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A Crystallographic, Conformational Energy, and Biological Study of Actodigin (AY-22,241) and Its Genin

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SUMMARY

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A multidisciplinary (crystallographic, conformational energy, biological) study of Actodigin and digitoxigenin was completed, and the data analyzed using the NIH PROPHET computer system. These data were compared to Na^+,K^+-ATPase inhibition studies on Actodigin genin, digitoxigenin β-D-glucoside, digitoxin, and digitoxigenin β-D-digitoxide. This work has shown that Actodigin genin's ability to inhibit Na^+,K^+-ATPase can be largely explained by its lactone carbonyl oxygen position (5.22 Å displaced from the carbonyl oxygen of digitoxigenin, both molecules in their crystallographically observed energy minima) and molecular conformation. The ring D of Actodigin, for example, was found to be in a half chair, unlike those of natural digitalis ring D's, which exist in an envelope. However, the β-D-glucose makes an unexpectedly large contribution to Actodigin's activity—much larger than with digitoxigenin glucoside. Actodigin genin has very low activity ($I_{50} = 7 \times 10^{-5}$ M), nearly the least active genin we have studied. These findings were not predicted by a recently proposed Actodigin binding model, and they give new insight into the glycoside binding model proposed by Yoda and Yoda.

INTRODUCTION

Two molecular features distinguish Actodigin[1] (I) (1–3) from naturally occurring cardenolide glycosides such as digoxin (III), digitoxin (IV), and ouabain (V). These are: (1) a glucose is at C3 instead of digitoxoses or rhamnose (see Table 1); and (2) Actodigin has a "rotated" lactone ring that is attached to C17 by C22, not C20. These two structural features must combine in some manner to produce Actodigin's rapid onset and reversal of pharmacological and toxicological effects. Some investigators have also reported that Actodigin may actually be less toxic than the clinically used glycosides (1). However, more recent studies have challenged this concept (2, 3).

X-Ray crystallography has been very helpful in our digitalis analog studies (4–7) as well as in the study of steroids of other pharmacological classes (8). Since interatomic distance measurements of even relatively rigid steroid molecules have been shown to be incorrect by 0.9 Å or more using molecular models (9), X-ray crystallography permits structure–activity studies to begin with a high degree of precision.

Because of its modified biological properties, altered geometry, and resulting stereochemistry, we have applied these techniques to a study of Actodigin. Our previous studies of this type on genins of natural and modified structure (e.g., **VI–XIII, XVII–XIX**) have shown that side group carbonyl position is a primary determinant of genin activity (4–6). Thus, in contrast to previous reports, the C20–C22 double bond is most likely not directly contributing to Na^+,K^+-ATPase inhibition and/or binding. This bond appears to just keep the carbonyl in the correct position for maximal effect (4–6). Studies by Yoda (10) and Wallick *et al.* (11) have emphasized the importance of the sugar directly attached to the genin C3β-OH as another major factor in the binding of glycosides to Na^+,K^+-ATPase.

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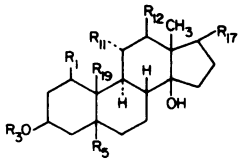
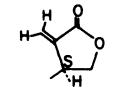
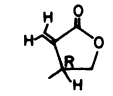
[1] The synthesis of Actodigin was first reported by Ferland, J. M., Y. Lefebure, R. Deghenghi and K. Wiesner. Synthetic new cardenolides. *Tetrahedron Lett.*, 3617–3620 (1966); and later patented by Lefebure, Y. and J. M. Ferland. U. S. 3,398,138, August 20, 1968.

0026-895X/80/010043-09$02.00/0

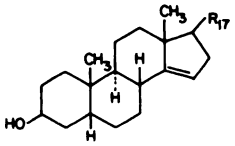
TABLE 1

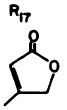


Compound	R_3	R_1	R_5	R_{11}	R_{12}	R_{19}	R_{17}
I. Actodigin	β-D-Glucose	H	H	H	H	CH_3	
II. Actodigin genin	H	H	H	H	H	CH_3	
III. Digoxin	β-D-Digitoxose$_3$	H	H	H	OH	CH_3	
IV. Digitoxin	β-D-Digitoxose$_3$	H	H	H	H	CH_3	
V. Ouabain	α-L-Rhamnose	OH	OH	OH	H	CH_2OH	
VI. Digoxigenin	H	H	H	H	OH	CH_3	
VII. Digitoxigenin	H	H	H	H	H	CH_3	
VIII. Strophanthidin	H	H	OH	H	H	CHO	
IX.	H	H	H	H	H	CH_3	
X.	H	H	H	H	H	CH_3	
XI.	H	H	H	H	H	CH_3	
XII.	H	H	H	H	H	CH_3	
XIII.	H	H	H	H	H	CH_3	
XIV.		H	H	H	H	CH_3	
XV. Digitoxigenin glucoside	β-D-Glucose	H	H	H	H	CH_3	
XVI. Digitoxigenin digitoxide	β-D-Digitoxose	H	H	H	H	CH_3	

