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Photoaffinity Labeling of the Sodium- and Potassium-Activated Adenosinetriphosphatase with a Cardiac Glycoside Containing the Photoactive Group on the C-17 Side Chain[†]

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ABSTRACT: The synthesis and properties of a radiolabeled glycoside photoaffinity probe, [^{3}H]-(3β,5β,14β,20*E*)-24-azido-3-[(2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-14-hydroxy-21-norchol-20(22)-en-23-one, containing the photoactive group at the C-17 side chain of the steroid moiety are reported. The molecule binds to the sodium- and potassium-activated adenosinetriphosphatase from porcine kidney outer medulla under type II binding conditions [5 mM $MgCl_2$, 3 mM phosphate, 2 mM ethylenediaminetetraacetic acid, 30 mM tris(hydroxymethyl)aminomethane, pH 7.2, 37 °C] in the dark with an equilibrium dissociation constant of $(1.4 \pm 0.3) \times 10^{-7}$ M. Ultraviolet irradiation of a solution of enzyme plus ^{3}H-labeled probe, followed by analysis of covalently incorporated radiolabel, shows ouabain-displaceable labeling exclusively of the α subunit of the sodium- and potassium-activated adenosinetriphosphatase. These data indicate that the binding site of the C-17 side group of cardiac glycosides is located on or near the α subunit of this enzyme.

Digitalis glycosides are widely used in the treatment of cardiac dysrhythmias and congestive heart failure (Fullerton, 1982). The physiological receptor for these drugs is thought to be Na,K-ATPase[1] (Schwartz et al., 1982; Akera, 1977).

While the precise quaternary structure of the Na,K-ATPase catalytic complex is not yet known (Kyte, 1981), the protein is thought to be composed of α (M_r 100 000) and β (M_r 40 000) subunits (Peterson & Hokin, 1981; Freytag & Reynolds, 1981; Peters et al., 1981) and perhaps a low molecular weight proteolipid component (M_r 12 000) (Forbush et al., 1978; Rodgers & Lazdunski, 1979a; Collins et al., 1982). The α subunit of the enzyme contains the site of phosphorylation by ATP (Post & Kume, 1973; Nishigaki et al., 1974); however, the exact location of the cardiac glycoside binding site remains somewhat unclear. Forbush et al. (1978), using photoaffinity labels of ouabain with the photoactive group attached to the sugar portion of the molecule, found covalent labeling of the α subunit as well as a low molecular weight proteolipid component. Rodgers & Lazdunski (1979a,b) showed that if the photoactive group was located on the sugar group of ouabain (NAP-ouabain), labeling occurred at both the α subunit and the low molecular weight peptide, while experiments utilizing a glycoside derivative with the group positioned between the steroid A and B rings (NAP-strophanthidin) gave covalent label incorporation only in the α subunit. In experiments probing the sugar binding sites of digitoxin, Hall & Ruoho (1980) synthesized $3'''$- and $4'''$-(diazomalonyl)digitoxin and found that both α and β subunits were covalently labeled, with enhanced labeling of the β subunit for the $4'''$-derivative as opposed to the $3'''$-derivative. At this time, there has been no report of successful photoaffinity-labeling or affinity-alkylating studies of the cardiac glycoside binding site where the alkylating moiety is located at or near the C-17 side group of the glycoside.

Previous work (Fullerton et al., 1979; Ahmed et al., 1983) has indicated that a strong correlation exists between the C-17 side group carbonyl oxygen position and the resulting inhibition of Na,K-ATPase activity. The purpose of this paper is to report the synthesis and binding properties of a radiolabeled cardiac glycoside photoaffinity probe with the photoactive group located on the C-17 side chain of the molecule. Photoaffinity-labeling experiments, followed by analysis for co-

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[1] Abbreviations: Na,K-ATPase, magnesium-dependent, sodium- and potassium-stimulated adenosinetriphosphatase (EC 3.6.1.3); ATP, adenosine 5′-triphosphate; NAP-strophanthidin, *N*-strophanthidinyl-*N*′-(2-nitro-4-azidophenyl)ethylenediamine; NAP-ouabain, *N*-ouabainyl-*N*′-(2-nitro-4-azidophenyl)ethylenediamine; 24-azidodigitoxoside (**1**), (3β,5β,14β,20*E*)-24-azido-3-[(2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-14-hydroxy-21-norchol-20(22)-en-23-one; digitoxigenin digitoxoside acetonide, (3β,5β)-3-[[2,6-dideoxy-3,4-*O*-(1-methylethylidene)-β-D-*ribo*-hexopyranosyl]oxy]-14-hydroxycard-20(22)-enolide; TLC, thin-layer chromatography on silica gel; IR, infrared; ^{1}H NMR, proton nuclear magnetic resonance; THF, tetrahydrofuran; DMF, dimethylformamide; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; solvent system A, CH_2Cl_2–ethyl acetate–methanol (20:2:1); solvent system B, CH_2Cl_2–ethyl acetate–methanol (10:3:1); solvent system F, CH_2Cl_2–acetone (1:1); EDTA, ethylenediaminetetraacetic acid.

