 11).

The sterols, glycosides, and other derivatives were dried overnight at 20 mm of Hg and 100° in the presence of P_2O_5 and paraffin. All compounds were determined to be homogeneous by thin-layer chromatography in two or more solvent systems (9, 10).

In order to facilitate solution, the sterols, glycosides, and acetates were dissolved in dimethylsulfoxide; then water was added to adjust the organic solvent concentration to 10%. This procedure permitted the preparation of 1×10^{-4} M solutions of gitoxigenin and several glycosides, and of 5×10^{-5} M solutions of the aglycone acetates. Other sterols and glycosides could be obtained at concentrations of 10^{-3} M or higher. Ten per cent dimethylsulfoxide was a convenient solvent, as it produced no measurable influence on the responsiveness of (Na+ + K+)-ATPase to ouabain.

Tris-ATP was prepared in a 5° cold room by passing 200 ml of 5% disodium ATP solution through a 2.6 × 23 cm column of Dowex 50-X8, analytical grade, 100–200 mesh, in the Tris form. The Tris-ATP was lyophilized and stored at 5° in a desiccator.

ATPase preparation and assay. Calf brain microsomal (Na+ + K+)-ATPase was prepared essentially according to the method of Skou (12).

The assay for ATPase was automated by adapting an existing Technicon technique for measurement of orthophosphate. "Combined" ATPase was measured at an enzyme concentration of 40 μg of protein per milliliter. In order to obtain a direct measurement of the initial reaction velocity, incubation was conducted at 40° for 17.5 min in the presence of 33 mM Tris-Cl buffer (pH 7.4), 3.3 mM ATP, 8 mM $MgCl_2$, 125 mM NaCl,

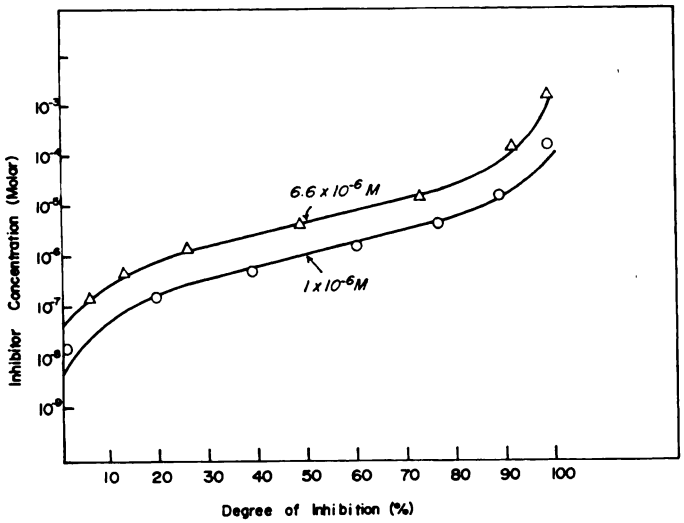


FIG. 1. *Concentration dependence of (Na+ + K+)-ATPase inhibition by ouabain* (○) *and dihydrooubain* (△)

Each point represents the average of three determinations. I_{50} values are listed in Tables 3 and 4.

and 25 mM KCl. Incubation of the membranal preparation as above, without NaCl and KCl, permitted direct determination of the Na^+-independent ATPase activity (13).

The (Na^+ + K^+)-ATPase was determined to comprise approximately the same percentage (88%) of the "combined" ATPase inhibited by 10^{-3} M ouabain. The dependence on temperature of both K_m and V was consistent with the observations of Swanson for the guinea pig brain enzyme (14). Maximum reaction velocity generally corresponded to about 36 μmoles of P_i produced per milligram of protein per hour for (Na^+ + K^+)-ATPase under the incubation conditions specified above.

The extent of enzyme inhibition was measured in the simultaneous presence of substrate, activators, and cardioactive sterols (13), as suggested by the observations of Albers *et al.* (15) and Matsui and Schwartz (16). As indicated in Fig. 1, the variation in the degree of (Na^+ + K^+)-ATPase inhibition with an exponential function of inhibitor concentration was linear in the concentration range permitting approximately 25–75% inhibition of the ATPase.