We have attempted to delineate the relative roles of Actodigin's side group orientation as well as its glucose on Na^+,K^+-ATPase inhibition. We have completed X-ray crystallographic and conformational analyses of Actodigin, and have related these analyses to the Na^+,K^+-ATPase inhibitory activities of Actodigin, Actodigin genin (II), digitoxigenin (VII), digitoxigenin glucoside (XV), digitoxin (IV), and digitoxigenin digitoxide (XVI). The

TABLE 1—*Continued*



Compound	R_{17}
XVII.	
XVIII.	
XIX.	

pharmacological effects of digitalis drugs and their analogs appear to be related to inhibition of this enzyme system (12–14).

These studies have shown that Actodigin's ability to inhibit Na^+,K^+-ATPase can be largely explained by its lactone carbonyl oxygen position and its molecular conformation and configuration. However, the β-D-glucose makes an unexpectedly large contribution to Actodigin's activity—much larger than with digitoxigenin glucoside (XV). Its genin (II) has very low activity—nearly the least active of the more than 15 genins we have studied. These findings were not predicted by a recently proposed Actodigin binding model (2), and they give new insight into the glycoside binding model proposed previously by Yoda and Yoda (10).

METHODS

Chemical methods. Actodigin (I) was generously provided by Dr. Romano Deghenghi, Director of Research, Ayerst Laboratories, Montreal, Canada. The genin (II) was prepared by hydrolysis of XIV. (Genin II was reported in the Ayerst patent[1] but not characterized.) Attempted acid hydrolysis of I gave largely the 14-dehydrated analog. A solution of XIV (100 mg), K_2CO_3 (100 mg), MeOH (4 ml), and water (1 ml) was refluxed for 2 hr. The solution was acidified with AcOH, diluted with water, and extracted with CH_2Cl_2. The CH_2Cl_2 layer was washed with water and evaporated. The resulting residue was purified by column chromatography on silica gel (10 g). Elution with CH_2Cl_2-EtOAc (4:1) gave II: yield 61 mg (68%), mp 108–110°. No 14-dehydrated product could be seen either on TLC or on NMR. Recrystallization from EtOAc-*n*-hexane gave the analytical sample of II: mp 114–115°. IR(KBr) 3435, 1735, 1645 cm^{-1}; NMR ($CDCl_3$) 7.27 (s, 1, C22-H), 4.78 (s, 2, C23-H), 4.13 (1, br s, C3-H.

Anal. Calcd for $C_{23}H_{34}O_4$: C, 73.76; H, 9.15. Found: C, 74.00; H, 9.37. *m/e* 374 (M^+), 356 (M^+-H_2O).

Digitoxigenin was prepared by dilute acid hydrolysis of digitoxin (IV) as previously reported (15). Digitoxigenin β-D-glucoside (XV) was prepared by reaction with tetra-*O*-acetyl-α-D-glucosyl bromide and hydrolysis of the glucose acetate groups as previously reported (16). Digitoxigenin digitoxide (XVI) was prepared by two methods: acid hydrolysis of digitoxin (IV) followed by chromatographic separation of the bis digitoxide and digitoxigenin; and the stepwise degradation method of Satoh and co-workers (17). The products obtained by each method were identical in all regards to XVI obtained from Boehringer–Mannheim.

The crystal of Actodigin used in the crystallographic studies was grown in ethyl acetate.

Crystallographic methods. The crystal data for Actodigin ($C_{29}H_{44}O_9$) were measured on a specimen crystal of dimensions 0.08 × 0.20 × 0.58 mm with an Enraf Nonius CAD-4 diffractometer using Ni-filtered Cu$K\alpha$ radiation. The space group was determined to be $P2_1$, from the systematic absences along the $0k0$ row. The lattice parameters were calculated to be a = 14.3671(7)Å, b = 7.3914(4)Å, c = 13.5833(8)Å, and β = 106.923(4)° from a least-squares procedure using the 2θ values of 38 reflections in the range $50° < 2\theta < 70°$. The integrated intensities for 3048 reflections having a $2\theta < 150°$ were measured using θ–2θ scans; 2421 of these were significantly above the background level ($I > 2\sigma_I$).

