yield) of the desired I, bp 151–153° (0.5 mm), $n^{20}D$ 1.5998; maleate salt, mp 141–142°, alone or mixed with authentic material.[2] This material was found to be homogeneous by paper chromatography and gave satisfactory analyses.

The residue from the distillation was recrystallized from acetone repeatedly to give 24 g (12% yield) of **2**, mp 123–125°. This material was homogeneous by paper chromatography and glpc.

Anal. Calcd for $C_{38}H_{40}N_4O_2$: C, 76.08; H, 7.51; N, 10.44; mol wt, 537. Found: C, 75.80; H, 7.78; N, 10.79; mol wt (osmometrically), 541.

The dimer did not give a crystalline salt with either HCl or maleic acid.

5-[o-(o-Acetoxy-N-acetylanilino)benzyl]-5,11-dihydrodibenz-[b,e][1,4]oxazepine.—A mixture of 4.90 g (0.025 mole) of 5,11-dihydrodibenz[b,e][1,4]oxazepine, 1.44 g (0.030 mole) of 50% NaH dispersion in mineral oil, and 50 ml of xylene was stirred and heated under reflux for 8 hr and filtered, and the filtrate was concentrated to dryness *in vacuo*. The residue was extracted with 500 ml of 1% aqueous NaOH. The insoluble brown amorphous polymeric material was separated by filtration. The filtrate was treated with 1 g of Darco and refiltered, and the filtrate was acidified with 10% aqueous H_3PO_4. The precipitated solid was collected, dried, dissolved in 15 ml of benzene, chromatographed on a 1 × 20 cm column of Florisil, and eluted with benzene to give 1.0 g of material, mp 75–84°. In the infrared this material showed a single strong broad OH plus NH absorption band centered at *ca.* 3330 cm^{-1}. The solid, 1.0 g, 10 ml of Ac_2O, and 0.5 ml of pyridine were kept 18 hr at room temperature and concentrated *in vacuo*, and the residue was dissolved in 20 ml of 1:10 benzene–acetone, chromatographed on Florisil, and eluted with the same solvent to give a crystalline product, mp 92–95° dec, ν 1670 and 1750 cm^{-1} with no absorption at 3330 cm^{-1}.

Anal. Calcd for $C_{30}H_{26}N_2O_4$: C, 75.28; H, 5.47; acetyl, 17.9. Found: C, 75.26; H, 5.58; acetyl, 15.4.

5-Benzyl-5,11-dihydrodibenz[b,e][1,4]oxazepine.—To 2.50 g (0.0125 mole) of 5,11-dihydrodibenz[b,e][1,4]oxazepine in 25 ml of anhydrous THF was added 4.28 g (0.025 mole) of benzyl bromide. To the stirred solution was added in 0.5 hr 0.96 g (0.02 mole) of 50% NaH dispersion. The mixture was stirred 18 hr at room temperature and filtered, the filtrate was concentrated *in vacuo*, and the residual solid was recrystallized from pentane to give 1.65 g (46% yield) of product, mp 84–86°.

Anal. Calcd for $C_{20}H_{17}NO$: C, 83.58; H, 5.97; N, 4.88. Found: C, 83.55; H, 6.14; N, 4.95.

O,N-Diacetyl-4-hydroxydiphenylamine.—A solution of 6.0 g (0.033 mole) of 4-hydroxydiphenylamine, 50 ml of Ac_2O, and 0.5 ml of pyridine was refluxed for 1 hr and concentrated to dryness *in vacuo*. The residue was recrystallized from hexane to give 7.2 g (83% yield) of product, mp 117–118°.

Anal. Calcd for $C_{16}H_{15}NO_3$: C, 71.35; H, 5.62; N, 5.21. Found: C, 71.13; H, 5.49; N, 5.26.

12-Benzyl-11,12-dihydro-6H-dibenz[b,f][1,4]oxazocine.—To a solution of 0.90 g (0.004 mole) of 11,12-dihydro-6H-dibenz[b,f]-[1,4]oxazocine in 40 ml of anhydrous THF was added 2.5 ml of 1.6 *N* butyllithium in hexane. The mixture was stirred 1 hr at room temperature, 0.70 g (0.004 mole) of benzyl bromide in 5 ml of anhydrous hexane was added dropwise, and the stirring at room temperature was continued for 48 hr. The solution was concentrated *in vacuo*, and the residue was recrystallized from petroleum ether (bp 30–60°) to give 0.4 g (33% yield) of product, mp 70–72°.

Anal. Calcd for $C_{21}H_{19}NO$: C, 83.64; H, 6.35; N, 4.64; neut equiv, 301. Found: C, 83.19; H, 6.81; N, 4.58; neut equiv ($HClO_4$ in glacial acetic acid), 298.

The Chemistry and Biological Activity of Derivatives of Strophanthidin[1]

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A number of new synthetic derivatives of the cardenolide, strophanthidin, have been prepared in an attempt to delineate and compare the structural requirements for activity in three biological systems; *i.e.*, cytotoxic activity against human carcinoma of the nasopharynx in tissue culture (KB), inhibition of a brain transport ATPase, and cardiotonic activity. Strophanthidin was converted to the monoanhydroacetate derivative Vb and the dianhydroacetate derivative VIb. Epoxidation of Vb yielded a mixture of epoxides (X and XI) in which the α-epoxide X predominated. Treatment of the mixture with hydrogen chloride in chloroform gave the 5α-hydroxy-6β-chloro derivative VII and the hemiacetal of the 5α-chloro-6β-hydroxy derivative IXa. Upon attempted acetylation, VII was largely recovered unchanged, whereas IXa gave the diacetate IXb. Potassium acetate treatment of VII gave pure X, and similar treatment of IXa gave XI. Epoxidation of VIb gave the 5,6α-14,15α-diepoxide XIII and the 5,6β-14,15α-diepoxide XIV. Diepoxide XIII was also obtained by dehydration of X to XII, followed by epoxidation of XII to XIII. Strophanthidin 3-iodoacetate (XVIIb) was converted to the azidoacetate XVIII; the latter was reduced to the 3-aminoacetate hydrochloride XIX, which was directly converted to the 3-diazoacetate XX. The results of the biological tests of the foregoing and other derivatives of strophanthidin indicate that similar structural features are important for activity in each of the three systems. These results indicate that the receptors for the cardenolides in the three systems may be structurally very similar.

The cardiotonic steroids exert a specific and powerful action on the heart muscle and have been successfully used in heart therapy for almost two centuries. Most therapeutically useful members of the group are glycosides, although many aglycones show potent activity. Extensive studies have shed considerable light upon relationships between cardiotonic activity and structure and configuration of the cardiotonic steroids.[3–5]

Cardiotonic steroids have also been shown to be specific and reversible inhibitors of adenosine triphosphatases (ATPases) involved in the active transport of Na^+ and K^+.[6–8] A preliminary study of structure–activity relationships among 21 digitalis derivatives revealed that, in most cases, the ability of a cardiotonic steroid to block a transport ATPase *in vitro* paralleled its activity and toxicity in the intact animal.[9]

(1) (a) Tumor Inhibitors. XXVI. Part XXV of the series: S. M. Kupchan and M. Mokotoff, *J. Med. Chem.*, **10**, 977 (1967). (b) This investigation was supported by grants from the National Institutes of Health (CA-04500 and NB-01730) and the American Cancer Society (T-275).

(2) (a) Fellow of the American Foundation for Pharmaceutical Education, 1963–1965. (b) Research Career Awardee of the National Institutes of Health (5-K6-GM-1347).

(3) K. K. Chen, Proceedings of the 1st International Pharmacological Meeting, Stockholm, 1961, Vol. 3, p 27.

(4) Ch. Tamm, ref 3, p 11.

(5) F. G. Henderson and K. K. Chen, *J. Med. Chem.*, **8**, 577 (1965).

