mals (ten eye swabs) and five TK$^-$-inoculated animals (ten eye swabs) (Table 2, left).

The isolation of similar amounts of TK$^+$ and TK$^-$ HSV from eye swabs but decreased amounts of TK$^-$ HSV in ganglion homogenates suggests that TK$^-$ virus may not be transmitted via the trigeminal nerve to the ganglion as efficiently as TK$^+$ virus or, more likely, that TK$^-$ virus may not survive or replicate as well as TK$^+$ virus in ganglionic neurons. These results indicate that HSV-induced TK may play a role in viral pathogenesis in neural but probably not in nonneural tissues and that the presence of the TK gene in HSV may be important in the pathogenesis of natural HSV infection of ganglion neurons.

The TK$^+$ HSV phenotype was found to be important in in vitro infection of nondividing cells (*3*), and may be analogous to infection in vivo in nondividing cells, that is, neurons. While TK$^-$ HSV was easily isolated from ocular tissues where many dividing or potentially dividing cells are present, TK$^-$ HSV was not isolated from ganglion tissue. Nonneuronal supporting and sheathing cells are capable of division, but for virus to reach these cells, it probably must first pass through ganglionic neurons. While the role of other possible defects in the TK$^-$ viruses cannot be excluded, our results indicate the probable importance of the HSV TK$^+$ phenotype in infection of the trigeminal ganglion.

RICHARD B. TENSER
Departments of Medicine (Neurology) and Microbiology and Specialized Cancer Research Center, Pennsylvania State University College of Medicine, Hershey 17033
RICHARD L. MILLER
Riker Laboratories, 3M Center, St. Paul, Minnesota 55101
FRED RAPP
Department of Microbiology and Specialized Cancer Research Center, Pennsylvania State University College of Medicine

References and Notes

1. J. R. Baringer and P. Swoveland, *Lab. Invest.* **30**, 230 (1974).
2. M. L. Cook, V. B. Bastone, J. G. Stevens, *Infect. Immun.* **9**, 946 (1974).
3. A. T. Jamieson, G. A. Gentry, J. H. Subak-Sharpe, *J. Gen. Virol.* **24**, 465 (1974).
4. S. Kit, G. N. Jorgensen, D. R. Dubbs, S. Chan, W. Leung, *Virology* **69**, 179 (1976).
5. H. Yamamoto, M. A. Walz, A. L. Notkins, *ibid.* **76**, 866 (1977).
6. W. Munyon, E. Kraiselburd, D. Davis, J. Mann, *J. Virol.* **7**, 813 (1971).
7. J. F. Aswell, G. P. Allen, A. T. Jamieson, D. E. Campbell, G. A. Gentry, *Antimicrob. Agents Chemother.* **12**, 243 (1977).
8. Virus was adsorbed to RK monolayer cells at a multiplicity of 0.1 for 1 hour at 37°C. Medium (1 ml of Eagle's minimum essential medium containing antibiotics and 5 percent calf serum) with or without ara-T (50 μg/ml) was added. After 48 hours, CPE was recorded, and tube cultures were frozen at −70°C. Cultures were thawed, and duplicate cultures were pooled and centrifuged at 2500 rev/min for 10 minutes at 4°C. Supernatants were titrated on RK monolayer cells in 60-mm dishes under a 0.5 percent methylcellulose overlay. After incubation for 4 days, monolayers were stained with neutral red and plaques were counted.
9. Homogenates of uninfected TK$^+$ and TK$^-$ mouse fibroblastic cells (N clA and N clA cl10, respectively, provided by R. Goldberg of NIH) and of TK$^-$ cells lytically infected with TK$^+$ and TK$^-$ HSV were tested. Monolayer cells in 60-mm dishes were infected at a multiplicity of 1 to 2. After being allowed to absorb for 40 minutes at 37°C, virus was removed and 5 ml of Dulbecco's medium supplemented with antibiotics and 5 percent fetal calf serum was added. After the cells were incubated at 37°C for 16 hours, medium was decanted and homogenates of infected cells as well as those of uninfected TK$^+$ and TK$^-$ cells were prepared. To each dish, 0.5 ml of homogenization buffer (tris, 0.01*M*, pH 7.9; 2-mercaptoethanol, 0.0014*M*; KCl, 0.025*M*; $MgCl_2$, 0.02*M*) was added. Cells were harvested with a rubber policeman, put into centrifuge tubes, and frozen at −70°C. After thawing, the homogenized cells were centrifuged at 16,000 rev/min (31,000*g*) for 1 hour at 4°C. Supernatants were tested for TK activity. Supernatant (20 μl) and reaction buffer (20 μl) (homogenization buffer plus 0.1*M* adenosine triphosphate, 0.05 m*M* thymidine, and ^{3}H at 2 μCi/ml) were inoculated on 1.25-cm squares of DEAE-81 filter paper and incubated for 20 minutes at 37°C in a humidified incubator. Filter squares were washed three times with 2-ml portions of 0.03*M* ammonium formate, three times with distilled water, and once with absolute ethanol. Filters were then dried, and residual ^{3}H activity was determined after addition of 5 ml of toluene-based scintillation fluid and counting in a liquid scintillation counter (Beckman LS-250). The amount of thymidine phosphorylated was determined after subtracting background activity.
10. R. B. Tenser and G. D. Hsiung, *Infect. Immun.* **16**, 69 (1977).
11. H. J. Field and P. Wildy, *J. Hyg.* **81**, 267 (1978).
12. Supported in part by contract NO1 CP 53516 within the Virus Cancer Program of the National Cancer Institute, NIH grants CA 18450 and 1 R01 NS 14568, and NIH Teacher-Investigator award 1 KOS NS 00248. We thank Marie Miller for technical assistance.