The direct methods program MULTAN (18) and the negative quartet figure of merit procedure (19) were used to phase an E-map showing clearly 28 of the 38 nonhydrogen atoms. The missing atoms were located from electron density maps calculated from the positions of the 28 atoms. The details of the structural refinement follow those given previously (20). The final values of the residual, $R = \Sigma \parallel F_o \mid - \mid F_c \parallel / \Sigma \mid F_o \mid$, were 0.047 for the observed data and 0.065 for all the measured data. Table 2 lists the positional parameters and esd's.[2] A stereoscopic ORTEP view (Fig. 1) of Actodigin gives an overall view of its conformation. (Stereoscopic drawings have become increasingly popular in biologically related crystallographic studies. The three-dimensional character of the drawing is seen by viewing through glasses available from Hubbard Scientific Co., P. O. Box 105,

[2] Thermal parameters and structural factor amplitudes are available on request from the Medical Foundation of Buffalo, Inc., 73 High St., Buffalo, N. Y. 14203.

TABLE 2

Fractional atomic coordinates (×10⁴) for the nonhydrogen atoms of Actodigin and for the hydrogen atoms (×10³)

Standard deviations (×10⁴ and 10³, respectively) are shown in parentheses.

Atom	X/A ( )	Y/B ( )	Z/C ( )	Atom	X/A ( )	Y/B ( )	Z/C ( )
C (1)	6178 (2)	3819 (5)	3197 (2)	H (1B)	656 (2)	465 (6)	273 (2)
C (2)	6702 (2)	2060 (6)	3573 (2)	H (1A)	615 (2)	455 (5)	372 (2)
C (3)	6790 (2)	893 (5)	2695 (2)	H (2A)	633 (2)	127 (4)	404 (2)
C (4)	5796 (2)	615 (5)	1942 (2)	H (2B)	747 (2)	222 (4)	406 (2)
C (5)	5262 (2)	2381 (5)	1547 (2)	H (3A)	700 (2)	−18 (4)	287 (2)
C (6)	4279 (2)	2006 (7)	766 (2)	H (4A)	541 (2)	−10 (4)	231 (2)
C (7)	3570 (2)	1155 (6)	1285 (2)	H (4B)	590 (2)	−13 (4)	139 (2)
C (8)	3437 (2)	2256 (4)	2161 (2)	H (5B)	567 (2)	293 (4)	120 (2)
C (9)	4420 (2)	2759 (4)	2947 (2)	H (6A)	439 (2)	99 (5)	31 (2)
C (10)	5152 (2)	3595 (5)	2423 (2)	H (6B)	411 (2)	339 (6)	66 (2)
C (11)	4237 (2)	3924 (5)	3800 (2)	H (7A)	385 (1)	3 (3)	160 (2)
C (12)	3582 (2)	2911 (5)	4326 (2)	H (7B)	300 (2)	96 (4)	88 (2)
C (13)	2575 (2)	2386 (4)	3601 (2)	H (8B)	315 (2)	345 (4)	193 (2)
C (14)	2717 (2)	1370 (4)	2652 (2)	H (9A)	473 (1)	160 (3)	323 (2)
C (15)	2951 (2)	−561 (5)	3052 (2)	H (11B)	415 (2)	491 (5)	369 (2)
C (16)	2239 (2)	−893 (5)	3678 (2)	H (11A)	497 (2)	399 (5)	446 (2)
C (17)	2131 (2)	939 (5)	4180 (2)	H (12B)	349 (2)	373 (5)	490 (2)
C (18)	1944 (2)	4098 (5)	3298 (1)	H (12A)	392 (2)	180 (5)	470 (2)
C (19)	4820 (2)	5485 (6)	1990 (1)	H (15A)	365 (2)	−49 (4)	352 (2)
C (20)	269 (2)	1206 (5)	3576 (2)	H (15B)	295 (2)	−143 (4)	238 (2)
C (21)	−516 (2)	1689 (7)	4025 (3)	H (16B)	248 (2)	−187 (4)	427 (2)
C (22)	1131 (2)	1296 (5)	4257 (2)	H (16A)	156 (2)	−122 (5)	318 (2)
C (23)	963 (2)	1842 (6)	5220 (2)	H (17A)	258 (1)	109 (4)	492 (2)
O (3B)	7442 (1)	1848 (−)	2247 (1)	H (18A)	229 (2)	495 (6)	298 (2)
O (14B)	1785 (1)	1135 (3)	1872 (1)	H (18B)	132 (2)	378 (5)	280 (2)
O (21)	17 (2)	2067 (4)	5101 (2)	H (18C)	196 (2)	458 (6)	401 (2)
O (23)	1575 (2)	2056 (5)	6043 (1)	H (19A)	530 (2)	583 (4)	158 (2)
C (1′)	7671 (2)	1012 (5)	1443 (2)	H (19B)	403 (2)	582 (5)	153 (2)
C (2′)	8165 (2)	2356 (5)	930 (2)	H (19C)	492 (2)	614 (5)	251 (2)
C (3′)	8428 (2)	1472 (5)	36 (2)	H (20)	16 (2)	76 (6)	281 (2)
C (4′)	9056 (2)	−165 (5)	423 (2)	H (21A)	−99 (2)	277 (5)	377 (2)
C (5′)	8585 (2)	−1400 (5)	1046 (2)	H (21B)	−96 (2)	74 (6)	417 (2)
C (6′)	9229 (2)	−2932 (5)	1584 (2)	H (14O)	158 (2)	192 (4)	165 (2)
O (2′)	7558 (2)	3855 (4)	553 (2)	H (1′)	708 (1)	52 (3)	101 (1)
O (3′)	8928 (1)	2687 (3)	−444 (1)	H (2′)	879 (1)	284 (3)	146 (1)
O (4′)	9185 (1)	−1150 (4)	−441 (1)	H (3′)	776 (1)	108 (3)	−55 (1)
O (5′)	8337 (1)	−427 (3)	1837 (1)	H (4′)	978 (2)	33 (4)	87 (2)
O (6′)	8740 (2)	−4076 (3)	2100 (2)	H (5′)	804 (2)	−182 (4)	66 (2)
				H (6′A)	995 (2)	−249 (5)	201 (2)
				H (6′B)	945 (2)	−371 (4)	112 (2)
				H (3′O)	841 (2)	360 (7)	−95 (2)
				H (4′O)	972 (2)	−157 (5)	−23 (2)
				H (6′O)	861 (2)	−371 (4)	246 (2)
				H (2′O)	758 (2)	457 (6)	112 (2)