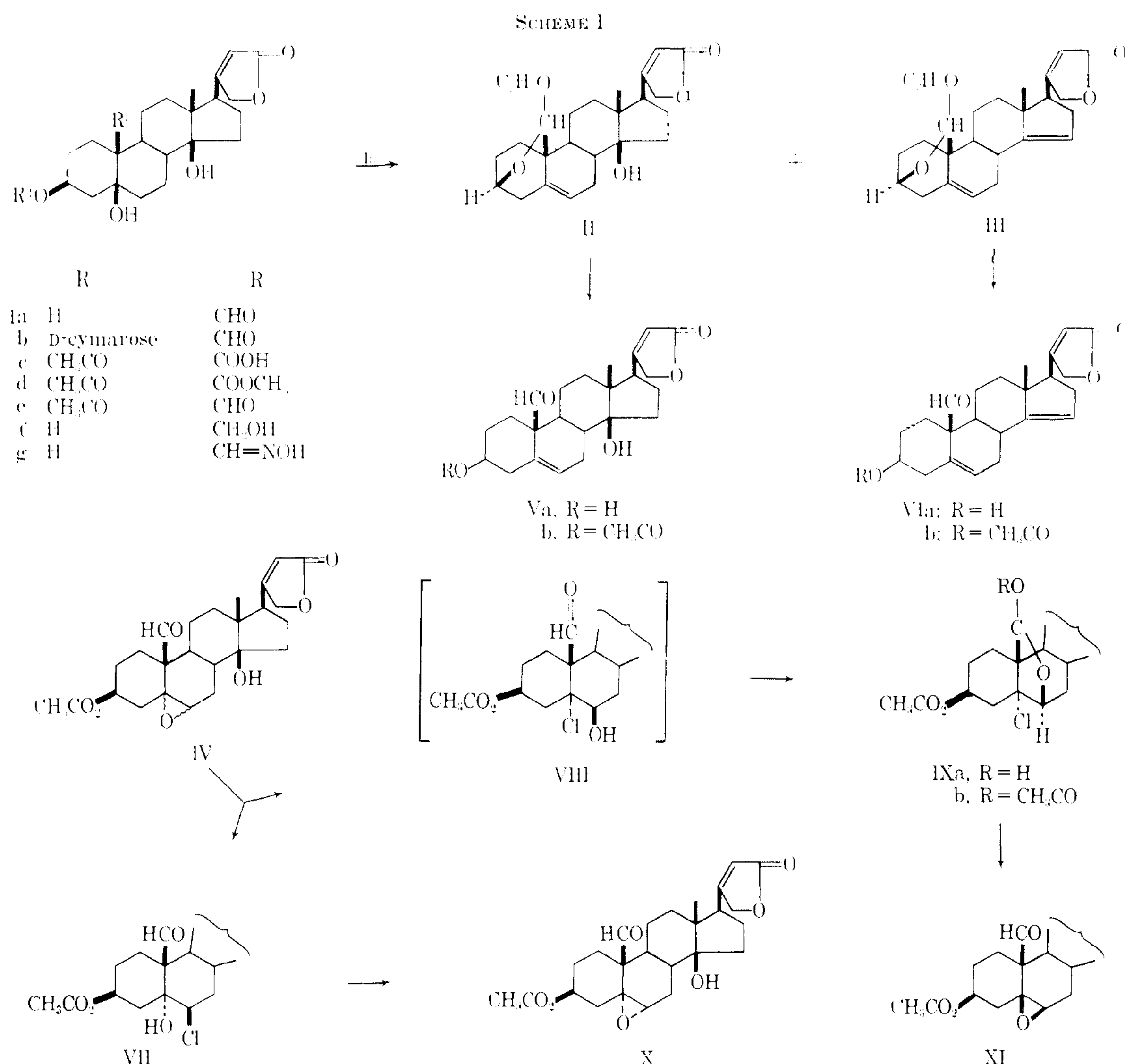
(6) H. J. Schatzmann, *Helv. Phys. Acta*, **11**, 346 (1953).

(7) I. M. Glynn, *Pharmacol. Rev.*, **16**, 381 (1964).

(8) J. C. Skou, *Physiol. Rev.*, **45**, 596 (1965).

(9) K. Repke in "Drugs and Enzymes," B. B. Brodie and J. R. Gillette, Ed., Pergamon Press Ltd., Oxford, 1965, p 65.

SCHEME I



In a third line of investigation, cardiotonic steroids have recently been shown to possess strong cytotoxic activity against human carcinoma of the nasopharynx carried in cell culture (KB).[10-12]

The present report describes a study of the synthesis and biological properties of derivatives of strophanthidin. The cardenolide strophanthidin (Ia) was chosen as a suitable starting material for chemical modification for several reasons. First, the aglycone can be prepared easily by mild acid hydrolysis of the commercially available glycoside, strophanthin-k. Second, strophanthidin has been shown to possess both cardiotonic activity[3] and effectiveness in inhibiting coupled Na^+ and K^+ transport.[6] Third, strophanthidin constitutes the aglycone portion of the cytotoxic glycoside, cymarin (Ib).[10] The strophanthidin derivatives were evaluated for cytotoxic activity against KB, inhibitory activity against brain transport ATPase, and cardiotonic activity in guinea pig atrial muscle. The study was undertaken to further delineate and compare structural requirements for each type of biological activity.

Strophanthidin (Ia) was dehydrated by the method of Jacobs and Collins[13] to yield 3β,19-oxido-14-hydroxycarda-5,20(22)-dienolide 19-ethylal (II) and 3β,19-oxidocarda-5,14,20(22)-trienolide 19-ethylal (III). Compounds II and III were separately hydrolyzed with 2% HCl to afford 3β,14-dihydroxy-19-oxocarda-5,20-(22)-dienolide (Va) and 3β-hydroxy-19-oxocarda-5,14,-20(22)-trienolide (VIa). Acetylation of Va and VIa gave the respective C-3 acetates, Vb and VIb.

To evaluate derivatives which could act as enzyme alkylating agents, the next targets chosen were epoxides of both the monoanhydro (Vb) and dianhydro (VIb) compounds. The acetate Vb was oxidized by monoperphthalic acid in ethereal solution to yield 3β,14-dihydroxy-5,6ξ-epoxy-19-oxo-5ξ-card-20(22)-enolide 3-acetate (IV). Thin layer chromatography on both silica gel and alumina suggested that the product IV was homogeneous. However, earlier investigators[14] have found that epoxidation of Δ^5-3β-steroid alcohols and their esters leads to mixtures of epoxides in which the α-epoxides predominate. Careful analysis of the product obtained by epoxidation of Vb indicated that

(10) S. M. Kupchan, R. J. Hemingway, and R. W. Doskotch, *J. Med. Chem.*, **7**, 803 (1964).

(11) S. M. Kupchan, J. R. Knox, J. E. Kelsey, and J. A. Saenz Renauld, *Science*, **146**, 1685 (1964).

(12) R. B. Kelly, E. G. Daniels, and L. B. Spaulding, *J. Med. Chem.*, **8**, 547 (1965).

(13) W. A. Jacobs and A. M. Collins, *J. Biol. Chem.*, **59**, 713 (1924).

(14) *E.g.*, A. Bowers, L. Cuellar Ibanez, and H. J. Ringold, *Tetrahedron*, **7**, 138 (1959).

a mixture had, indeed, resulted, and that the α-epoxide was the predominant product.

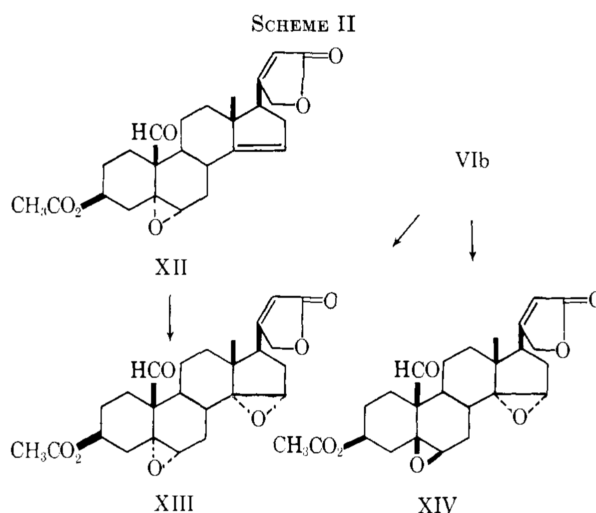
Treatment of IV with HCl in chloroform gave a mixture of two components. The mixture was separated by thick layer chromatography, to give, as major component, 3β,5,14-trihydroxy-6β-chloro-19-oxo-5α-card-20(22)-enolide 3-acetate (VII), in 45% yield, and, as the more polar minor component, 3β,14,19-trihydroxy-5-chloro-6β,19-oxido-5α-card-20(22)-enolide 3-acetate, (IXa), in 5% yield (Scheme I). The infrared spectrum of the minor component (IXa) resembled that of the major component (VII), but there was no evidence of the C–H stretching at 3.62 μ (characteristic of aldehydes). Compound IXa is presumed to have originated by acid-catalyzed hemiacetal formation *via* intermediate VIII.

The assignment of the structures for VII and IXa was based on the following chemical and physical evidence. The chlorohydrin VII, upon treatment with pyridine–acetic anhydride solution, gave recovered starting material, with only a trace of recyclized epoxide (IV). Tertiary hydroxyl groups are known not to be acetylated under the conditions utilized, but secondary hydroxyls readily react.[15] However, the hemiacetal IXa, upon treatment with pyridine–acetic anhydride, readily formed an acetylated product, 3β,14,19-trihydroxy-5-chloro-6β,19-oxido-5α-card-20(22)-enolide 3,19-diacetate (IXb), in 88% yield. The nmr spectrum of IXb showed signals for CH_3CO_2 at τ 7.83 (C-19) and 7.95 (C-3), a vinyl proton at 4.12 (C-22), a hemiacetal proton at 3.85 (C-19), and a 6α-proton at 5.70 (as a multiplet); no signal for an aldehyde proton was observed. The assignments are in accord with those made recently for a chloro hemiacetal acetate in the cholestane series.[16]

Steroid epoxides usually open to give *trans*-diaxial products.[17] Upon treatment of a steroidal 5,6α-epoxide with HCl, the product expected is the 5α-hydroxy-6β-chloro compound; since the hydroxyl is tertiary, it would not acetylate under pyridine–acetic anhydride conditions. This was found to be the case with VII. On the other hand, a steroidal 5,6β-epoxide would normally yield a 5α-chloro-6β-hydroxy compound upon treatment with hydrogen chloride. However, in the case of VIII, the secondary 6β-hydroxyl apparently formed a hemiacetal with the C-19 aldehyde. The latter results supported the conclusion that the monoepoxide IV was a mixture of α-epoxide and β-epoxide in which the α-epoxide was the major component. This was further borne out when the chlorohydrin VII was converted to homogeneous 3β,14-dihydroxy-5,6α-epoxy-19-oxo-5α-card-20(22)-enolide 3-acetate (X), in 67% yield. The hemiacetal IXa was similarly converted to pure 3β,14-dihydroxy-5,6β-epoxy-19-oxo-5β-card-20-(22)-enolide 3-acetate (XI), in 60% yield.[18]

The dianhydro acetate (VIb) was also oxidized with monoperphthalic acid, to afford a product with two major components (Scheme II). Chromatographic separation on neutral alumina afforded the less polar 3β-acetoxy-5,6β-14,15α-diepoxy-19-oxo-5β,14α-card-20(22)-enolide (XIV), in 18% yield, and the more polar

SCHEME II



isomer, 3β-acetoxy-5,6α-14,15α-diepoxy-19-oxo-5α,-14α-card-20(22)-enolide (XIII), in 25% yield. The infrared spectra of both isomers were almost identical and showed absorption at 3.70 (aldehyde proton), 5.60, 5.72 (carbonyl of lactone), 5.76–5.80 (carbonyl of acetate and aldehyde), and 6.15 μ (C=C of α,β-unsaturated lactone).