Spectral measurements. NMR spectra were obtained using a Varian HA-100 instrument with a decoupling accessory. Spectra were collected using 4% solutions in deuterochloroform. Infrared spectra were obtained on a Perkin-Elmer model 621 instrument, using KBr discs containing 1% sterol or glycoside.

RESULTS

Stoichiometry of enzyme-inhibitor interactions. Observations by other investigators have indicated that the membranal Na^+-independent ATPase may be heterogeneous (17). Although the enzyme cannot be completely purified at the present time, we have assumed that calf brain microsomal (Na^+ + K^+)-ATPase comprises a single molecular species, in accordance with the indications of Kahlenberg *et al.* (18) and Uesugi *et al.* (19).

Inhibition of calf brain membranal (Na^+ + K^+)-ATPase by ouabain was examined according to the technique of Ackerman and Potter (20) and was determined to be reversible. The enzyme-inhibitor complex dissociation constant was found to be approximately 1×10^{-6} M.

In order to determine the number of molecules of cardiac glycoside required to inhibit 1 molecule of (Na^+ + K^+)-ATPase, the approach of Johnson *et al.* (21), as mentioned by Douville and Warren (22), was adopted. The reversible interaction between the complementary binding site (CBS) on the ATPase and an inhibitor, I, is expressed as follows.

$$\mathrm{CBS} + nI \rightleftharpoons \mathrm{CBS}\cdot I_n \qquad (1)$$

A complex of inhibitor plus responsive enzyme binding site is represented by $\mathrm{CBS}\cdot I_n$. The association constant is

$$K_A = \frac{[\mathrm{CBS}\cdot I_n]}{[\mathrm{CBS}][I]^n} \qquad (2)$$

Assuming that the inhibitor completely inactivates the enzyme upon interaction at the complementary binding site, the activity that remains in the presence of inhibitor, V_i, is proportional to the concentration of the binding sites. As V_0 represents activity in the absence of inhibitor, the activity lost as a result of binding of inhibitor, $V_0 - V_i$, is proportional to [CBS · I_n]. Equation 2 may thus be rewritten as follows.

$$K_A = \left(\frac{V_0 - V_i}{V_i}\right) \cdot \frac{1}{[I]^n} \qquad (3)$$

Equation 3 may be linearized as follows

$$\log K_A = \log\left(\frac{V_0 - V_i}{V_i}\right) - n \log [I] \qquad (4)$$

or, it may be rearranged to permit a Hill plot.

$$n \log [I] + \log K_A = \log\left(\frac{V_0 - V_i}{V_i}\right) \qquad (5)$$

A plot of $\log (V_0/V_i - 1)$ with respect to $\log [I]$ gives a straight line with a slope of n, while $1/K_A = [I]^n$ when $\log (V_0/V_i - 1) = 0$. From Fig. 2 it may be seen that the slopes of the lines $\cong 1$; thus n equals 1 for the two sterols and the glycoside, ouabain. This relationship correlated with the observation reported by Matsui and Schwartz (16) that ^{3}H-labeled ouabain forms a 1:1 complex with cardiac membranal (Na^+ + K^+)-ATPase.

In order to determine whether the cardioactive sterols effect inhibition by combining with a single type of responsive site on the $(Na^+ + K^+)$-ATPase, mixed inhibition studies were conducted. Assuming identical binding sites, inhibitors A and B should act additively, giving the following expression (22) for remaining enzyme activity in the presence of both compounds.

$$V_{i_{A+B}} = \frac{V_0}{1 + K_A[A] + K_B[B]} \quad (6)$$

For independent and noninteracting sites, the enzyme activity remaining after binding of the stronger inhibitor A may be considered essentially the initial velocity for inhibitor B. Assuming that the enzyme–inhibitor A complex has no more affinity than the native enzyme for inhibitor B, the final activity in the presence of both inhibitors is

$$V_{i_{AB}} = \frac{V_0}{(1 + K_A[A])(1 + K_B[B])} \quad (7)$$

Table 2 presents results of inhibition studies involving combinations of three distinctly different cardioactive sterols. Experimental V_i values (average of six determinations) were compared with V_i values calculated using Eqs. 6 and 7. Statistical treatment assumed normality and absence of bias in the data. The discrimination indicated by the statistical tests in Table 2 suggested that the identical site theory is at least 24 times more likely than the nonidentical site theory.