22 February 1979; revised 25 May 1979

Digitalis Genin Activity: Side-Group Carbonyl Oxygen Position Is a Major Determinant

Abstract. *The Na$^+$,K$^+$-adenosine triphosphatase-inhibiting activity of digitalis genins and their analogs is a function of side-group carbonyl (C=O) oxygen position. For each 2.2 angstroms that this oxygen is displaced from its position in digitoxigenin, activity drops by one order of magnitude. This quantitative relation resolves previously proposed models which have attempted to describe the molecular basis of genin activity. A multidisciplinary (crystallographic, conformational energy, synthetic, biological) approach to structure-activity relations is described.*

Digitalis preparations remain a major therapeutic modality in the management of cardiovascular disease. The pharmacological effects of the digitalis glycosides [digoxin (**1**) and digitoxin (**2**)] and their genins [digitoxigenin (**3**) and digoxigenin (**4**)] appear to be the result of inhibition of membrane-bound Na$^+$, K$^+$-adenosine triphosphatase (Na$^+$, K$^+$-ATPase) (*1, 2*).

An array of often conflicting models has been proposed to describe the chemical and geometric characteristics that determine the capacity of a given genin to inhibit the Na$^+$,K$^+$-ATPase (*3*). However, the activity of a number of modified genins has been unexplained by, or is inconsistent with, important portions of these models. For example, aldehyde **5** was predicted to be up to 123 times more active than digitoxigenin, on the basis of proposed models, but it was found to be slightly less active (*4*). The C20 stereoisomers **8** and **9** were unexpectedly found to have greatly different activities (*5, 6*). The glucose glycoside actodigin (AY-22,241) with an unusual genin lactone linkage (**7**) has been of interest because of its unpredicted rapid reversibility of Na$^+$,K$^+$-ATPase inhibition and toxicity (*7*). Finally, although it has been presumed that the 14β-hydroxyl group enhances inhibition of Na$^+$, K$^+$-ATPase, the 14-anhydro analog **8** was found to be more active than the 14β-hydroxyl analog **6** and its C20 stereoisomer (*8, 9*).