The configurational assignments of the two diepoxides were based on physical and chemical evidence. The nmr spectrum of the dianhydro acetate (VIb) showed signals at τ 4.08 (broad, C-22 and C-6 vinyl protons) and at 4.65 (multiplet, C-15 vinyl proton), which disappeared (except the C-22 vinyl proton) upon epoxidation. Hofer, *et al.*,[19] reported that, in epoxidation of Δ^{14}-17β-steroids, perbenzoic acid gives *exclusively* the 14,15α-epoxide. Therefore, it was expected that oxidation of VIb with the bulky reagent monoperphthalic acid would give two products which contained the 14,15α-epoxide group, but differed in the configuration of the 5,6-epoxide. The less polar or higher R_f component (XIV) showed signals in the nmr at τ 4.16 (C-22 vinyl proton), 6.40 (C-15 epoxide proton), and 6.72 (C-6 epoxide proton, doublet, with J = 1.5–2.0 cps), whereas the more polar or lower R_f component (XIII) showed signals at τ 4.19 (C-22 vinyl proton), 6.58 (C-15 epoxide proton), and 6.89 (C-6 epoxide proton, doublet with J = 2.5 cps). Tori, *et al.*,[20] have reported that the signals of steroidal epoxide protons of α isomers generally appear at a higher field than that of β isomers. Cross[21] reported the J values for 6β-protons (α-epoxides) as 3.3–4.1 cps (doublet) and for 6α-protons (β-epoxides) as 2.1–2.7 cps (doublet) in steroids with a C-19 methyl group. The foregoing led to the tentative formulation of the less polar compound (XIV) as the 5,6β-14,15α-diepoxide and the more polar compound (XIII) as the 5,6α-14,15α-diepoxide. To seek confirmation, the synthesis of one of the diepoxides by another route was undertaken. Since the configuration of the 5,6α-monoepoxide (X) had been established, this appeared to be a suitable starting material. Compound X was smoothly dehydrated to afford a 72% yield of 3β-acetoxy-5,6α-epoxy-19-oxo-5α-carda-14,20(22)-dienolide (XII). The nmr spectrum of XII showed sig-

(15) P. A. Plattner and W. Lang, *Helv. Chim. Acta*, **27**, 1872 (1944).
(16) M. E. Wolff and J. A. Munoz, *J. Org. Chem.*, **30**, 920 (1965).
(17) R. E. Porter and N. S. Isaacs, *Chem. Rev.*, **59**, 737 (1959).
(18) *Cf.* M. Akhtar and D. H. R. Barton, *J. Am. Chem. Soc.*, **86**, 1528 (1964).
(19) P. Hofer, H. Linde, and K. Meyer, *Helv. Chim. Acta*, **45**, 1041 (1962).
(20) K. Tori, T. Komeno, and T. Nakagawa, *J. Org. Chem.*, **29**, 1136 (1964).
(21) A. D. Cross, *J. Am. Chem. Soc.*, **84**, 3206 (1962).

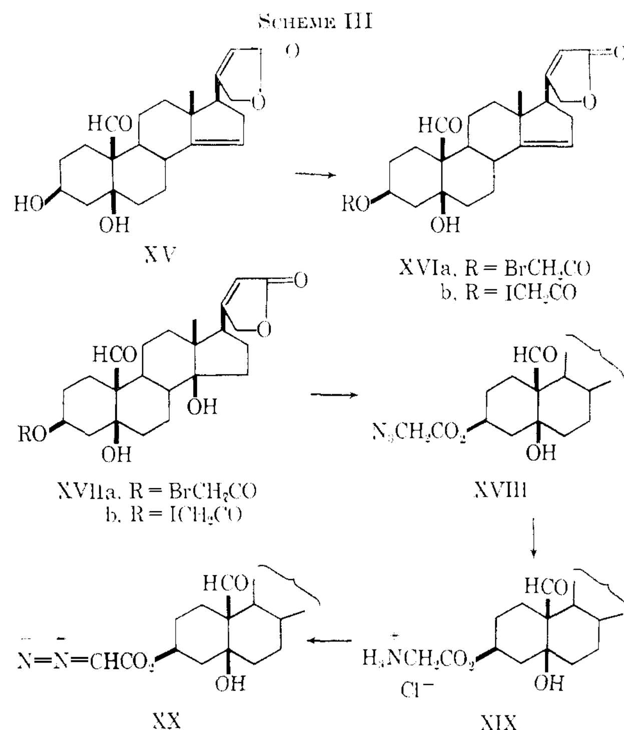
nals at τ 0.10 (aldehyde proton), 4.12 (C-22 vinyl proton), 4.68 (C-15 vinyl proton), and 6.79 (C-6 epoxide proton, doublet, J = 3 cps). Oxidation of XII with monoperphthalic acid afforded a 50% yield of a diepoxide which was identical with XIII (ir, mixture melting point, and nmr). The latter result offered strong support for the configurational assignments to XIII and XIV.

Another class of enzyme alkylating agents is comprised of haloacetate esters.[22] In recent years, such compounds have not only been advanced as potential chemotherapeutic agents,[23] but have found use as active-site-directed irreversible inhibitors of enzymes.[24] The preliminary results of biological evaluation of the above-described strophanthidin derivatives indicated that almost all variations in structure other than in ring A led to compounds with lessened biological activity. Consequently, the synthesis of 3-haloacetate esters of strophanthidin was undertaken.

Strophanthidin 3-bromoacetate (XVIIa), mp 191–194°, and strophanthidin 3-iodoacetate (XVIIb), mp 127–128°, were prepared as described previously.[25] The respective haloacetates had been reported to have been prepared earlier; the melting point of the 3-bromoacetate was recorded as 220–222° and that of the 3-iodoacetate as 186–188°.[26] Further study has shown that the material of mp 220–222° is 3β,5-dihydroxy-19-oxo-5β-carda-14,20(22)-dienolide 3-bromoacetate (XVIa). Strophanthidin (Ia) was treated with thionyl chloride in pyridine to yield 3β,5-dihydroxy-19-oxo-5β-carda-14,20(22)-dienolide (XV).[27,28] Treatment of XV with bromoacetyl bromide and pyridine gave an 86% yield of XVIa, mp 214–215° dec. Direct comparison of XVIa with a sample of the bromoacetate of Welles, *et al.*,[26] confirmed the identity of the materials. Treatment of XV with iodoacetyl chloride in dioxane readily gave 3β,5-dihydroxy-19-oxo-5β-carda-14,20(22)-dienolide 3-iodoacetate (XVIb), mp 162–163° dec, in 73% yield. The melting point differed from that reported for the iodoacetate of Welles, *et al.*[26] Since a sample of the iodoacetate of Welles, *et al.*, is no longer available, no explanation of the discrepancy in melting point is readily apparent. However, on the basis of the fact that our iodoacetate of mp 127–128° was convertible to the same strophanthidin 3-azidoacetate (XVIII) as was obtained from our strophanthidin 3-bromoacetate of mp 191–194°, it is highly probable that our iodoacetate is 3β,5,14-trihydroxy-19-oxo-5β-card-20-(22)-enolide 3-iodoacetate (XVIIb).

Encouraged by the biological activity of XVIIa and XVIIb as irreversible inhibitors of the transport ATPase, we sought next to prepare a diazoacetate derivative. Upon treatment with sodium azide in aqueous dioxane, strophanthidin 3-iodoacetate (XVIIb) was converted to 3β,5,14-trihydroxy-19-oxo-5β-card-20(22)-enolide 3-azidoacetate (XVIII), in 74% yield. The infrared spectrum showed absorption at 3.61 (C–H of aldehyde) and 4.72 μ (azide). The azidoacetate XVIII could also be prepared in 65% yield from the bromoacetate XVIIa. While the catalytic reduction of azides is known to be fast,[29] the reduction of the α,β-unsaturated lactone of cardenolides is sluggish when a Pd catalyst is used.[30] It was therefore feasible to selectively reduce the azide (XVIII) with a catalyst of 6% Pd–$SrCO_3$ under 1 atm of hydrogen. The product isolated was converted with HCl to 3β,5,14-trihydroxy-19-oxo-5β-card-20(22)-enolide 3-aminoacetate hydrochloride (XIX), which was not purified but used directly for the next step. The hydrochloride (XIX) was diazotized to afford 3β,5,14-trihydroxy-19-oxo-5β-card-20(22)-enolide 3-diazoacetate (XX), in 30% yield from the azidoacetate (XVIII). The infrared spectrum showed absorption at 3.63 (aldehyde proton), 4.72 (diazo), and 5.96 μ (ester carbonyl conjugated with diazo group). The ultraviolet spectrum of XX showed maxima at 249 mμ (ϵ 14,700), assigned to the ester carbonyl conjugated with the diazo group, and 217 mμ (ϵ 18,100), assigned to the α,β-unsaturated lactone (see Scheme III).