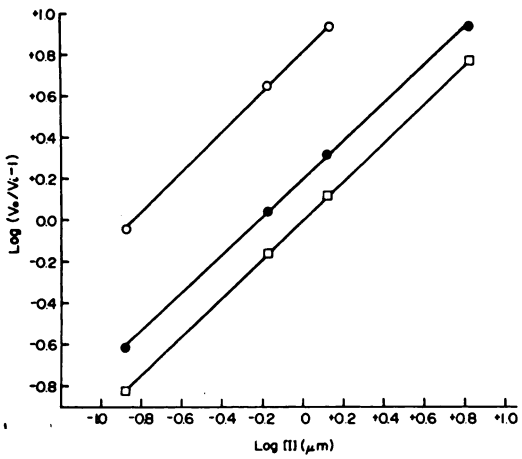


FIG. 2. *Plots for inhibition of* $(Na^+ + K^+)$-*ATPase by three different cardioactive sterols*

Each point represents the average of six determinations. Line slopes (n) were obtained using the method of least squares to fit the linear relationship given by Eq. 5. ○, gamabufotalin, $n = 0.95$; ●, digitoxigenin, $n = 0.90$; □, ouabain, $n = 0.90$.

TABLE 2

Specificity of cardioactive sterol-binding site of calf brain microsomal $(Na^+ + K^+)$-*ATPase*

Cardioactive sterol	Concentration	K_A[a]	Calculated V_i(A)[b]		Experimental V_i(B)	B/A[c]	
			Nonidentical sites	Identical sites		Nonidentical sites	Identical sites
	μM	μM^{-1}					
Ouabain	0.50	1.01	16.04	18.06	17.57	1.095	0.972
Digitoxigenin	0.30	1.65					
Ouabain	0.50	1.01	15.86	17.88	17.88	1.128	1.000
Gamabufotalin	0.075	6.85					
Digitoxigenin	0.30	1.65	15.85	17.86	17.47	1.102	0.978
Gamabufotalin	0.075	6.85					
Average ± SD						1.108 ± 0.043	0.983 ± 0.037

[a] K_A values were obtained from the data in Fig. 2.

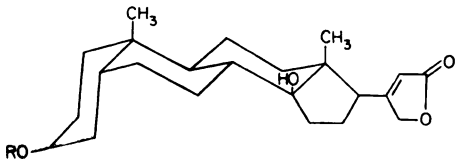
[b] V_0 was 36 mμmoles of P_i per milligram of protein per hour.

[c] Ninety-five per cent confidence limits are given for the average ratios. Application of the t-test indicated that the average value for nonidentical site ratios differed from 1.000 with a probability of $p = 0.008$, while that for the identical site ratios differed from 1.000 with a probability of $p = 0.19$.

Model for complementary binding site. Since the $(Na^+ + K^+)$-ATPase may be inhibited quite effectively by digitoxigenin, it was assumed that this aglycone or its dihydro analogue represents one of the simplest cardioactive sterol inhibitors capable of acting at the complementary binding site. Accordingly, the A component is designated as that component of the binding site that interacts with the 3β-hydroxyl group of the sterol and/or with alcohol groups of a glycoside derivative attached to the 3β-hydroxyl group. The B component is postulated to interact with the 14-hydroxyl group of the sterol inhibitor. The lactone ring substituted at C-17 of the sterol is proposed to interact with one or more functional groups at the C component of the binding site.

TABLE 3

Responsiveness of $(Na^+ + K^+)$-ATPase to cardiotonic compounds, particularly those with variations in sugar substitution