A multidisciplinary approach, including x-ray crystallography, conformational energy calculations, organic synthesis and Na$^+$,K$^+$-ATPase inhibition studies, has been used to find the straightforward relation between genin structure and activity which we report. Nine genins (**3** to **11**) (Fig. 1) differing widely in construction, stereochemistry, and geometry were selected for study (*10*). In compounds **3** to **7** and **11**, C14 is tetrahedral and (except for **6**) C20 is planar. The reverse is true for **8** and **9**. In **10**, C14 and C20 are both planar. Aldehyde **5** is illustrative of the group of active genins that do not have a lactone ring for a side group. Six have the presumably important 14β-hydroxyl, and three do not. The genin portion (**7**) of AY-22,241 was included in our study, as was strophanthidin (**11**), with its additional oxygen substituents on C5 and C19.

Although the steroid backbone of these molecules is quite rigid, the 17β side group can rotate about the C17–C20 bond. In order to explore this conformational freedom, relative conformational

0036-8075/79/0831-0917$00.50/0

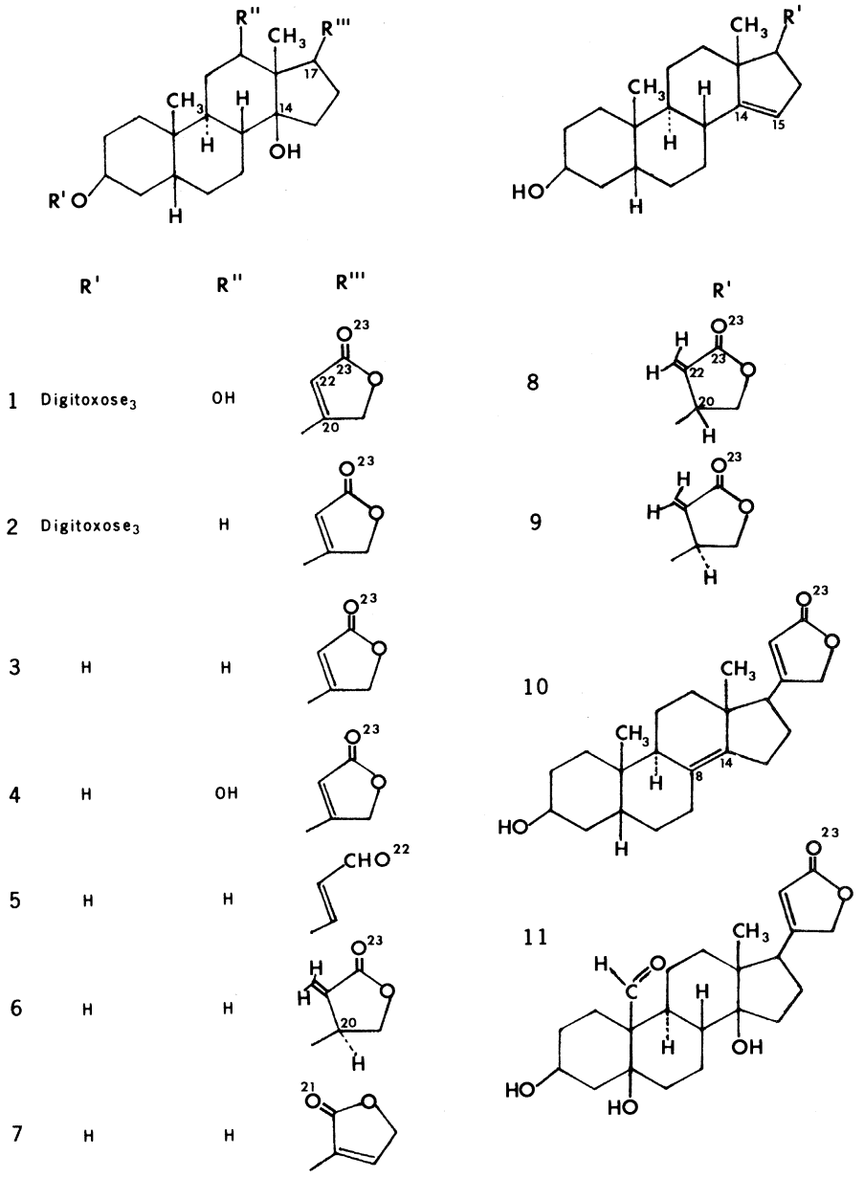

Fig. 1. The genins digitoxigenin **3** and its analogs **5** to **10**, digoxigenin (**4**), and strophanthidin (**11**) were studied. The naturally occurring glycosides, digoxin (**1**) and digitoxin (**2**) are shown for comparison.

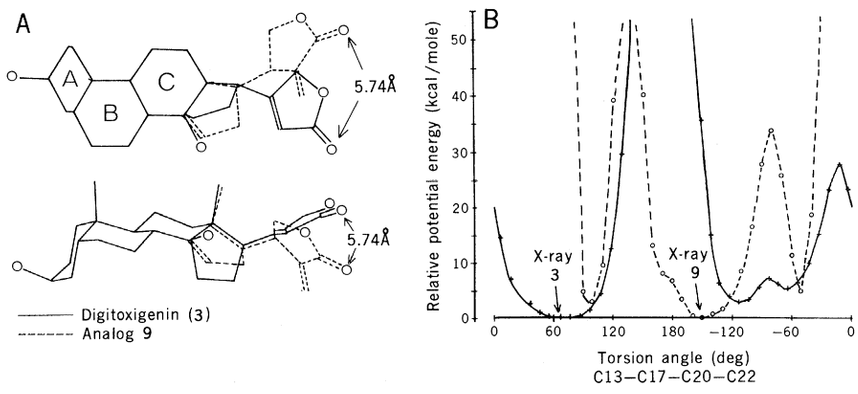

Fig. 2. (A) Top and side views of the superimposed A, B, and C rings of analog **9** and digitoxigenin (**3**) obtained from FITMOL (*14*). The x-ray crystallographically observed conformations of the lactone rings and distance between carbonyl oxygens are shown. (B) The relative calculated potential energies for **9** (dashed line) and **3** (solid line) obtained from CAMSEQ (*11*) for rotation of the C17 side group. The observed x-ray crystallographic conformations of **9** and **3** are indicated.