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valent incorporation of radioactivity with SDS–PAGE, indicated that the binding site for the C-17 side-group portion of the molecule appears to be located on the α subunit of Na,K-ATPase.

Experimental Procedures

Elemental analyses were performed by MHW Laboratories, Phoenix, Az, and are within ±0.3% unless otherwise noted. The 100-MHz NMR spectra were taken at the Oregon State University NMR Spectroscopy Laboratory, Department of Chemistry. Samples were dissolved in $CDCl_3$ with 2% Me_4Si as an internal standard. Melting points were obtained on a Thomas Hoover apparatus and are uncorrected. IR spectra were run as KBr pellets or as liquid films on NaCl disk in a Beckman Model 7 spectrophotometer with polystyrene as reference. UV spectra were recorded in 95% EtOH on a Varian CARY 219 UV spectrophotometer. Optical rotations were taken in absolute MeOH on a Perkin-Elmer 243 polarimeter. Thin-layer chromatographies were on 0.25-mm EM silica gel 60 F254 glass TLC plates (EM reagents) with techniques previous described (Yoshioka et al., 1978). Additional details for digitoxoside synthesis are given by Kihara et al. (1984).

Preparative TLC plates were coated with silica gel 60 PF 254 with $CaSO_4$ (EM reagents (20 × 20 nm, 0.75 mm thickness). The same solvent systems used in analytical TLC were used for developing preparative TLC. Samples were extracted from the gel in a mixture of CH_2Cl_2–EtOAc–MeOH (1:1:1). Visualization on preparative TLC was done by UV light at 254 nm. Analytical TLC plates were visualized by either iodine vapor, UV light at 254 nm, or by heat after spraying with a solution of 2% $CeSO_4$ in 2 N H_2SO_4. On preparative TLC, no more than 50 mg of sample was spotted per plate.

Flash column chromatography was performed according to the procedure described by Still et al. (1978). Flushing of samples was done with nitrogen gas instead of air to avoid oxidation reactions. Dry column chromatography was also used for some intermediates. Solvents were dried by in either CaH_2 or $LiAlH_4$ for 24 h with stirring at room temperature, distilled, and stored under nitrogen in a round-bottom flask containing molecular sieves (type 5A, $^1/_{16}$ in.) with a rubber stopper. All the light-sensitive reactions (azide compounds) are done in the dark; reaction vessels were covered with aluminum foil. After recrystallization, these light-sensitive compounds were covered with aluminum foil, stored in a brown bottle, and kept at −20 °C in a desiccator with Drierite.