Compound	I_{50}
	μM
Digitoxigenin (3β,14-dihydroxy-5β-card-20[22]-enolide)	0.60
Digitoxigenin 3β-monodigitoxoside	0.15
Digitoxigenin 3β-bisdigitoxoside (β1 → 3)	0.30
Digitoxin (β1 → 3-tridigitoxoside)	0.55
Digitoxigenin 3β-acetate	5.00
Digoxigenin (3β,12β,14-trihydroxy-5β-card-20[22]-enolide)	1.10
Digoxigenin 3β-monodigitoxoside	0.50
Digoxigenin 3β-bisdigitoxoside (β1 → 3)	0.60
Digoxin (β1 → 3-tridigitoxoside)	0.90
Lanatoside C (digoxin acetylglucoside)	1.70
Digoxigenin 3β,12β-diacetate	150.00[a]
Gitoxigenin (3β,14,16β-trihydroxy-5β-card-20[22]-enolide)	2.00
Gitoxin (β1 → 3-tridigitoxoside)	1.30
16-Acetylgitoxin	1.10
Gitoxigenin 3β,16β-diacetate	6.00
Strophanthidin (3β,5,14-trihydroxy-16-al 5β-card-20[22]-enolide)	0.70
Cymarin (strophanthidin 3β-monocymaroside)	0.30

[a] The value represents an estimate due to limitations relative to solubility of this compound in 10% dimethylsulfoxide in water.

Characteristics of the A component of the sterol-binding site. Table 3 shows that the micromolar concentrations of compounds required for 50% inhibition of the $(Na^+ + K^+)$-ATPase indicate a preferred order of affinity of sterol glycosides for the complementary binding site. This order—aglycone monosaccharide > disaccharide > trisaccharide > aglycone—was consistent for each of four different sterols and their glycoside derivatives. This affinity order suggested that substitution of one sugar on the 3β-hydroxyl group permitted optimal opportunity for hydrogen bonding or other dipole-dipole interaction with the A component of the binding site, while additional sugar residues restricted access to that component.

The apparent reduction in accessibility to the A component of the binding site is attributed to the bulk effect of the addition of sugar groups to the sterol monosaccharide. Although it is recognized that hydrogen bonding involving the second, third, or fourth sugar group of the glycosides could also influence the affinity of the glycoside for the binding site, it is difficult to rationalize a diminution in enzyme-inhibitory responsiveness to additional hydrogen-bonding capacity.

The observation that digitoxigenin acetate was significantly less inhibitory than the aglycone or corresponding glycosides was interpreted as an indication that a hydroxyl hydrogen may be a preferred characteristic of the 3β-substituent on the sterol inhibitor.

The B component of the sterol-binding site. Gitoxigenin had a much higher I_{50} for the $(Na^+ + K^+)$-ATPase than did digitoxigenin (Table 3). This result was consistent with the hypothesis that intramolecular hydrogen bonding between the hydroxyl groups on carbons 14 and 16 resulted in specific orientation of the 14-hydroxyl group, thus, per-

haps, lowering the affinity of the aglycone for the B component of the binding site. Intramolecular hydrogen bonding in gitoxigenin is strongly suggested by a broad infrared absorption band in the 3540–3300 cm^{-1} region and is demonstrable using Corey-Pauling-Koltun models.

Gitoxin had a lower I_{50} than gitoxigenin, but an appreciably higher I_{50} than digoxin. Acetylation of the 16β-hydroxyl group (16-acetylgitoxin) increased the inhibitory effectiveness. Attempted correlations from these observations were complicated by the possibility of intramolecular interactions involving the lactone ring and either the 16β-hydroxyl of gitoxigenin or the acetoxy group of 16-acetylgitoxin.

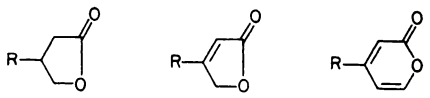
Since relative configuration about carbons 16 and 17 of gitoxigenin trimethylsilyl ether had previously been questioned (10), the NMR spectra of gitoxigenin acetate were examined to determine whether the accepted configuration for the free sterol (23) had been retained in this derivative. The NMR spectra in a deuterochloroform solution of gitoxigenin acetate revealed that the symmetrical doublet ($J_{HH'}$ = 8.5 cps at 3.8 ppm), corresponding to the C-17 hydrogen, collapsed upon irradiation of the C-16 hydrogen (5.50 ppm). The magnitude of this coupling constant was consistent with a dihedral angle of about 20 degrees (24) for the vicinal hydrogens at carbons 16 and 17; thus, these two hydrogens possess the accepted *cis*-relationship.