energies were calculated with a version of the CAMSEQ (*11*) program, which was specially modified to be used in conjunction with the NIH PROPHET computer system (*12*).

With the crystallographic coordinates (*13*) as a starting point, the energy was evaluated at 10° rotation steps of the 17β side group, with the use of the nonbonded and electrostatic potential built into the program. No differences were found in the locations of minima or in the shape of the curve when solvent (that is, water) interactions were included in these calculations. The result was a plot of potential energy for each genin illustrated by the plots for **3** and **9** in Fig. 2B. In each case, the crystallographically observed conformation was at a potential energy minimum calculated by this method. In agreement with previous crystallographic observations (*6, 9*) more than one energy minima for digitoxigenin (**3**), digoxigenin (**4**), and strophanthidin (**11**) were found. Since all these studies showed an excellent correlation between the observed crystallographic conformation of the genins with their calculated energy minima in solution, the crystallographically determined atomic positions of each genin were used in subsequent measurements.

The PROPHET procedure FITMOL (*14*) was used to superimpose the structurally similar portions of the steroid backbone of genins **4** through **11** on the corresponding portion of the most active genin in the series, digitoxigenin (**3**). In every case, the average separation between the corresponding atoms in the superimposed portions of the two molecules was less than 0.1 Å. Figure 2A shows the superimposition of digitoxigenin (**3**) and genin **9** obtained in this manner. The distance between atoms such as the carbonyl oxygens was calculated by FITMOL and is shown in Fig. 2A for **3** and **9**.