Northbrook, Ill.) The bond distances and angles of Actodigin are given in Fig. 2.

Conformational energy calculations. The conformational energy diagrams of Actodigin and digitoxigenin were generated using a version of the molecular mechanics program CAMSEQ (21), which was specially modified to be used in conjunction with the NIH PROPHET Computer system (22). The CAMSEQ program uses empirical potential functions for steric, electrostatic, and torsional interactions, as well as a molecule-solvent term and a molecular dipole-solvent term to evaluate the relative energies associated with molecular geometry. The energies thus obtained provide a useful measure of the conformational preferences of a complex molecule, particularly since a rigorous *ab initio* calculation is presently impossible even with modern computer technology (23). Each diagram was calculated using the crystallographic structure as a starting model. The published structural data of Karle and Karle (24) were used as the basis of the CAMSEQ calculations for digitoxigenin. The conformation of the lactone ring relative to the steroid backbone was varied by rotation about the C17–C20 bond in steps of 10°. The energy for each point was calculated using the nonbonded and electrostatic potentials built into the CAMSEQ program. Each diagram was then shifted to place the minimum at the zero energy level, and then displayed using the graphics display features of PROPHET as shown in Fig. 3.

The crystallographically observed orientation of the lactone ring in both digitoxigenin (C13–C17–C20–C22 = 76.3°) and Actodigin (C13–C17–C22–C23 = −106.9°) correspond to the minimum energy conformations from

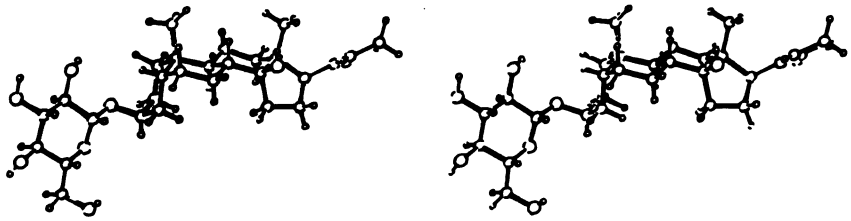


FIG. 1. *Stereoscopic ORTEP view of the molecular structure of Actodigin*
Viewing information is contained in METHODS.