Synthesis of 24-Azidodigitoxoside (**1**) (*See Figure 1*). Digitoxigenin digitoxoside acetonide (**2**) was prepared from digitoxin by the procedure of Satoh & Aoyama (1970). Two grams (3.67 mmol) of **2** was dissolved in CH_2Cl_2 and ozonized at −78 °C for 5 h in an Ozone Research and Equipment Corp. Model 03V10-0 ozonator. After being flushed with N_2 to remove excess ozone, the solution was reduced by adding 4 mL of dimethyl sulfide and stirring at −78 °C overnight. A total of 100 mL of CH_2Cl_2 was then added, and the mixture was washed in a separatory funnel 3 times with H_2O to remove Me_2SO, dried over anhydrous $MgSO_4$, and evaporated in vacuo to yield 2 g of **3**, which migrated as a single spot, R_f 0.65, on TLC in solvent system B. Compound **3** was not extremely stable and was therefore utilized immediately after preparation. A total of 316 mg (8.35 mmol) of sodium borohydride was slowly added to 2.0 g of **3** in 200 mL of methanol with stirring. Monitoring the reaction by TLC in solvent system B (two new spots, corresponding to C-20 diastereomers, appear, $R_f \sim 0.45$) indicated that the reduction was complete in 1 h, after which the mixture was evaporated to about half the volume, 100 mL of H_2O was added, and the mixture was extracted 3 times with 100 mL of ether. The ether extract was washed with H_2O and saturated NaCl, dried over anhydrous $MgSO_4$, and evaporated in vacuo, giving 2.46 g of diol **4** as an oil. The 2.46 g of **4** were dissolved in 100 mL of 2% w/v KOH in methanol and refluxed for 30 min on a steam bath. After being cooled to room temperature, the solution was evaporated to about 50-mL volume. The pH was adjusted to 5–6 with glacial acetic acid, and diol **5** (R_f 0.4, solvent system B) was precipitated by gradual addition of H_2O. The mixture was then cooled in an ice bath and filtered, and the precipitate was washed with water and dried under vacuum to give 1.4 g of **5**. To a solution of 1.4 g of (**5**) in 60 mL of methanol was added 1.4 g of sodium metaperiodate dissolved in 14 mL of H_2O. The reaction was complete after 1 h at room temperature as monitored by the disappearance of **5** by TLC in solvent system B (crude **6** has an R_f of 0.85). The reaction mixture was then filtered to remove $NaIO_3$ that had precipitated, and the precipitate was washed with a small volume of methanol. The combined filtrates were evaporated to about half of the volume, and the crude produce (**6**) was precipitated by gradual addition of H_2O. After being cooled on ice, the mixture was filtered, and the precipitate was recrystallized from CH_2Cl_2–CH_3OH to give 1.3 g of unlabeled **6**. The overall yield from **3** to **6** was 72%. Compound **6** showed the following: mp 75–80 °C; IR (KBr) ν_{max} 3470 (OH), 1705 (CHO), 2730 (CHO) cm^{-1}; ^1H NMR ($CDCl_3$) δ 4.73 (dd, 1 H, J = 9.3 Hz, C-1′ H), 4.37 (m, 1 H, C-3′ H), 3.60 (dd, 1 H, J = 9.5 Hz, C-4′ H), 3.39 (m, 1 H, C-5′ H), 1.16 (d, 1 H, J = 6 Hz, C-6′ H), 3.97 (m, 1 H, C-3 H), 9.7 (d, 1 H, J = 3 Hz, CHO), 0.95 (s, 3 H, C-18 CH_3), 0.85 (s, 3 H, C-19 CH_3), 1.39 and 1.27 (both s, 3 H each, methyl protons of acetonide group); $[\alpha]^{22}{}_D$ −14.66 (c 0.3, CH_3OH). Anal. ($C_{29}H_{46}O_6$) C, H.

Synthesis of **6** *Tritium-Labeled at C-20*. A total of 8.4 mg (0.22 mmol) of $[^3H]NaBH_4$ (New England Nuclear, 9 Ci/mmol) was added to 64 mg (11 mmol) of **3** in 5 mL of methanol. After being stirred for 1 h at room temperature, the mixture was evaporated to dryness and the oil dissolved in 5 mL of 2% w/v KOH in methanol. After being refluxed for 30 min, the mixture was evaporated to half-volume and adjusted to pH 5–6 with 5% acetic acid, and 3 mL of H_2O was added. The milky suspension was extracted 3 times with ether. The ether extracts were washed and dried as above and evaporated to give 58 mg of ^3H-labeled diol **5**. All of **5** was dissolved in 5 mL of methanol, and 58 mg of sodium metaperiodate in 1 mL of H_2O was added with stirring. The reaction was complete in 15 min, after which the solution was filtered, the precipitate was washed with methanol, and the combined filtrates were evaporated to about 1-mL volume. H_2O was added and the suspension extracted with CH_2Cl_2. After the CH_2Cl_2 was dried over $MgSO_4$, the CH_2Cl_2 was evaporated to dryness to give 42.2 mg (77%) of $[^3H]$**6** as an oil. $[^3H]$**4**, -**5**, and -**6** were all shown to comigrate with the respective unlabeled compounds on TLC in solvent system B.

Synthesis of Unlabeled Compound **1**. A total of 25.62 mL of 1.6 M butyl lithium in hexane (Aldrich) (41 mmol) was added to 5 g (40.32 mmol) of dimethyl methylphosphonate (Aldrich) in 50 mL of dry THF under N_2. The phospholithium base was then transferred under N_2 to a solution of 4.2 g of methyl bromoacetate (Aldrich, 41 mmol) in 25 mL of dry THF at −78 °C. The reaction mixture was stirred overnight, and the temperature was allowed to gradually rise to 25 °C as the dry ice evaporated. After addition of 50 mL