Resibufogenin, a 14,15β-epoxide, was markedly less inhibitory (Table 4) than telocinobufagin or gamabufotalin; however, the lack of other structural analogues may lessen the significance of this observation regarding involvement of a binding role of the 14-hydroxyl group.

The significance of the role of the 14-hydroxyl group could be demonstrated more clearly if an aglycone were available with a 14-hydrogen in place of the hydroxyl group. In this regard, Repke (6) reported a 30-fold decrease in inhibitory effectiveness of a mixture of $\Delta^{8:14}$- and $\Delta^{14:15}$-anhydrodigitoxigenins relative to digitoxigenin. Even so, the relevance of this observation was complicated because of the alterations in ring conformation which accompany elimination of the alcohol group at C-14.

TABLE 4

Responsiveness of $(Na^+ + K^+)$-ATPase to cardiotonic compounds, particularly those with variations in the lactone ring



γ-Butyrolactone (in sterol, cardanolide) | Crotonolactone (in sterol, card-20 [22]-enolide) | α-Pyrone (in sterol, bufa-20,22-dienolide)

Compound	I_{50}
	μM
Ouabain (1β,3β,5β,11α,14,19-hexahydroxy-5β-card-20[22]-enolide 3-L-rhamnoside)	1.00
Dihydroouabain (γ-butyrolactone ring at 17β-position of ouabain)	6.60
Strophanthidin (3β,5β,14-trihydroxy-19-al 5β-card-20[22]-enolide)	0.70
Hellebrigenin (3β,5β,14-trihydroxy-19-al 5β-bufa-20,22-dienolide)	0.10
Oleandrigenin (3β,14-dihydroxy-16-acetoxy-5β-card-20[22]-enolide)	0.60
Bufotalin (3β,14-dihydroxy-16-acetoxy-5β-bufa-20,22-dienolide)	0.30
Gamabufotalin (3β,11α,14-trihydroxy-5β-bufa-20,22-dienolide)	0.15
Telocinobufagin (3β,5β,14-trihydroxy-5β-bufa-20,22-dienolide)	0.12
Resibufogenin (3β-hydroxy-14,15-epoxy-5β-bufa-20,22-dienolide)	4.50
Uzarigenin (3β,14-dihydroxy-5α-card-20[22]-enolide)	2.40

The C component of the sterol-binding site. As indicated in Table 4, ouabain was 7 times more inhibitory than dihydroouabain, while hellebrigenin was 7 times more inhibitory than strophanthidin. In addition, bufotalin was twice as inhibitory as oleandrigenin. That bufotalin was not a more potent inhibitor relative to oleandrigenin may be related to intramolecular interaction involving the C-16 carbonyl group and the 17β-lactone, resulting in restriction of freedom of lactone ring rotation and/or shielding of a portion of the π-electron system of the ring.

The above results qualitatively resemble those previously reported by Ruoho *et al.*

(4), who found that hellebrigenin was 25–30 times more inhibitory to guinea pig brain ($Na^+ + K^+$)-ATPase than was strophanthidin, and by Repke (6), who found digitoxin to be 12 times more inhibitory than dihydrodigitoxin.

Other structure-activity considerations. In confirmation of Repke's observations (6), the I_{50} for uzarigenin was 4 times greater than that for digitoxigenin. This indicated that a more desirable spatial distribution of functional groups and/or a more desirable steroid backbone continuity is afforded by *cis*, rather than *trans*, fusion of the A:B ring junction.

As indicated in Table 4, hellebrigenin and telocinobufagin possess hydroxyl groups at C-5, and they possess similar inhibitory effectiveness even though C-19 of hellebrigenin exists as an aldehyde rather than a methyl group. The affinity of the cardioactive sterols for the binding site appeared to be very similar, whether hydroxylation occurred at C-5 in telocinobufagin or at the 11α-position in gamabufotalin.

Comparison of the I_{50} values of ouabain and digitoxigenin monodigitoxoside indicated that hydroxylation at positions 1β, 5, 11α, and 19 markedly reduced the inhibitory effectiveness of the glycosides, if it is assumed that the differences in the sugar portion of the glycosides are relatively unimportant.