The I_{50} (molar concentration for 50 percent inhibition) in vitro Na^+,K^+-ATPase inhibitory activities of the genins were determined with the use of rat brain Na^+,K^+-ATPase (E.C. 3.6.1.3), the preparation and assay of which have been reported (*15, 16*). Rat brain enzyme was used in these studies for two reasons: (i) the preparation of high activity enzyme is easy and (ii) the sensitivity of most heart and brain enzyme preparations is of the same order (*16*). The I_{50} ranges for the poorly water-soluble genins **9** and **10** were extrapolated from their dose-response curves at concentrations where they were completely soluble. Each genin was first incubated with the enzyme for 10 minutes, that is, mixing

steroid, enzyme, and media lacking K^+ before the addition of KCl to maximize inhibitory effects (*17*).

Comparison of relative carbonyl oxygen separations obtained from FITMOL and Na^+,K^+-ATPase-inhibiting activities revealed a striking correspondence. For each 2.2 Å that this atom is displaced from that of digitoxigenin, activity drops by one order of magnitude. A simple linear regression model was used to test the relation between carbonyl oxygen separation and Na^+,K^+-ATPase I_{50}. The nine points were found to fit the line shown in Fig. 3 (*18*):

$$\log I_{50} = +0.457\,D - 6.47$$

where D = carbonyl oxygen separation in angstroms.

$$r^2 = .994$$

For test of the regression relationship, $P = .0001$. The linear nature of the relationship illustrated in Fig. 3 also suggests that Na^+,K^+-ATPase preferentially binds to low energy conformers of each genin.

This simple relationship provides an explanation for the lower-than-predicted (*4*) activity of aldehyde **5** (opening the lactone ring moves O22); the greater activity of the 20(*R*) form **8** than that of its 20(*S*) stereoisomer **9** [changing C14 from tetrahedral, as with **3**, to planar as with **8** and **9** moves O23, but this move is partially compensated for by the 20(*R*) stereochemistry]; and the genin contribution to the modified biological activity of AY-22,241 (the genin **7** being a weak Na^+,K^+-ATPase inhibitor because of its unusual O21 position). The better activity of 14-anhydro **8** than that of either 14β-hydroxyl **6** or its C20 stereoisomer suggests that the 14β-hydroxyl group may not be directly contributing to Na^+,K^+-ATPase inhibition as much as previously believed (*3*, *19*).

It has also been assumed in previous models that the side-group carbon-carbon double bond plays an active role, in Na^+,K^+-ATPase inhibition (*3*). It would appear from Fig. 3 that this double bond's primary role is a passive one—keeping the carbonyl oxygen pointed in the appropriate direction for maximal Na^+,K^+-ATPase effect. This has been confirmed in separate chemical and biological studies in which the two C20 stereoisomers of dihydrodigitoxigenin have been characterized and tested for Na^+,K^+-ATPase inhibition (*8*).

The role of the stereochemistry relation between rings A and B on carbonyl oxygen position and a structural correlation of unusual Na^+,K^+-ATPase inhibitors, such as prednisolone bisguanylhydrazones (*3*) await further study. However, our results clarify and simplify the molecular pharmacology of digitalis genins (*19*).

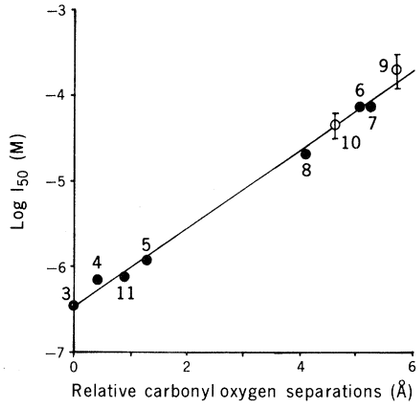

Fig. 3. Correlation between carbonyl oxygen position relative to digitoxigenin (**3**) and Na^+,K^+-ATPase inhibiting activity. (●) I_{50} measured; (○) I_{50} extrapolated from lower concentrations in which the analog was completely soluble in the enzyme media.