Scheme III

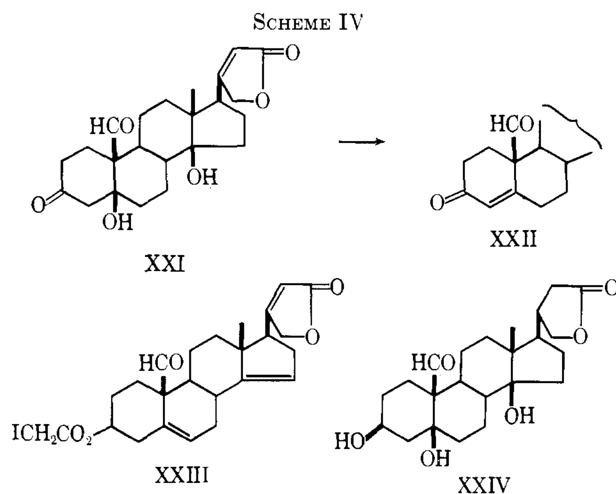


Catalytic oxygenation of strophanthidol (If) in the presence of platinum black gave the 3,19-dioxo derivative XXI. Dehydration of XXI with trifluoroacetic acid afforded the known 3,19-dioxo-14-hydroxy-carda-4,20(22)-dienolide (XXII) (Scheme IV).

A control experiment, designed to determine whether the two irreversible inhibitors XVIIa and XVIIb were acting at or near the cardiotonic steroid site, required the preparation of an iodoacetate of a strophanthidin derivative known *not* to inhibit the transport ATPase. Therefore, 3β-hydroxy-19-oxo-carda-5,14,20(22)-trienolide 3-iodoacetate (XXIII) was prepared as reported elsewhere.[25] The nmr spectrum confirmed the structure of XXIII, with signals at τ 0.27 (aldehyde proton),

(22) M. Dixon and E. C. Webb in "Enzymes," 2nd ed, Academic Press Inc., New York, N. Y., 1964, p 341.
(23) S. P. Kramer, *et al.*, *J. Natl. Cancer Inst.*, **31**, 297 (1963).
(24) B. R. Baker, *J. Pharm. Sci.*, **53**, 347 (1964).
(25) L. E. Hokin, M. Mokotoff, and S. M. Kupchan, *Proc. Natl. Acad. Sci. U. S.*, **55**, 797 (1966).
(26) J. S. Welles, R. C. Anderson, and K. K. Chen, *Proc. Soc. Exptl. Biol. Med.*, **65**, 218 (1947).
(27) P. A. Plattner, A. Segre, and O. Ernst, *Helv. Chim. Acta*, **30**, 1432 (1947).
(28) G. K. Makarichev and N. K. Abubakirov, *J. Gen. Chem. USSR*, **32**, 2338 (1962).
(29) R. Schröter, "Methoden der Organischen Chemie" Houben-Weyl Vol. 11/1, 1957, p 539.
(30) W. A. Jacobs and M. Heidelberger, *J. Biol. Chem.*, **54**, 253 (1922).

Scheme IV



4.04 (multiplet, C-22 and C-6 vinyl protons), 4.65 (C-15 vinyl proton), and 6.34 (singlet, methylene of iodoacetate).

Experimental Section

Melting points were determined on a Fisher-Johns melting point stage which had been calibrated with standard samples; melting points above 220° are uncorrected. Ultraviolet absorption spectra were determined in 95% ethanol on a Beckman (Model DK2A) recording spectrophotometer. Infrared absorption spectra were recorded in KBr on a Beckman (Model 5A) double-beam infrared recording spectrophotometer. Microanalyses were performed by Mr. J. F. Alicino, P. O. Box 267, Metuchen, N. J., and Mr. A. Spang, P. O. Box 111, Ann Arbor, Mich. Nmr spectra were recorded on a Varian Associates recording spectrometer (Model A60) at 60 Mc/sec in $CDCl_3$ (unless otherwise stated), Me_4Si as internal standard. Coupling constants (J) are recorded in cps, while chemicals shifts are recorded in τ values (ppm).[31] Skellysolve B refers to petroleum ether fraction boiling at 60–68°. Thin layer chromatography (tlc) and thick layer chromatography (1.0-mm thick) were carried out with silica gel G and silica gel $HF_{254+366}$ (unless stated otherwise), Brinkmann Instruments. Tlc plates were sprayed with 3% ceric sulfate solution in 3 N H_2SO_4 and were heated to locate the spots. All solvents were evaporated under reduced pressure. Optical rotations were determined in $CHCl_3$ (unless stated otherwise) in a 1.0-dm tube on a Zeiss-Winkel polarimeter and are approximated to the nearest degree.

3β,19-Oxido-14-hydroxycarda-5,20(22)-dienolide 19-Ethylal (II) and 3β,19-Oxidocarda-5,14,20(22)-trienolide 19-Ethylal (III).—Dehydration of strophanthidin (2.000 g, 4.95 mmoles) by the procedure of Jacobs and Collins[13] afforded 1.139 g of crystalline material (A) which consisted of two components, according to tlc. A second crop (B) of 0.123 g was obtained, which was shown to consist of only the higher R_f component, according to tlc (solvent, 3% CH_3OH in $CHCl_3$). The two-component mixture was chromatographed on 50 g of silica gel packed as a slurry in $CHCl_3$. The higher R_f component was eluted with $CHCl_3$ (yield 0.479 g) and was identified as III, mp 226–237° dec, $[\alpha]^{26}D$ −126° (c 0.61) (lit.[13,32] mp 249–251°, 229–235°, and $[\alpha]D$ −142°, −126°, respectively). The second crop (B) was combined with these fractions (total yield 0.602 g, 30%). The lower R_f component was eluted by increasing the polarity to 1% CH_3OH in $CHCl_3$; yield 0.560 g (27%) of II, mp 220–226° dec, $[\alpha]^{26}D$ −39° (c 0.51) (lit.[13,32] mp 223–230°, 197–200° dec, and $[\alpha]D$ −50°, −44°, respectively).

3β,14-Dihydroxy-19-oxocarda-5,20(22)-dienolide (Va) and acetate (Vb) and 3β-hydroxy-19-oxocarda-5,14,20(22)-trienolide (VIa) and acetate (VIb) were prepared according to Jacobs and Collins.[13]

3β,14-Dihydroxy-5,6ξ-epoxy-19-oxo-5ξ-card-20(22)-enolide 3-Acetate (IV).—The monoanhydroacetate (Vb) (1.60 g, 3.7 mmoles) was dissolved in 200 ml of $CHCl_3$ and concentrated to a volume of about 150 ml. The solution was treated initially with 20 ml of monoperphthalic acid in ether solution[33] (0.044 g/1.0 ml, titrated[34]) and then with another 10 ml 11 hr later, and the mixture was allowed to stand for another 9 hr. The mixture was filtered and the filtrate was washed (H_2O, twice with saturated $NaHCO_3$, H_2O). The organic layer was dried (Na_2SO_4) and evaporated to a colorless solid foam. The foam was crystallized from $CHCl_3$–Et_2O; yield 1.14 g of colorless crystals (IV), which appeared as one component on tlc (5% CH_3OH–$CHCl_3$).

3β,5,14-Trihydroxy-6β-chloro-19-oxo-5α-card-20(22)-enolide 3-Acetate (VII) and 3β,14,19-Trihydroxy-5-chloro-6β,19-oxido-5α-card-20(22)-enolide 3-Acetate (IXa).—A solution of 0.704 g (1.58 mmoles) of the monoepoxide IV in 70 ml of $CHCl_3$ (cooled in ice) was treated dropwise with a solution of dry HCl in $CHCl_3$ (approximately 0.4 N by titration). The solution, cooled in ice, was stirred for 2 hr and allowed to stand for another 1 hr and then washed (H_2O, 1% $NaHCO_3$, H_2O). The organic layer was dried (Na_2SO_4) and concentrated to an oil which crystallized upon treatment with a few drops of acetone. The crystals were recrystallized from acetone containing a few drops of HCl in $CHCl_3$ to afford 0.284 g of colorless crystalline VII, mp 227–230° dec. The mother liquor was evaporated to dryness and chromatographed on several silica gel HF thick layer plates (about 0.06 g/plate) with 8% CH_3OH–$CHCl_3$ as the mobile phase. Two bands were extracted, and the upper band afforded another 0.063 g of VII; total yield 0.347 g (45%). Recrystallization of VII from acetone containing a trace of HCl in $CHCl_3$ afforded the analytical sample: mp 233–235° dec; $[\alpha]^{26}D$ −11° (c 0.64, CH_3OH); λ_{max} 3.62 (aldehyde proton), 5.62, 5.72–5.90 (C=O of lactone, aldehyde and acetate), 6.19 (C=C of α,β-unsaturated lactone), and 7.97–8.07 μ (C–O–C of acetate).

Anal. Calcd for $C_{25}H_{33}ClO_7$: C, 62.42; H, 6.93. Found: C, 62.46; H, 6.89.