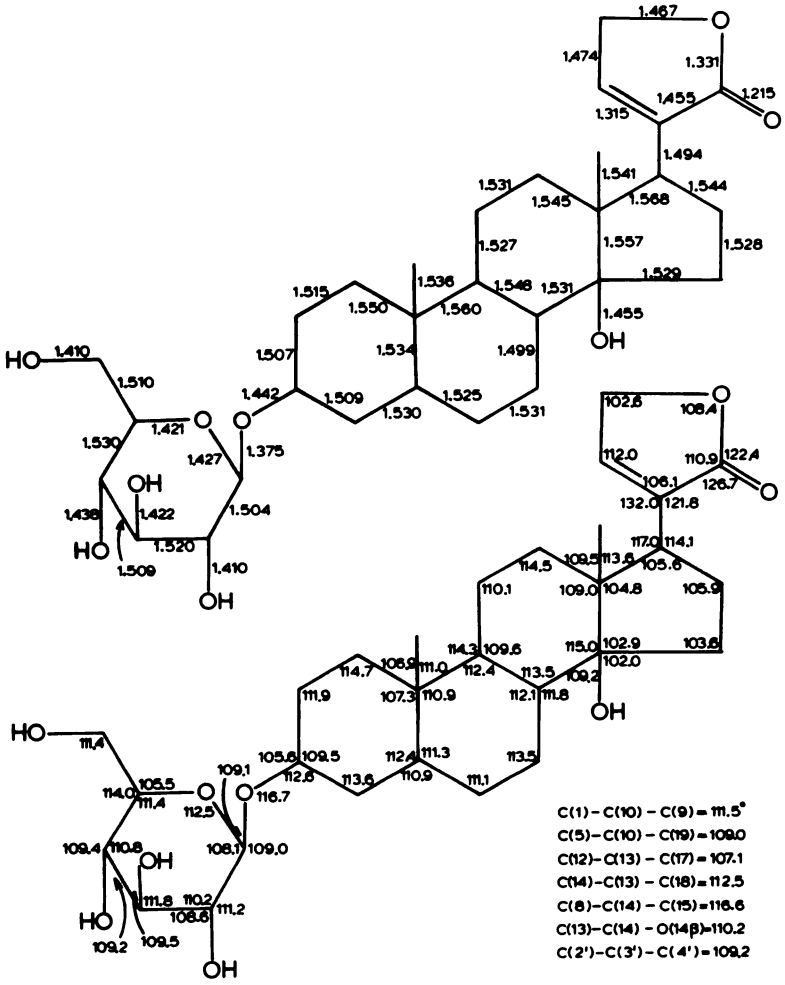


FIG. 2. *Intramolecular dimensions of Actodigin*
Top: Bond distances (Å); σ range = 0.003 to 0.005 Å. Bottom: Bond angles (degrees); σ range = 0.2 to 0.3°.

these calculations. Furthermore, no differences were found in the locations of minima or the shape of the curves when solvent (water) interactions were included in these calculations. Therefore, the crystallographically determined atomic positions of each were used in the biological activity and structural comparisons described in the next sections.

Structural comparisons using PROPHET. The PROPHET system provides programming and graphics procedures for direct comparison, measurement, and manipulation of molecular structures. On an atom by atom basis, the PROPHET program FITMOL (25) was used to superimpose structurally similar portions of the steroid backbones of Actodigin and digitoxigenin. The program was then used to measure distances between relative positions of specific atoms in the two molecules. Such measurements are impossible with plastic or steel molecular models because, of course, such models cannot be completely superimposed. The FITMOL superposition is illustrated in Fig. 4 wherein the atomic positions of C1 through C19 and 03β of Actodigin in its crystallographic conformation were fit to the corresponding atoms in digitoxigenin in its crystallographic conformation. The average separation between fit atoms was 0.09 Å.

Na^+,K^+–ATPase inhibition studies. Partially purified Na^+,K^+-ATPase preparation from rat brain was utilized for these studies (26). The method of testing the inhibitory effect of various drugs on the Na^+,K^+-ATPase was

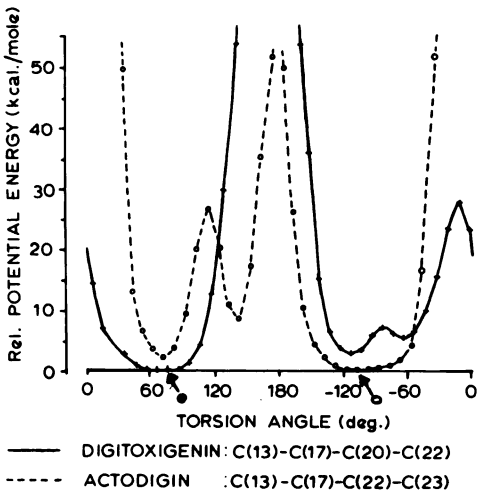


FIG. 3. *The relative nonbonded potential energies calculated by PROPHET CAMSEQ, associated with rotation of the lactone rings of digitoxigenin and Actodigin*

Crystallographically observed torsion angles for (●) digitoxigenin (76.3°) and for (○) Actodigin (−106.9°) are marked with arrows.