Relative to digitoxigenin monoacetate or gitoxigenin diacetate, the dramatic 50–100-fold decrease in inhibitory effectiveness of digoxigenin diacetate indicated that acetylation of the 12β-hydroxyl group resulted either in hindrance of accessibility of the lactone to the C component of the binding site or in a complicated conformational change in the ($Na^+ + K^+$)-ATPase, thereby rendering complex formation between the binding site and inhibitor ineffective in influencing enzyme activity. Assuming a 17β absolute configuration (23), the lactone ring is sterically prohibited from interacting with the 12β-acetoxy group of digoxigenin diacetate. As may be seen using Corey-Pauling-Koltun models (10), the 14-hydroxyl group is also sterically prohibited from interacting with a 12β-substituent.

Determination of relative polarities of lactone rings. The physical properties of the various types of lactone rings in the cardioactive sterols have not received sufficient study to permit prediction of their group dipole moments. The thin-layer chromatographic behavior of several of these sterols provided a reflection of the sum of all differences in group dipoles and polarizabilities of structurally similar compounds.

Thin-layer chromatography was conducted using a pair of glycosides and a pair of aglycones in which the only differences resided in the lactone rings. Trimethylsilylation resulted in derivatization of the primary and secondary alcohol groups of these compounds, thus, permitting solubilization in the developing solvents.

When separations were effected, R_F values from thin-layer chromatography clearly indicated that the compounds containing the γ-butyrolactone ring or the α-pyrone ring behaved similarly relative to structural analogues possessing the crotonolactone ring. The chromatographic behavior of these compounds thus suggested that known differences between dipole moments of γ-butyrolactone and the crotonolactone rings [μ = 4.13 and 4.62, respectively (25)] provided significant indication of probable dipole moment differences between α-pyrone and crotonolactone rings of the sterols.

DISCUSSION

It was mentioned in the INTRODUCTION that both cardiac aglycone toxicity to the KB carcinoma cell and heart muscle inotropic responsiveness correlated well with relative ($Na^+ + K^+$)-ATPase inhibitory responsiveness to the sterols. Additionally, studies by Chen (26) indicated some interesting relationships between the structures of several derivatives of two bufadienolides, (namely, hellebrigenin and its C-5 epimer, bovogenin) and their toxicities in the cat. The rhamnose glycoside and the glucose-rhamnose glycoside derivatives were 106% as potent as hellebrigenin, based on aglycone equivalents of each compound. The thevetose glycoside derivatives were 125–130% as potent as bovogenin. These results correlated very closely with those in Table 3 relating to specificity at the A component of the ($Na^+ + K^+$)-ATPase binding site.

The peculiarly influential properties of conjugated cardioactive sterol lactone rings have previously been interpreted as suggesting that inhibition of $(Na^+ + K^+)$-ATPase may result from a stronger interaction than hydrogen bonding at the C component of the complementary binding site. Therefore, the physical properties of the sterol lactone rings were considered to deserve additional attention.

The strength of binding at the C component of the binding site was expected to depend on one or more of the following sterol lactone ring characteristics: (a) ring size, (b) group dipole moment, (c) group polarizability, (d) extensiveness of the ring π-electron system, and (e) ring shape or, perhaps, the degree of ring planarity.

The alkali metal ion transport system of red blood cells (27) responds to cardioactive sterols in a fashion which qualitatively simulates that of the calf brain $(Na^+ + K^+)$-ATPase. The observation that hexahydroscillaren A is much less effective than scillaren A or digoxin in inhibiting potassium ion influx in red blood cells (28) would appear to indicate that slight differences in lactone ring size are probably unrelated to the inhibitory effectiveness of the sterols. This argument concerning ring size could also be developed by comparing the I_{50} values for calf brain $(Na^+ + K^+)$-ATPase of ouabain and dihydroouabain (Table 4).

Although behavior on thin-layer chromatography provided only an indirect measure of lactone ring properties, R_F differences among the structurally related sterols were consistent with the argument that the dipole moment of the crotonolactone ring exceeds that of either the γ-butyrolactone or α-pyrone rings (RESULTS and Table I).