The lower band from the aforementioned thick layer chromatography yielded 0.067 g of IXa, which, on crystallization from acetone–Skellysolve B, yielded 0.041 g (5%) of crystalline IXa, mp 214–216°. Repeated recrystallizations gave the analytical sample, mp 214–217°, $[\alpha]^{26}D$ +3° (c 0.78, methanol).

Anal. Calcd for $C_{25}H_{33}ClO_7$: C, 62.42; H, 6.93; Cl, 7.37. Found: C, 62.35; H, 6.97; Cl, 7.58

3β,14,19-Trihydroxy-5-chloro-6β,19-oxido-5α-card-20(22)-enolide 3,19-Diacetate (IXb).—A solution of 0.025 g (0.052 mmole) of hemiacetal IXa in 1.0 ml of pyridine was treated with 1.0 ml of Ac_2O. The resulting solution was allowed to stand at room temperature for 21 hr and was then concentrated to dryness The residue was crystallized from acetone–Skellysolve B to yield 0.025 g (88%) of IXb, mp 270–273° dec. Repeated recrystallizations gave the analytical sample of IXb: mp 272–274° dec; $[\alpha]^{25}D$ +23° (c 0.71); nmr, τ 3.85 (1 H, C-19 hemiacetal H), 4.12 (1 H, C-22 vinyl H), 4.85 (1 H, m, 3α-H), 5.12 (2 H, C-21), 5.70 (1 H, m, 6α-H), 7.83 (3 H, C-19 $COCH_3$), 7.95 (3 H, C-3 $COCH_3$), and 9.07 (3 H, C-18).

Anal. Calcd for $C_{27}H_{35}ClO_8$: C, 62.00; H, 6.75; Cl, 6.78. Found: C, 62.14; H, 6.87; Cl, 6.90.

3β,14-Dihydroxy-5,6α-epoxy-19-oxo-5α-card-20(22)-enolide 3-Acetate (X).—Chlorohydrin VII (0.100 g, 0.21 mmole) in 95% ethanol (8 ml) was refluxed for 5 hr with 0.050 g (0.50 mmole) of KOAc. The mixture was concentrated to dryness and the colorless residue was extracted with acetone. The acetone suspension was filtered free of inorganic salt and concentrated to dryness. The resulting residue was crystallized from acetone-Skellysolve B to yield 0.061 g (67%) of the α-epoxide X, mp 265–268° dec. Repeated recrystallizations afforded the analytical sample, mp 268–271° dec, $[\alpha]^{26}D$ −33° (c 0.93, dioxane).

Anal. Calcd for $C_{25}H_{32}O_7$: C, 67.55; H, 7.26. Found: C, 67.47; H, 7.32.

3β,14-Dihydroxy-5,6β-epoxy-19-oxo-5β-card-20(22)-enolide 3-Acetate (XI).—A solution of IXa (0.025 g, 0.052 mmole) and 0.025 g (0.25 mmole) of KOAc in 95% ethanol (5 ml) was refluxed for 22 hr. Work-up as for X and crystallization from acetone-Skellysolve B afforded 0.014 g (60%) of XI, mp 227–230° dec. Repeated recrystallizations from acetone-Skellysolve B gave the analytical sample: mp 229–232° dec; $[\alpha]^{27}D$ −46° (c 0.50); λ_{max} 3.68 (aldehyde proton), 5.60, 5.70–5.90 (C=O of lactone, aldehyde and acetate), 6.17 (C=C of α,β-unsaturated lactone), and 7.97–8.08 μ (C–O–C of acetate). The infrared

(31) G. V. D. Tiers, *J. Phys. Chem.*, **62**, 1151 (1958).

(32) L. F. Fieser and T. Goto, *J. Am. Chem. Soc.*, **82**, 1697 (1960).

(33) G. D. Payne, *J. Org. Chem.*, **24**, 1354 (1959).

(34) H. Bohme, *Org. Syn.*, **20**, 70 (1940).

spectra for the α- and β-epoxides (X and XI) are virtually identical between 2.0 and 8.0 μ.

Anal. Calcd for $C_{25}H_{32}O_7$: C, 67.55; H, 7.26. Found: C, 67.41; H, 7.35.

3β-Acetoxy-5,6α-epoxy-19-oxo-5α-carda-14,20(22)-dienolide (XII).—The epoxide X (0.050 g, 0.11 mmole) in 1.0 ml of pyridine was cooled in ice. $SOCl_2$ (0.10 ml) in 0.5 ml of pyridine was also cooled in ice. The two solutions were mixed and kept in a freezer (temperature about −20°) for 2 hr. The resulting yellow suspension was taken up in cold $CHCl_3$ and washed with cold 5% HCl (twice) and with water (twice). Evaporation of the dried (Na_2SO_4) solution afforded an oil which on repeated recrystallizations from acetone–Skellysolve B afforded the analytical sample of XII: mp 185–187°; nmr, τ 0.10 (1 H, C-19 aldehyde), 4.12 (1 H, C-22 vinyl H), 4.68 (1 H, C-15 vinyl H), 5.05 (1 H, m, 3α-H), 5.26 (2 H, d, J = 2 cps, C-21), 6.79 (1 H, d, J = 3 cps, 6β-H), 8.00 (3 H, C-3 $COCH_3$), and 9.28 (3 H, C-18).

Anal. Calcd for $C_{25}H_{30}O_6$: C, 70.40; H, 7.09. Found: C, 70.24; H, 7.05.

3β-Acetoxy-5,6β-14,15α-diepoxy-19-oxo-5β,14α-card-20(22)-enolide (XIV) and 3β-Acetoxy-5,6α-14,15α-diepoxy-19-oxo-5α,14α-card-20(22)-enolide (XIII).—A solution of 3β-acetoxy-19-oxocarda-5,14,20(22)-trienolide (VIIb, 0.150 g, 0.36 mmole) in 5 ml of $CHCl_3$ was treated initially with 2.0 ml of monoperphthalic acid in ethereal solution[33] (0.089 g/ml, titrated[34]). After standing at room temperature for 4.5 hr, another 1.0 ml of the monoperphthalic acid solution was added, and the mixture was allowed to stand for another 20 hr. The mixture was filtered and the filtrate was washed (H_2O, twice with saturated $NaHCO_3$, H_2O). The organic layer was dried (Na_2SO_4) and concentrated to a colorless solid foam (0.159 g). The solid showed two close spots upon tlc on aluminum oxide ($CHCl_3$). The solid foam (0.110 g) was chromatographed on a column of 7.0 g of Woelm neutral alumina (activity grade I) packed in 3% $CHCl_3$ in C_6H_6. The higher R_f component (0.020 g, 18%) was eluted with 20% $CHCl_3$ in C_6H_6, while the lower R_f component (0.028 g, 25%) was eluted with 40% $CHCl_3$ in C_6H_6. Somewhat better yields were obtained when the solid foam was chromatographed on 0.5-mm thick alumina HF (Brinkmann) thick layer plates ($CHCl_3$ as mobile phase). The edge of the plate was sprayed with ceric sulfate reagent to locate the bands. The higher R_f component was crystallized from acetone–Skellysolve B to afford XIV: mp 227–230°; $[\alpha]^{29}D$ −60° (c 0.55); nmr, τ 0.11 (1 H, C-19 aldehyde), 4.16 (1 H, C-22 vinyl H), 5.10 (1 H, m, 3α-H), 5.31 (2 H, C-21), 6.40 (1 H, C-15), 6.72 (1 H, d, J = 1.5–2.0 cps, C-6), 8.00 (3 H, C-3 $COCH_3$), and 9.23 (3 H, C-18).

Anal. Calcd for $C_{25}H_{30}O_7$: C, 67.85; H, 6.83. Found: C, 67.91; H, 6.76.

The lower R_f component was crystallized from $CHCl_3$–Et_2O to afford XIII: mp 194–197°; $[\alpha]^{24}D$ −55° (c 0.55); nmr, τ 0.02 (1 H, C-19 aldehyde), 4.19 (1 H, t, J = 1.5 cps, C-22 vinyl H), 5.10 (1 H, m, 3α-H), 5.31 (2 H, d, J = 1.5 cps, C-21), 6.58 (1 H, C-15), 6.89 (1 H, d, J = 2.5 cps, C-6), 8.02 (3 H, C-3 $COCH_3$), and 9.32 (3 H, C-18).

Anal. Calcd for $C_{25}H_{30}O_7$: C, 67.85; H, 6.83. Found: C, 67.73; H, 6.73.

The 5,6α-14,15α-diepoxide (XIII) was prepared *via* an alternate method by epoxidation of 3β-acetoxy-5,6α-epoxy-19-oxo-5α-carda-14,20(22)-dienolide (XII) with monoperphthalic acid in ether. The mixture melting point with a sample of XIII prepared as above was undepressed, and the infrared and nmr spectra of the respective samples were identical.