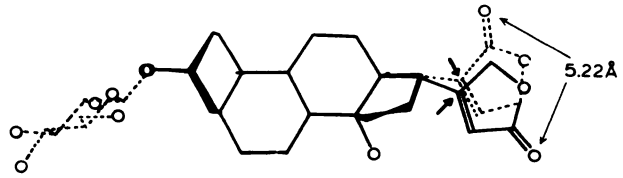


FIG. 4. *PROPHET FITMOL superpositions of atoms C1 through C19 and 03 in Actodigin (dashed line) and digitoxigenin (solid line) in their crystallographically observed conformations*

The distance between carbonyl oxygens is shown. The distance between the superimposed C20 and C22 atoms (at arrows) is 0.43 Å.

precisely as described in our previous work (5, 26, 27). In brief the small, standard reaction medium in a final volume of 2 ml consisted of 110 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 3 mM ATP (Tris salt), 30 mM Tris-HCl buffer, pH 7.45 at 37°, and about 5 μg of enzyme protein in 0.1 to 0.2 ml of a medium consisting of 0.25 M sucrose/1 mM EDTA/10 mM imidazole-HCl, pH 7.45, at 37°. The reaction time was adjusted to keep the amount of ATP hydrolyzed under 10% of the total substrate concentration. The reaction was started by adding the enzyme to the assay media with and without the presence of the drugs. Alternately, the system was allowed to preincubate for 10 minutes as above but in the absence of added K^+ to allow maximal binding of the drug to the enzyme system. Appropriate amounts of KCl were then added and the reaction was allowed to proceed as usual to assay the ATPase activity. Na^+,K^+-ATPase was measured as micromoles of P_i formed per milligram of protein per hour in the presence of Mg^{2+} + Na^+ + K^+ minus that in the presence of Mg^{2+} + Na^+. I_{50} values for the drugs (with and without preincubation) were estimated as described previously (5, 26, 27). The specific activity of the Na^+,K^+-ATPase used in these experiments was 400–500 μmol P_i/mg of protein/hr, of which some 95% was ouabain-sensitive. The rat brain enzyme was used in these studies for two reasons: (1) the ease of preparation of the enzyme with high activity; and (2) the *in vitro* sensitivity of this brain preparation to cardiac glycosides is comparable to that of heart Na^+,K^+-ATPase preparations from other species (compare, e.g., Refs. (27–29)).

RESULTS AND DISCUSSION

The potential energy plot for rotation of Actodigin's lactone ring (Fig. 3) as calculated by CAMSEQ shows that the crystallographically observed conformation (C13–C17–C22–C23 = −106.9°) is in the widest and deepest energy minimum. Since the plot did not change when solvent effects of water were introduced into the CAMSEQ program, this crystallographically observed minimum should therefore represent the largest conformational population in solution. The X-ray crystal structure of Actodigin (stereoscopic view, Fig. 1) is thus very useful for studying Actodigin's molecular pharmacology. It provides (1) precise atomic coordinates, bond lengths, and positions (e.g., for conformational energy studies) and (2) as will be discussed, unexpected insights into Actodigin's molecular geometry relative to natural digitalis cardenolides such as digitoxigenin (VII).

The conformational energy plot of both Actodigin and digitoxigenin reflect a variety of intramolecular interactions. The high energy regions of the plot for Actodigin around 0 and 180° result primarily from close nonbonded contacts between 023 or H20 with the hydrogens on C18. The smaller peak around 110° results from the close approach of 023 to the C16β hydrogen. The potential energy plot for digitoxigenin shows two large peaks around 0 and 180° resulting from close approach of the C21 and C22 hydrogens to the hydrogens on C18. The smaller peak around −80° is caused by the nonbonded interaction between the C21 hydrogens and the C16β hydrogen. Again the crystallographically observed conformation (C13–C17–C20–C22 = 76.3°) is in the lowest energy region of the plot.

FITMOL overlays (Fig. 4) of atoms C1 to C19 and 03β of Actodigin and digitoxigenin reveal the following:

(1) The average fit of all these atoms is good, less than 0.1 Å. This is consistent with the assumption that the changes in molecular pharmacology of Actodigin relative to natural glycosides are from its altered lactone ring attachment and its β-D-glucose. That is, the modified properties are clearly *not* from any major change in shape of the interior of the steroid ring system. However, rings A and D show the largest deviations—0.1 Å for C3 and C4 and 0.2 Å for C17. The altered positions of these atoms in ring A of Actodigin is obviously from the influence of the β-D-glucose. However, the change in C17 position is the result of an important conformational change in ring D.

(2) We have found that ring D in naturally occurring cardenolides such as digitoxigenin (VII), digoxigenin (VI), and strophanthidin (VIII) is in the C14β-envelope conformation[3] (Fig. 5). However, Actodigin's ring D is in

[3] Discussions of cyclopentane conformation are contained in most organic chemistry textbooks, as well as in *The Atlas of Steroid Structure* (W. L. Duax and D. A. Norton, eds.), Vol. 1. Plenum, New York, 20, (1974).

a C14β | C15α-half chair conformation[3] (Fig. 5) as are, for example, the analogs **XII** and **XI**. Although other analogs will need to be examined, it is clear that the conformation of ring D of Actodigin and other analogs can be greatly changed by the side group. Ring D conformation of genins may also be predictable (6), a major aid in the design of new cardenolide analogs.