DWIGHT S. FULLERTON*
KOUICHI YOSHIOKA
School of Pharmacy, Oregon State University, Corvallis 97331
DOUGLAS C. ROHRER
Medical Foundation of Buffalo, Inc., Buffalo, New York 14203
ARTHUR H. L. FROM
Cardiovascular Division, Veterans Administration Medical Center, and *University of Minnesota, Minneapolis 55455*
KHALIL AHMED
Department of Laboratory Medicine and Pathology, Veterans Administration Medical Center, and *University of Minnesota*

References and Notes

1. Recent reviews: A. Askari, Ed., *Ann. N.Y. Acad. Sci.* **242** (1974); T. Akera, *Science* **198**, 569 (1977); A. Schwartz, G. E. Lindenmayer, J. C. Allen, *Pharmacol. Rev.* **27**, 3 (1975); H. Flasch and N. Heinz, *Naunyn-Schmiedebergs Arch. Pharmacol.* **304**, 37 (1978); T. Akera and T. M. Brody, *Pharmacol. Rev.* **29**, 187 (1978).
2. Changes in levels of cyclic adenosine monophosphate (AMP) may also be involved with the effects of these drugs. For example, see A. Schwartz, M. L. Entman, E. C. Ezrailson, D. C. Lehotay, G. Levey [*Science* **195**, 988 (1977)].
3. Recent reviews: T. W. Guntert and H. H. Linde, *Experientia* **33**, 697 (1977); R. Thomas, J. Boutagy, A. Gelbart, *J. Pharm. Sci.* **63**, 1391 (1974); D. S. Fullerton, in *Textbook of Organic Medicinal and Pharmaceutical Chemistry*, C. O. Wilson, R. F. Doerge, O. Gisvold, Eds. (Lippincott, Philadelphia, ed. 7, 1977).
4. D. S. Fullerton, M. C. Pankaskie, K. Ahmed, A. H. L. From, *J. Med. Chem.* **19**, 1330 (1976).
5. D. S. Fullerton, D. M. Gilman, M. C. Pankaskie, K. Ahmed, A. H. L. From, W. L. Duax, D. C. Rohrer, *ibid.* **20**, 841 (1977).
6. D. C. Rohrer, W. L. Duax, D. S. Fullerton, *Acta Crystallogr. Sect. B* **32**, 2893 (1976).
7. A recent review and study: R. Thomas, J. Allen, B. J. Pitts, A. Schwartz, *Eur. J. Pharmacol.* **53**, 227 (1979). We thank Professor Schwartz for sending us a copy of the accepted manuscript.
8. D. S. Fullerton, K. Yoshioka, D. C. Rohrer, A. H. L. From, K. Ahmed, *J. Med. Chem.* **22**, 529 (1979); K. Yoshioka, D. S. Fullerton, D. C. Rohrer, *Steroids* **22**, 511 (1978).
9. D. C. Rohrer and D. S. Fullerton, *Abstracts*, American Crystallographic Association Meeting, Norman, Okla., March 1978; D. S. Fullerton, D. C. Rohrer, K. Ahmed, A. H. L. From, *Acad. Pharm. Sci. Abstr.* **8**, 182 (1976).
10. All the genins have been synthesized in our laboratories [see (*4*, *5*, *8*)] from digitoxin (**1**), except for **7** (obtained from hydrolysis of the C3 acetate of **7**, a gift from Dr. Romano Deghenghi, Ayerst Laboratories, Montreal); **10**, a gift from Dr. Mitsuru Yoshioka, Shionogi Research Laboratories, Osaka, Japan; and **11** and **4**, which were obtained commercially.
11. H. J. R. Weintraub and A. J. Hopfinger, *Int. J. Quantum Chem. Quantum Biol. Symp.* **2**, 203 (1975).
12. For example, C. M. Weeks, V. Cody, S. Pokrywiecki, D. C. Rohrer, W. L. Duax, *Proc. Natl. Comput. Conf.* **43**, 469 (1977); *Chem. Eng. News* **51**, 20 August 1973, pp. 20–22; *Fed. Proc. Fed. Am. Soc. Biol. Med.* **32**, 1744 (1973).
13. All x-ray studies except for **3**, **10**, and **11** were completed at the Medical Foundation of Buffalo, Inc., by methods previously described (*6*). Reports on these structures are in preparation. The structure of **3** was reported by I. L. Karle and J. Karle, *Acta Crystallogr. Sect. B* **25**, 434 (1969); that of **10** by R. D. Gilardi and I. L. Karle, *ibid.* **26**, 207 (1970); and that of **11** by R. D. Gilardi and J. L. Flippen, *ibid.* **29**, 1842 (1973).