3β,5,14-Trihydroxy-19-carboxy-5β-card-20(22)-enolide 3-Acetate (Ic) and Methyl Ester (Id).—Strophanthidin 3-acetate (Ie)[35] in dioxane was oxidized with 30% H_2O_2 by the procedure of Barber and Ehrenstein[36] to yield strophanthidinic acid 3-acetate (Ic). The methyl ester (Id) was prepared according to Koechlin and Reichstein.[37]

3,19-Dioxo-5,14-dihydroxy-5β-card-20(22)-enolide (XXI).—Strophanthidol[38] (If, 0.400 g, 0.99 mmole) in 60 ml of 50% aqueous acetone was oxidized in an atmosphere of O_2 in the presence of Pt black (prepared from 0.30 g of PtO_2).[39] The acetone was removed by concentration and the remaining aqueous solution was extracted with four 100-ml portions of $CHCl_3$. The combined $CHCl_3$ extracts were dried (Na_2SO_4) and the solvent was evaporated. The resulting solid foam was crystallized from $CHCl_3$–Et_2O, to afford 0.278 g (69%) of XXI, mp 206–208°. Recrystallization of XXI from acetone–Skellysolve B gave the analytical sample: mp 217–220°; $[\alpha]^{26}D$ +32° (c 0.75, methanol); λ_{max} 216 mμ (ϵ 17,000); nmr, τ −0.03 (1 H, C-19 aldehyde) in hexadeuterioacetone.

Anal. Calcd for $C_{23}H_{30}O_6$: C, 68.63; H, 7.51. Found: C, 68.14; H, 7.39.

Compound XXI was dehydrated with trifluoroacetic acid to the known 3,19-dioxo-14-hydroxy-carda-4,20(22)-dienolide (XXII). The infrared spectrum was superimposable upon that of an authentic sample.[40]

Strophanthidin 19-oxime (Ig) was prepared by the method of Jacobs and Heidelberger.[30]

3β,5-Dihydroxy-19-oxo-5β-carda-14,20(22)-dienolide (XV) was prepared by the sequence of Plattner, *et al.*,[27] and Makarichev and Abubakirov.[28]

3β,5-Dihydroxy-19-oxo-5β-carda-14,20(22)-dienolide 3-Bromoacetate (XVIa).—A solution of 0.030 g (0.078 mmole) of 14-monoanhydrostrophanthidin (XV), 0.013 ml (0.16 mmole) of pyridine, and 3.0 ml of dioxane was treated with bromoacetyl bromide (0.014 ml, 0.16 mmole) whereupon a granular precipitate formed. The yellow suspension was stirred for 1.5 hr and then slowly diluted with 7 ml of water. The water initially caused the solubilization of the granular precipitate (most probably a pyridinium salt) and then the separation of a light yellow crystalline product. The crystals were collected with water and air dried; yield 0.034 g (86%) of XVIa, mp 200–203° dec. Repeated recrystallizations from 95% ethanol gave colorless crystals of analytical purity: mp 214–215° dec; $[\alpha]^{28}D$ +16° (c 0.99); nmr, τ −0.13 (1 H, C-19 aldehyde), 4.08 (1 H, C-22 vinyl H), 4.67 (2 H, m, C-3α H and C-15 vinyl H), 5.24 (2 H, d, J = 2 cps, C-21), 6.12 (2 H, $BrCH_2CO$), and 9.18 (3 H, C-18).

Anal. Calcd for $C_{25}H_{31}BrO_6$: C, 59.17; H, 6.16; Br, 15.75. Found: C, 59.09; H, 6.12; Br, 15.84.

The product was shown to be identical with the bromoacetate ester prepared by Welles, *et al.*,[26] by acylation of strophanthidin with bromoacetyl chloride. A sample of the latter ester (kindly provided by Dr. R. C. Anderson) showed mp 209–211°, and the mixture melting point with XVIa was not depressed. The infrared spectra of the respective samples in $CHCl_3$ were superimposable. The melting point and infrared spectrum differed from that of strophanthidin 3-bromoacetate.[25]

3β,5-Dihydroxy-19-oxo-5β-carda-14,20(22)-dienolide 3-Iodoacetate (XVIb).—A solution of 14-monoanhydrostrophanthidin (XV, 0.031 g, 0.080 mmole) in 3.0 ml of 50% dioxane–benzene was treated with 0.1 ml of iodoacetyl chloride[41] and warmed to 45–50°. After 1 hr the solution was added dropwise to cold Skellysolve B (75 ml). The resulting yellow precipitate was collected, air-dried, and dissolved in $CHCl_3$. The $CHCl_3$ solution was washed once with $Na_2S_2O_3$, once with water, and dried (Na_2SO_4). $CHCl_3$ was evaporated to leave a light yellow residue. Upon crystallization from 95% ethanol, colorless crystals (0.032 g, 73%) of XVIb, mp 152° dec, were obtained. Repeated recrystallizations gave the analytical sample: mp 162–163° dec; $[\alpha]^{26}D$ +8° (c 0.48); nmr, τ −0.12 (1 H, C-19 aldehyde), 4.10 (1 H, C-22 vinyl H), 4.72 (2 H, m, C-3α-H and C-15 vinyl H), 5.25 (2 H, d, J = 1.5 cps, C-21), 6.27 (2 H, ICH_2CO), and 9.19 (3 H, C-18).

Anal. Calcd for $C_{25}H_{31}IO_6$: C, 54.16; H, 5.64; I, 22.89. Found: C, 54.29; H, 5.68; I, 22.82.

3β,5,14-Trihydroxy-19-oxo-5β-card-20(22)-enolide 3-Azidoacetate (XVIII).—Strophanthidin 3-iodoacetate[25] (XVIIb, 0.200 g, 0.35 mmole) in 20 ml of dioxane was treated with a solution of 0.056 g (0.88 mmole) of NaN_3 in 4 ml of H_2O. The mixture was allowed to stand in the dark for 40 hr, after which time it was diluted with 20 ml of H_2O and the dioxane was evaporated. The crystalline precipitate was collected with water and dried in a vacuum desiccator over P_2O_5; yield 0.147 g. The precipitate was crystallized from acetone–Skellysolve B to afford a total yield of XVIII of 0.129 g (74%). Repeated recrystallizations gave

(35) T. Reichstein and H. Rosenmund, *Pharm. Acta Helv.*, **15**, 157 (1940).

(36) C. W. Barber and M. Ehrenstein, *J. Org. Chem.*, **26**, 1230 (1961).

(37) B. Koechlin and T. Reichstein, *Helv. Chim. Acta*, **30**, 1673 (1947).

(38) A. Hunger and T. Reichstein, *Ber.*, **85**, 635 (1952).

(39) R. P. A. Sneeden and R. B. Turner, *J. Am. Chem. Soc.*, **77**, 190 (1955).

(40) S. M. Kupchan, C. J. Sih, N. Katsui, and O. El Tayeb, *ibid.*, **84**, 1752 (1962).

(41) E. Abderhalden and M. Guggenheim, *Ber.*, **41**, 2852 (1908).

an analytical sample of XVIII: mp 213–215° dec; $[\alpha]^{27}D$ +22° (*c* 0.55, methanol); λ_{max} 3.61 (C–H of aldehyde) and 4.72 μ (azide).

Anal. Calcd for $C_{27}H_{33}N_3O_7$: C, 61.58; H, 6.82; N, 8.62. Found: C, 61.61; H, 6.75; N, 8.71.

3β,5,14-Trihydroxy-19-oxo-5β-card-20(22)-enolide 3-Aminoacetate Hydrochloride (XIX).—A suspension of 0.120 g of 6% Pd–$SrCO_3$ in 5 ml of absolute ethanol was equilibrated for 30 min with H_2 at atmospheric pressure. Strophanthidin 3-azidoacetate (XVIII, 0.224 g, 0.46 mmole) in 30 ml of absolute ethanol was added and washed in with another 5 ml of absolute ethanol. The hydrogenation proceeded for 3 hr, the catalyst was removed by filtration, and the filtrate was evaporated to dryness. The residue was dissolved in purified THF, cooled in ice, and treated with an ethanolic solution of dry HCl (about 10 drops). The resulting white suspension was concentrated to dryness and used directly in the next step.

3β,5,14-Trihydroxy-19-oxo-5β-card-20(22)-enolide 3-Diazoacetate (XX).—The aminoacetate hydrochloride (XIX) was dissolved in 20 ml of water, filtered free of insoluble material, and cooled in ice. The cold solution was treated with 0.080 g (1.2 mmoles) of $NaNO_2$, while stirring. The reaction mixture was kept in the refrigerator overnight. The resulting suspension was filtered to yield 0.078 g of a peach-colored precipitate (A). The filtrate was extracted ($CHCl_3$) until a drop of the extract no longer gave a positive test with 3,5-dinitrobenzoic acid.[42] The combined extracts were washed once with water and dried (Na_2SO_4). The $CHCl_3$ solution was evaporated to an oil which crystallized with a few drops of acetone; yield 0.078 g (B). Product B consisted mostly of two spots on tlc (5% CH_3OH–$CHCl_3$), while product A corresponded to the upper component of B. Product B was chromatographed on two silica gel HF thick layer plates (10% CH_3OH–$CHCl_3$ as mobile phase). The product from the upper band was crystallized from methanol to afford 0.015 g of XX, mp 213–215° dec. Product A was also crystallized from methanol and, in two crops, yielded 0.051 g of diazoacetate XX, mp 215–217° dec, combined yield 0.066 g (30%), based on the quantity of azidoacetate used. The lower band from the chromatography of product B was obtained as an almost colorless solid foam (0.018 g) and was shown to be strophanthidin (Ia) by comparison of its infrared spectrum with that of an authentic sample. Repeated recrystallizations of XX from methanol gave an analytical sample: mp 218–220° dec; $[\alpha]^{26}D$ +39° (*c* 0.38, dioxane); λ_{max} 3.63 (C–H of aldehyde), 4.72 (diazo), and 5.96 μ (ester C═O conjugated with the diazo group); λ_{max} 249 mμ (ε 14,700) (C═O conjugated with diazo group) and 217 mμ (ε 18,100) (α,β-unsaturated lactone).