According to a second argument, the 2,6-dimethyl-γ-pyrone carbonyl oxygen has greater "proton-accepting power" than that of 2,4-dimethyl-α-pyrone, as reflected by their infrared C=O and C=C stretching vibrations (29). Greater "proton-accepting power," an expression of relative electronegativity, provides an indirect measure of dipole moment differences for these two substituted lactones. The dipole moments of 2,6-dimethyl-γ-pyrone and of γ-pyrone are 4.58 and 3.72 debyes, respectively (30). Since the dipole moment of crotonolactone exceeds that of 2,6-dimethyl-γ-pyrone, it appears consistent to interpret the infrared spectral correlations as also supporting the argument that the dipole moment of crotonolactone exceeds that of α-pyrone.

In view of the probable nature of the binding interaction(s) at the C component of the complementary binding site, the suggestion that the lactone ring is involved as a result of hydrogen bonding (6, 29) is not inconsistent with observations resulting from the present study. It should be noted that dipole induction in response to the lactone ring dipole moment might also be expected to represent a significant interaction force in the biological system. Dipole induction could be considered significant, as this type of interaction has been found to be a critical determinant of gas chromatographic behavior of cardioactive sterol derivatives under conditions excluding dipole-dipole interactions (10).

However, discriminatory responsiveness of the $(Na^+ + K^+)$-ATPase to sterols possessing various types of lactone ring characteristics appeared to correlate best with the extensiveness of the ring π-electron system (which is necessarily accompanied by an increased polarizability and an increased tendency toward ring planarity). The types of bonding interaction which would be favored by an increase in extensiveness of the lactone ring π-electron system include dipole induction, π-complex formation, and π-π system interaction (31, 32).

While π-complexation frequently occurs under conditions in which metals act as electron acceptors, alcoholic hydrogens or other relatively electropositive species may also assume this type of role (31, 33). However, acceptor-donor association such as that involved in intercalation of 3,4-benzpyrene with purine and/or pyrimidine bases in DNA (34) provided an analogy for the model depicted for the C component of the binding site in Fig. 3.

An attractive feature of the molecular association model in Fig. 3 is that the apparent free energy of binding may be 2-6 kcal $mole^{-1}$ (33, 34), an order of magnitude

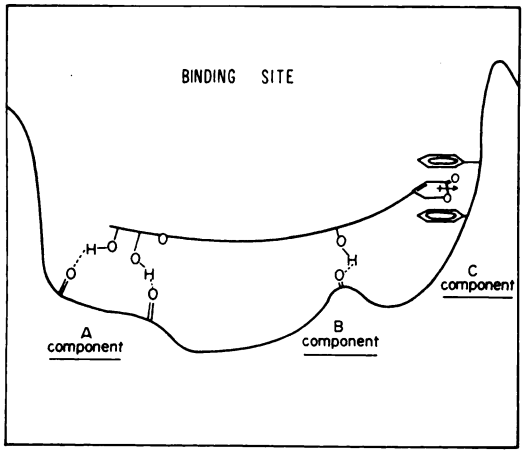


FIG. 3. *Model indicating optimal spatial distribution of functional group types at the binding site*

Functional group types at the A and B components simulate a portion of the lumen of a macrocyclic antibiotic (35). Phenyl groups are presented at the C component to emphasize complementarity with the nearly planar crotonolactone lactone ring; however, other functional group types may be present (see the text).

significant relative to alternative hydrogen-bonding interaction forces.

The representation of the C component of the binding site in Fig. 3 emphasizes involvement of the sterol lactone ring in π-complex formation with phenyl groups. In reality, involvement of other amino acid R groups—phenol, indole, imidazole—could possess equal or greater importance at the C component.

A more careful study of the weak physical forces interaction capabilities of the various lactone ring systems would obviously be desirable. The correlation of extensiveness of the lactone ring π-electron system with the inhibitory effectiveness of cardioactive sterols against (Na^+ + K^+)-ATPase may facilitate additional understanding of those molecular association phenomena which relate directly to determination of biological responsiveness to a variety of classes of compounds, such as steroid hormones, carcinogenic aromatic hydrocarbons, and microbial toxins, in addition to drugs.

The specificity of membranal (Na^+ + K^+)-ATPase responsiveness to structurally similar cardioactive sterols and sterol glycosides also appears to be correlated, to a large extent, with the specificity of the recognition process observed in the nuclear receptor proteins of target organ cells for the steroid hormones (36–38).

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