(3) The result of the conformational change of ring D is that the side group of Actodigin is moved, for example, 0.43 Å for C22 measured from C20 of digitoxigenin (see the difference of positions of these two atoms in Fig. 4). Consequently *all other* lactone ring atoms in Actodigin are displaced much more than would be predicted by studying molecular models.

(4) In previous studies (4–6) we have found that the farther the cardenolide's side group carbonyl is displaced from that of digitoxigenin, the more is the loss in its potency to inhibit the Na^+,K^+-ATPase. With both Actodigin and digitoxigenin in their crystallographically observed lactone conformations (Fig. 4), this distance is 5.22 Å measured from the carbonyl oxygens. Only analog **XIX** has been found to have a greater carbonyl oxygen displacement (5.74 Å). [Although in a previous study (7) we reported that the lactone rings of digitoxigenin (**VII**), digoxigenin (**VI**), and strophanthidin (**VIII**) can exist in an alternate conformation rotated by 180° (i.e., −104° for digitoxigenin), no correlation exists between carbonyl distances measured from those alternate conformations and Na^+,K^+-ATPase inhibitory potency (4, 5).]

The Na^+,K^+-ATPase I_{50} data (Table 3) reveal other unexpected results described as follows:

(1) Actodigin genin (**II**) is an extremely weak Na^+,K^+-ATPase inhibitor ($I_{50} = 7.0 \times 10^{-5}$ M). Of over 15 genins we have studied to date (4, 5) only analog **XIX** has been found to be less active ($I_{50} = 1\text{–}3 \times 10^{-4}$ M). Further, as noted in the previous section, only **XIX**'s carbonyl is displaced more than Actodigin's. Thus, we have found that Actodigin genins Na^+,K^+-ATPase inhibiting potency is consistent with other genins we have studied (4, 6). For each 2.2 Å a genin's carbonyl oxygen is displaced from that of digitoxigenin (both in their crystallographically observed conformational energy minima), activity drops by 10 ($n = 9$, $r^2 = .994$) (4). Since it is certainly possible to change carbonyl oxygen distance by changing the genin being studied and/or digitoxigenin to a higher energy C17-side group conformation, this relationship

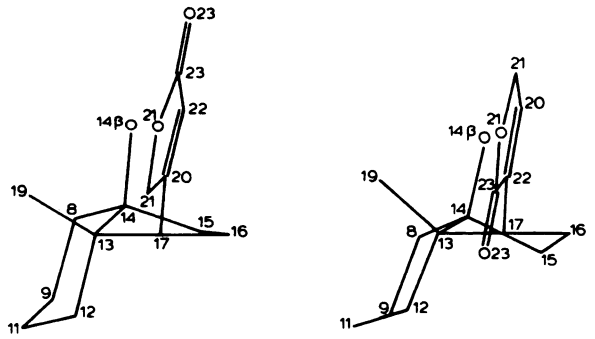


FIG. 5. *Ring D conformations with partial structures*
Left: Digitoxigenin, ring D in C14β-envelope conformation. Right: Actodigin, ring D in C14β/C15α-half chair conformation.

TABLE 3
Na^+, K^+ dependent ATPase inhibition studies[a]

Steroid[c]	I_{50} without preincubation	I_{50} with 10-min preincubation[b]
	(M)	(M)
I. Actodigin	1.0×10^{-6}	1.0×10^{-6}
II. Actodigin genin	7.0×10^{-5}	7.0×10^{-5}
VII. Digitoxigenin	3.5×10^{-7}	3.5×10^{-7}
XV. Digitoxigenin glucoside	3.1×10^{-7}	1.3×10^{-7}
XVI. Digitoxigenin digitoxide	1.1×10^{-7}	5.0×10^{-8}
IV. Digitoxin	4.0×10^{-7}	3.0×10^{-8}

[a] I_{50} values are established from two to four experiments. Appropriate Mg^{2+} + Na^+-containing tubes were included to determine the basal activity of the Na^+, K^+-ATPase in the presence and absence of the drugs. This was then subtracted from activity in the presence of Mg^{2+}, Na^+, and K^+ in the control and test systems.

[b] Steroid added to Na^+, K^+-ATPase medium lacking K^+ to allow the steady-state binding of steroid and enzyme. After 10 min, KCl was added to initiate the Na^+, K^+-ATPase reaction.