Anal. Calcd for $C_{25}H_{32}N_2O_7$: C, 63.54; H, 6.83; N, 5.93. Found: C, 63.34; H, 6.98; N, 5.88.

Preparation of ATPase.—Eight guinea pig brains were each homogenized in 10 ml of sucrose–EDTA.[43] The pooled homogenates were centrifuged at 13,600*g* for 10 min. The supernatant fluid and the loosely packed buff-colored layer were decanted into a flask and mixed with an equal volume of 1*M* NaCl and 0.2 vol. of 0.75% Tris deoxycholate. The mixture was homogenized briefly and then centrifuged at 78,500*g* for 1 hr. The residue was resuspended in 160 ml of sucrose–EDTA and centrifuged again for 1 hr at 78,500*g*. The pellet was suspended in 10–20 ml of sucrose–EDTA, and the enzyme was stored in a Dry Ice chest where it retained activity for at least 1 month. The "transport ATPase" averaged about 75 μmoles of ATP hydrolyzed/mg of protein per hr at 37°. The "nontransport ATPase" activity was about 10–20% of that of the "transport ATPase."

Incubation of ATPase with Cardenolide Derivatives.—The enzyme was thawed at room temperature just before use and suspended in 50 m*M* imidazole–HCl buffer, pH 7.1. Preincubation was carried out at 37° for 30 min with each cardenolide derivative. The preincubation vessels contained 125 m*M* imidazole–HCl, pH 7.1, approximately 125–250 μg of protein in a final volume of 1.0 ml, and the cardenolid derivative (DMF) at a final concentration of 10^{-4} *M*. Each derivative was tested for irreversible inhibition after incubation, by washing the enzyme suspension twice with sucrose–EDTA; the residue was then suspended in 2.0 ml of 100 m*M* imidazole–HCl, pH 7.1. ATPase activity was assayed by a slight modification of a previous method.[44] The assay tubes contained 0.50 ml of the incubated suspension (either washed or unwashed), 80 m*M* imidazole–HCl (pH 7.1), 2 m*M* $MgCl_2$, 2 m*M* Na_2ATP, 80 m*M* NaCl, and 16 m*M* KCl. Incubation was for 20 min at 37°. All assays were carried out in duplicate with the addition of either 0.05 ml of water or 0.05 ml of 1 m*M* ouabain. Protein was measured by the method of Lowry, *et al.*[45] The ATPase activity in the presence of 0.1 m*M* ouabain is due to the "nontransport ATPase," and the increment in activity on omitting ouabain is due to the "transport ATPase."

Evaluation for Cytotoxic Activity.—Assays were performed by A. D. Little, Inc., Cambridge, Mass., under contract to the Cancer Chemotherapy National Service Center (National Institutes of Health). The procedures have been described in detail.[46] The evaluation of assay results for cytotoxic activity toward cell cultures of human carcinoma of the nasopharynx (KB) by the CCNSC is such that a purified compound is considered active if the averaged ED_{50} of two tests is equal to or less than 4 μg/ml.

Evaluation for Cardiotonic Activity.—Healthy male guinea pigs (500–700 g) were killed by a blow on the back of the neck and the hearts were rapidly removed. The atria were quickly freed of ventricular muscle, fat, and connective tissue and suspended in 50 ml of modified Krebs–Henseleit solution (pH 7.4 ± 0.1)[47] into which was bubbled a mixture of 95% O_2 and 5% CO_2 at 37°. The modified Krebs–Henseleit solution was of the following composition: NaCl (6.92 g), KCl (0.35 g), anhydrous $CaCl_2$ (0.28 g), KH_2PO_4 (0.16 g), $MgSO_4 \cdot 7H_2O$ (0.29 g), $NaHCO_3$ (2.1 g), glucose (1.28 g), and enough distilled water to make 1 l.

Following immersion in the bath, the atria were allowed to stabilize for 30 min before use. In performing the test, the spontaneous contractions were recorded for 5 min before addition of the cardenolide to be tested. The cardenolide was added as a solution in DMF (final concentration of 10^{-5} *M*, unless stated otherwise) and the contractions again were recorded for 5 min. The amplitude of the isometric contractions was recorded on a Sanborn recorder (Model No. 150) *via* a force displacement transducer. There was a direct linear relationship between the amplitude of the stylus deflection and the force of contraction applied to the lever (resting tension of approximately 1 g). The Krebs–Henseleit solution in the bath was removed and replaced with fresh solution. The atria were washed in five changes of Krebs–Henseleit solution and allowed to beat for about 20 min, during which time the amplitude had diminished to its original height. The same atrium can respond to give satisfactory results for a large number of compounds tested.

Biological Results and Discussion

A series of strophanthidin derivatives have been evaluated for activity in three distinct biological systems in an attempt to explore possible parallels in relative effectiveness and modes of action. Certain of the compounds were selected in order to compare structure–activity relationships in one or more of the biological systems with results reported in the literature. For example, it has been reported that oxidation of the C-19 aldehyde group of the strophanthus glycosides destroys the cardiac activity, but that the activity is usually retained when the aldehyde group is reduced.[48] To evaluate the generality of the latter observations, strophanthidin was converted to strophanthidinic acid 3-acetate (Ic), strophanthidinic acid 3-acetate methyl ester (Id), and strophanthidol (If). Similarly, it has been reported that in cardenolides, saturation of the double bond in the lactone ring, or dehydrogenation

(42) I. E. Bush and D. A. H. Taylor, *Biochem. J.*, **52**, 643 (1952).

(43) L. E. Hokin and A. Yoda, *Proc. Natl. Acad. Sci. U. S.*, **52**, 454 (1964).

(44) L. E. Hokin and D. Reasa, *Biochim. Biophys. Acta*, **90**, 176 (1964).

(45) O. H. Lowry, N. J. Rosenbrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(46) *Cancer Chemotherapy Rept.*, **25**, 1 (1962).

(47) W. T. Salter and E. A. Runels, *Am. J. Physiol.*, **165**, 520 (1951).

(48) J. W. Wilson in "Medicinal Chemistry," A. Burger, Ed., 2nd ed, Interscience Publishers, Inc., New York, N. Y., 1960, Chapter 31.

of the 3β-hydroxyl group, affords a molecule which has decreased ability to inhibit transport ATPase and to stimulate cardiac muscle.[9] Strophanthidol (If) was accordingly converted to 3-dehydrostrophanthidin (XXI), and strophanthidin was converted to 3β,5,14-trihydroxy-19-oxo-5β-cardanolide (XXIV);[49] both were tested for cytotoxic activity toward KB.

The results of the biological tests of the strophanthidin derivatives are tabulated in Table I. The first test was an evaluation for cytotoxic activity against cell cultures of human carcinoma of the nasopharynx (KB). The second test evaluated the compounds for reversible and irreversible inactivation of transport ATPase. Transport ATPase activity in the presence of cardenolide was expressed as a per cent of the activity of the control sample incubated without cardenolide. Irreversible inhibition of the transport ATPase was demonstrated by a failure to regain the control activity after washing. The third biological test was an evaluation of the cardiotonic activity of the cardenolides. Trevan, *et al.*,[50] and Bhatt and MacDonald[51] have used isolated rabbit auricles for the estimation of digitalis activity. In the present work, it was found satisfactory to use isolated, spontaneously beating atria from guinea pigs in testing for cardenolide activity. The responses are expressed in Table I as per cent change in the amplitude of the contraction due to the cardenolide derivative (final concentration of 10^{-5} *M*, unless stated otherwise). The control amplitude of contraction was recorded just prior to the addition of the cardenolide derivative.

The results in Table I shed light on the effects of changes in structure among derivatives of strophanthidin on biological activity. It appears that cytotoxic activity in KB is lost when the *cis* configuration about rings A/B is altered. This is seen in the relative inactivity of compounds Vb, VII, IXa, X, and XI, as compared to the parent strophanthidin (Ia). Alteration of the *cis* C/D junction also results in loss of activity, as evidenced by the results for XV. The reduction of the C-19 aldehyde to the C-19 carbinol (If) or conversion to an oxime (Ig) causes little change in activity, whereas conversion to the C-19 carboxylic acid (Ic) or its methyl ester (Id) causes loss in activity. The latter observation is in agreement with earlier reports concerning structural requirements for cardiotonic activity.[48] Saturation of the double bond in the lactone (XXIV) also diminishes activity. The one position in which various changes can be made without loss of cytotoxic activity, and, in fact, with enhancement of activity, is C-3 (*e.g.*, Ie, XVIIa, XVIIb, XVIII, XX, and XXI).

Direct comparison of the cytotoxic activity (KB) with ATPase inhibitory activity indicates essentially parallel results. Of the eleven compounds which are "active" in KB, only two (XVIa and XVIb) fail to show at least a 70% inactivation of the transport ATPase.

Two compounds (XVIIa and XVIIb) were shown to be irreversible inhibitors of the transport ATPase, as evidenced by only partial reversal of the ATPase inhibitory activity upon washing. Detailed results of the study of these two compounds have been reported elsewhere.[25]

Comparison of the results of activity in the guinea pig atrial preparation with KB cytotoxic activity and transport ATPase inhibitory activity indicates a close parallel in the relative effectiveness of the compounds in each test. It is noteworthy that strophanthidol (If) and strophanthidin 19-oxime (Ig) are extremely active in increasing the strength of contraction of the atrial muscle.