14. J. J. Wood, Ed., "Public procedures—A program exchange for PROPHET users," NIH Biotechnology Resources Branch, Section 7-46 (FITMOL), October 1978.
15. G. Quarfoth, K. Ahmed, D. Foster, *Biochim. Biophys. Acta* **526**, 580 (1978).
16. K. Ahmed and B. S. Thomas, *J. Biol. Chem.* **246**, 103 (1971); although only a partially purified enzyme preparation was used in these experiments, it should not influence the results. It has been reported that the I_{50} for cardiac glycoside inhibition of Na^+,K^+-ATPase with purification does not change substantially [B. J. R. Pitts, L. K. Lane, A. Schwartz, *Biochem. Biophys. Res. Commun.* **53**, 1060 (1973); K. Ratanabanagkoon, J. F. Dixon, L. E. Hokin, *Arch. Biochem. Biophys.* **150**, 342 (1973)].
17. There was little or no difference in I_{50} with or without prior incubation for any of the genins except digoxigenin (**4**) and strophanthidin (**11**). The added OH group of these genins decreases lipophilicity with the result that binding to the enzyme is concommitantly slower.
18. C. Hollister, Ed., *Users Manual for* PROPHET *Users*, NIH Biotechnology Resources Branch, Section 7-14 (FITLINE), October 1978. If just the five points at longer carbonyl oxygen separations are used to fit a linear regression model, a slightly steeper curve results: $\log I_{50} = +0.567\,D - 7.01$, where D = carbonyl oxygen separation in angstroms; $r^2 = .97$ and $P = .001$. This is largely due to the point for **9**, obtained by extrapolation. When just the four points at smaller carbonyl oxygen separations are used to fit a linear regression model, a slightly less steep curve results: $\log I_{50} = +0.373\,D - 6.40$; $r^2 = .91$ and $P = .001$. We conducted a test for equality of these regression lines, and, with a P value of .7867, we concluded that they are the same. We are designing compounds that should have intermediate (about 3 Å) carbonyl oxygen separations, thus to absolutely resolve whether or not there is a small change in slope in this mid-range.
19. B. K. Naidoo, T. R. Witty, W. A. Remers, H. R. Besch, *J. Pharm. Sci.* **63**, 1391 (1974). Professor Remers has learned that his 14β-H analog of digitoxigenin "contained a fair amount of the 14(15)-ene." (Personal communication to D.S.F., January 1979.)

20. The financial assistance of the National Institutes of Health (grants HL 21457 and LM 02325), the Oregon and Minnesota Heart Associations, and the Veterans Administration Medical Research Fund is acknowledged. We also appreciate the technical assistance of G. Quarforth and R. Roelofs in the biological studies; S. Randall in 100-mHz nuclear magnetic resonance studies; Professor D. Griffin in mass spectral studies, and S. Maresh in statistical analyses. The organization and analysis of the data base associated with this investigation were carried out in part with the use of the PROPHET system, a unique national resource sponsored by NIH. Information about PROPHET, including how to apply for access, can be obtained from the director, Chemical/Biological Information-Handling Program, Division of Research Resources, NIH, Bethesda, Md. 20205.

23 January 1979; revised 4 May 1979