[c] The steroids were added in ethanol to the assay tubes. In no case was more than 20 μl of ethanol added per tube. Independent studies showed that significant inhibition (over 3%) of the enzyme preparation by ethanol does not occur until over 25 μl of ethanol was added.

suggests that the receptor site on Na^+,K^+-ATPase seems to prefer the side groups in their conformational energy minima (6). Part of the steroid ring system of the genin would appear to be held in place (or "constant") by the Na^+,K^+-ATPase, thus "fixing" the carbonyl oxygen position. Ring D is probably not involved because the analogs previously reported (4) differ in ring D structure and their activities are explained by carbonyl oxygen position alone. [Further studies are in progress to determine the cause of the decreased activity of A/B *trans* digitalis analog. If the A/B *trans* stereochemistry prevents the steroid ring system from being held in place by the Na^+,K^+-ATPase, then the carbonyl oxygen position would not be fixed. Alternatively, it may be that the good binding of the steroid rings is needed to activate a site on the Na^+,K^+-ATPase which associates with the carbonyl oxygen.]

(2) These data (Table 3) show the remarkable effect of the 3β-glucose on Actodigin's Na^+,K^+-ATPase inhibiting potency. Actodigin is 70 times more active than its genin. In contrast, digitoxigenin glucoside (**XV**) is only *slightly* more active than its genin, digitoxigenin (**VII**). Thus, the glucose of Actodigin is having a much greater effect on its Na^+,K^+-ATPase inhibitory activity than the glucose of digitoxigenin glucoside (**XV**).

These data extend the observations of Yoda (10). Yoda has concluded that binding of a glycoside to the Na^+,K^+-ATPase proceeds in two steps: first, binding of the genin to the enzyme and, second, binding of the sugar to the enzyme. His studies of monoglycoside association rate constants indicate the first is probably the rate limiting step. Yoda and Yoda concluded with digitoxin acetates that (a) cardenolide Na^+,K^+-ATPase binding is determined more by the sugar directly attached to the genin C3 than by other sugars (i.e., with glycosides with two sugars or the naturally occurring ones with three such as

III and IV; and (b) sugars such as digitoxose and rhamnose having a 3′β-OH bind much better to the enzyme than sugars that have a 3′α-OH (such as glucose, although glucose was not specifically included in these studies) or no 3′-OH at all.

Our results, described above, are consistent with the first two conclusions. For example, digitoxigenin digitoxide (XVI) was much more active than digitoxigenin glucoside (XV) after allowing 10 min preincubation which would be expected to allow "second step" binding of the sugar. Further, digitoxin with three digitoxoses was only slightly more active than digitoxigenin digitoxide (XVI) with just one digitoxose, illustrating the pivotal role of the first sugar in Na^+,K^+-ATPase binding.

However, our data indicate that if the genin does activate a sugar binding site (as suggested by Yoda), even extremely weak genins such as Actodigin genin (II) may be able to cause the activation. A normally weakly binding sugar such as glucose can have a major effect on I_{50} if its genin is so geometrically altered as to be unable to significantly inhibit Na^+,K^+-ATPase by itself. It is interesting to note that ten minutes preincubation of Actodigin (Table 3) does not change its I_{50}—in contrast to the effect of preincubation (predicted by the Yoda model) with digitoxigenin digitoxide (XVI), digitoxigenin glucoside (XV) or digitoxin (IV). It is possible that the unusually large increase of potency by the glucose in Actodigin may originate from additional effects other than glucose–receptor binding. Further, the K_d of the glucose–sugar receptor interaction may be somewhat decreased by the conformational and geometrical abnormalities of the genin of Actodigin. The Actodigin glucose could also be binding at a different sugar site, but one wonders if there is enough room right next to the C3 end of the genin binding site for two completely different sugar sites. Other causes are possible and further studies will be needed to explore these effects.

These data also indicate that a previous Actodigin binding model (2) may be partially incorrect. According to this model the genin portion of Actodigin binds well to the Na^+,K^+-ATPase, attached to the genin lactone. The altered geometry of the lactone in turn causes the rest of the genin to be directed somewhat *away* from Na^+,K^+-ATPase, and therefore too far for the glucose to bind. Clearly, the I_{50} data presented in Table 3 suggest this is not the case. Actodigin genin (II), as reflected in its I_{50}, is almost certainly binding weakly to the Na^+,K^+-ATPase—or if strongly, it is nevertheless unable to appreciably inhibit the enzyme. The addition of glucose to Actodigin genin causes a remarkable increase in Na^+,K^+-ATPase inhibiting potency. The glucose clearly can still interact with the enzyme, apparently much more effectively than with more active genins.

Further studies are in progress on Actodigin and other cardenolide glycosides to delineate the role of sugar conformation on I_{50} and glycoside binding.

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