The parallelism between cytotoxic activity and inhibition of transport ATPase with the different cardenolides merits comment. It has been shown that the active transport of many amino acids is inhibited by cardenolides, possibly because of an interrelationship between amino acid transport and sodium transport.[52] Inasmuch as tumor cells are very active in accumulating amino acids,[53] presumably because of their high amino acid requirements for growth, it is possible that the in-

TABLE I[a]

BIOLOGICAL EVALUATIONS OF THE CARDENOLIDE DERIVATIVES

Compd	Cytotoxic act. ED_{50}, μg/ml	Transport ATPase act. (% of control) Unwashed	Washed	Guinea pig atrial prepn % increase over control
Ia	0.24	11	105	50
Ic	32	71	107	No change
Id	34	100	96	No change
Ie	0.089	20	101	83
If	0.78	2	116	675
Ig	0.10	27	101	455
III	100	93	113	11
Vb	16	39	89	20
VIb	50	96	110	No change
VII	24	45	104	33
IXa	31	58	101	50
X	15	43	117	15 (decrease)
XI	100	51	106	No change
XIII	100	99	114	15 (decrease)
XIV	48	96	112	No change
XV	100	78	98	No change
XVIa	0.27	122	124	33
XVIb	1.9	78	80	14
XVIIa	0.17	2	8	75[b]
XVIIb	0.0014	3	21	...
XVIII	0.0032	14	97	70
XX	0.06	13	99	125
XXI	0.1	29	101	19
XXIII	28	111	100	No change[b]
XXIV	14	c	...	...

[a] Cytotoxic activity was determined by tests with cell cultures of human carcinoma of the nasopharynx (KB), and a compound is considered active if the ED_{50} (dose that inhibits growth to 50% of control growth) is equal to 4 μg/ml or less. Inhibitory activity of a guinea pig brain transport ATPase is expressed as per cent of control in unwashed and washed samples. Cardiotonic activity of the cardenolide derivatives was evaluated on isolated guinea pig atria, and the responses are expressed as per cent change (compared to the control) in the amplitude of the contraction due to the cardenolide derivative. [b] At 5×10^{-6} *M*. [c] It has been reported that the ATPase inhibitory activity of dihydrodigitoxin is 1/16 of that of digitoxin: K. Repke, ref 3, p 47.

(49) E. Rabald and J. Kraus, *Z. Physiol. Chem.*, **265**, 39 (1940).

(50) J. W. Trevan, E. Boock, J. H. Burn, and J. H. Gaddum, *Quart. J. Pharm.*, **1**, 6 (1928).

(51) J. G. Bhatt and A. D. MacDonald, *J. Pharm. Pharmacol.*, **12**, 733 (1960).

(52) T. Z. Csaky, *Federation Proc.*, **22**, 3 (1963).

(53) H. N. Christensen, T. R. Riggs, H. Fischer, and I. M. Palatine, *J. Biol. Chem.*, **198**, 1 (1952).

hibition in growth of nasopharyngeal carcinoma cells in culture by cardenolides is due to inhibition of amino acid accumulation.

In any event, the fact that similar structural features are important for inhibition of transport ATPase, KB cytotoxic activity, and atrial muscle inotropic activity lends support to the view that the receptors for the cardenolides in the three systems may be structurally very similar.

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Metabolism of (±)-Cotinine-2-^{14}C in the Rat[1a,2]

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A route to the synthesis of a number of nicotine metabolites bearing a ^{14}C label adjacent to the pyridine ring is described. This synthesis, which starts with the condensation of ethyl nicotinate-7-^{14}C and diethyl succinate, provides γ-(3-pyridyl)-γ-oxobutyric acid, γ-(3-pyridyl)hydroxybutyric acid, γ-(3-pyridyl)-γ-methylaminobutyric acid, cotinine, and demethylcotinine, as well as the two alkaloids nicotine and nornicotine. After administration of (±)-cotinine-2-^{14}C to the rat, the urine was examined chromatographically for radioactive and Koenig-positive (pyridine) substances. The general pattern of excretion of the radioactive Koenig-positive substances resembled those previously encountered in some other species and paralleled that found earlier with nonisotopic material. The radioactivity of administered (±)-cotinine was eliminated with a high degree of efficiency (90–97% of the administered dose), predominantly by way of the urine. Virtually no radioactivity was encountered in expired air, suggesting little or no conversion to nicotinic acid. Demethylcotinine and γ-(3-pyridyl)-γ-oxo-N-methylbutyramide were identified in the urine by isotopic dilution. Similar experiments where carrier nicotine was employed failed to provide evidence for the reversibility of the metabolic reaction nicotine → cotinine.

In the metabolism of (−)-nicotine in many mammalian species,[3] (−)-cotinine is one of the early metabolites that appears during the course of a long series of progressive oxidations leading eventually to the urinary excretion of 3-pyridylacetic acid. The experimental administration of large quantities of (−)-cotinine to the dog gives rise to (−)-demethylcotinine, one of the later intermediates in the series. Chromatographic studies on human urine after administration of (−)-nicotine suggest[3d] the presence of demethylcotinine, while administration of cotinine gives rise[4] to no, or insignificant, urinary excretion of the demethyl compound.

In view of the possible mammalian conversion of nicotine to nornicotine, a reaction[5] that was later to receive positive experimental support[3f,6] with rabbit liver and dog liver preparations, Wada, *et al.*,[7] administered nornicotine to dogs and studied the urinary metabolites. Among these, demethylcotinine was present. From these findings and additional data, there thus arose the possible alternate routes (Scheme I) to the formation of demethylcotinine in the dog.

Administration of (−)-nicotine-methyl-^{14}C to the rat gives[8] rise to significant quantities of ^{14}C activity in the respiratory CO_2. This excretion could presumably reflect several alternate routes of degradation, including (a) conversion of nicotine to nornicotine, (b) conversion of cotinine to demethylcotinine, and (c) oxidative attacks proceeding initially by way of the pyridine ring of nicotine.

Conversion of (−)-cotinine to (−)-demethylcotinine in the rat has already been established[9] on a semiquantitative basis using nonisotopic material.

We were thus led to consider and develop methods for the synthesis of pyrrolidone-ring-labeled cotinine-^{14}C and to determine some aspects of its metabolism and intermediary role as a nicotine metabolite in the rat.

Experimental Section

General Procedures.—Paper chromatograms were prepared on Whatman No. 1 paper and developed in solvent B,[10] 0.5 *N* NH_4OH–95% EtOH–*n*-BuOH (1:1:4 by volume), and solvent C,[10] 90% formic acid–*sec*-BuOH–H_2O (14:75:11 by volume). Thin-layer chromatograms were prepared on silica gel (Eastman Chromogram Sheet, Type K 301R) and developed in solvent K,[11,12] MeOH–$CHCl_3$ (15:85 by volume). Koenig-positive zones were disclosed as previously described.[7] Melting points were determined on the hot stage.

(1) (a) These studies were aided by grants from the American Medical Association Education and Research Foundation, The Council for Tobacco Research—U.S.A., and The American Tobacco Company. Presented in part at the 44th Annual Meeting of the Virginia Academy of Science, May 5, 1966. A preliminary report has appeared.[2] (b) To whom inquiries should be directed.

(2) P. L. Morselli, E. R. Bowman, H. H. Ong, and H. McKennis, Jr., *Virginia J. Sci.*, **17**, 345 (1966).

(3) (a) H. McKennis, Jr., L. B. Turnbull, and E. R. Bowman, *J. Am. Chem. Soc.*, **79**, 6342 (1957); (b) R. Truhaut and M. De Clercq, *Bull. Soc. Chim. Biol.*, **41**, 1693 (1959); (c) H. B. Hucker, J. R. Gillette, and B. B. Brodie, *Nature*, **183**, 47 (1959); (d) E. R. Bowman, L. B. Turnbull, and H. McKennis, Jr., *J. Pharmacol. Exptl. Therap.*, **127**, 92 (1959); (e) L. E. Appelgren, E. Hansson, and C. G. Schmiterlow, *Acta Physiol. Scand.*, **56**, 249 (1962); (f) K. Decker and R. Sammeck, *Biochem. Z.*, **340**, 326 (1964).

(4) E. R. Bowman and H. McKennis, Jr., *J. Pharmacol. Exptl. Therap.*, **135**, 306 (1962).

(5) The reversible nature of this reaction is suggested by experiments conducted with preparations from rabbit lungs by J. Axelrod, *ibid.*, **138**, 28 (1962).

(6) N. M. Papadopoulos and J. A. Kintzios, *ibid.*, **140**, 269 (1963).

(7) E. Wada, E. R. Bowman, L. B. Turnbull, and H. McKennis, Jr., *J. Med. Pharm. Chem.*, **4**, 21 (1961).

(8) H. McKennis, Jr., E. Wada, E. R. Bowman, and L. B. Turnbull, *Nature*, **190**, 910 (1961).

(9) H. McKennis, Jr., L. B. Turnbull, S. L. Schwartz, E. Tamaki, and E. R. Bowman, *J. Biol. Chem.*, **237**, 541 (1962).

(10) H. McKennis, Jr., L. B. Turnbull, and E. R. Bowman, *ibid.*, **239**, 1215 (1964).

(11) H. McKennis, Jr., E. R. Bowman, and M. S. Dar, *Virginia J. Sci.*, **18**, 14 (1967).

(12) J. C. Craig, N. Y. Mary, N. L. Goldman, and L. Wolf, *J. Am. Chem. Soc.*, **86**, 3866 (1964).