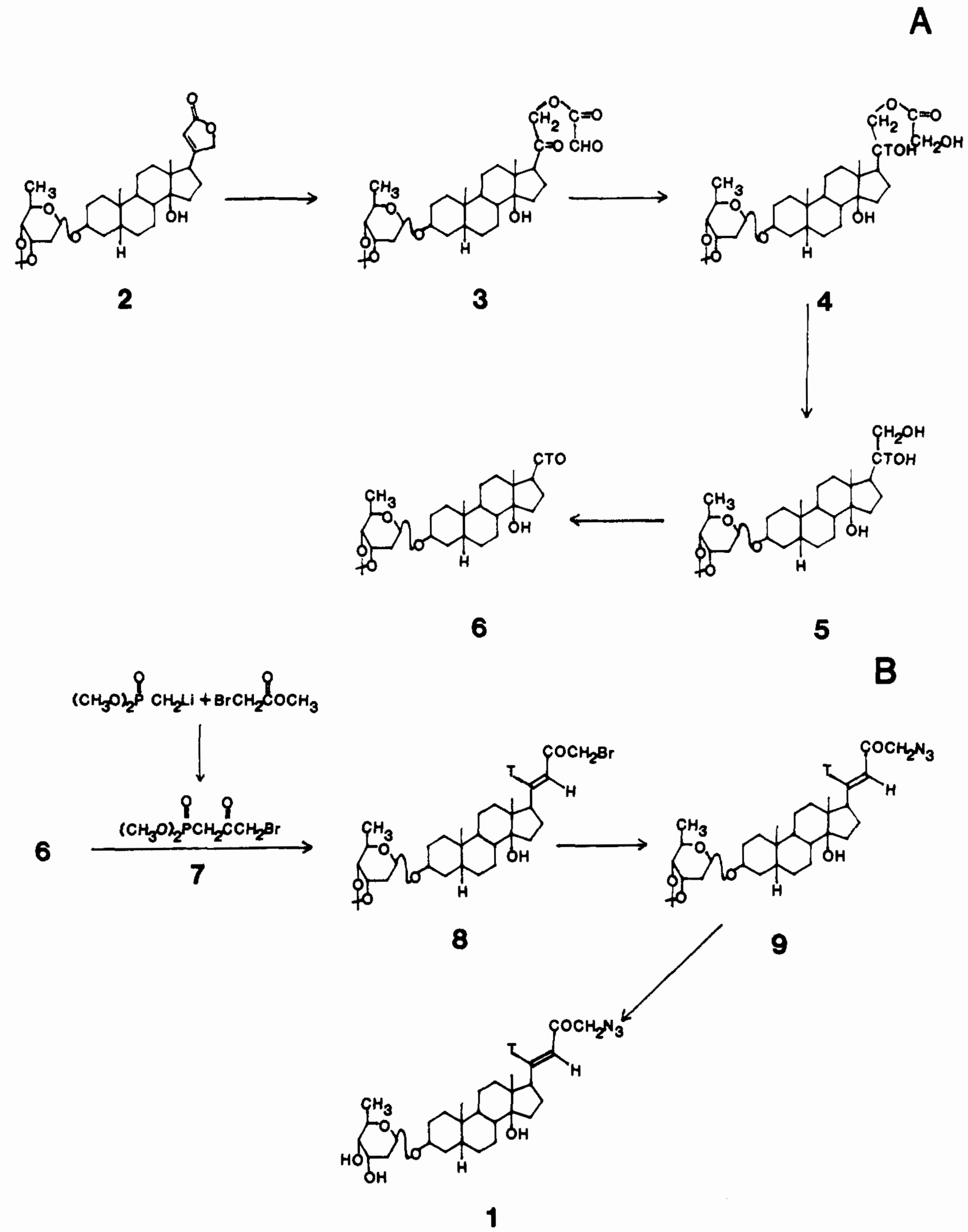

FIGURE 1: Synthesis of [^{3}H]-24-azidodigitoxoside (**1**): (A) reaction pathway up to synthesis of [^{3}H]**6**; (B) intermediates in the conversion of [^{3}H]**6** to **1**. The details are given under Experimental Procedures.

of H_2O, the reaction was acidified with 5% HCl and extracted 3 times with CH_2Cl_2. The CH_2Cl_2 was dried over $MgSO_4$ and evaporated to dryness, giving 6.35 g of a crude oil (two major spots, R_f 0.4 and 0.6, in solvent system F). The desired product (R_f 0.6) was purified on a silica gel column eluted with CH_2Cl_2–acetone (3:1) to give 3 g (31%) of **7**: IR (NaCl) ν_{max} 1725 (carbonyl), 1250 (P=O), 2850 and 2950 cm^{-1} (CH_3 and CH_2 asymmetrical stretching); ^{1}H NMR ($CDCl_3$) δ 3.4 [d, 2 H, J = 22 Hz, -P(O)–CH_2–C(O)], 3.8 (d, 6 H, J = 11 Hz, CH_3O–), 4.4 [s, 2 H, –C(O)–CH_2Br]. Compound **7** was stored at 4 °C under N_2 to avoid decomposition.

To synthesize unlabeled **8**, 1.0 g of **6** (2.04 mmol) and 1.5 g (6.12 mmol) of **7** in 30 mL of absolute ethanol were added to 416 mg (6.12 mmol) of freshly prepared sodium ethoxide in 30 mL of absolute ethanol. After this was stirred at room temperature for 3 h, TLC in solvent system A showed unreacted **6** (R_f 0.4) plus the appearance of a new spot with an R_f of 0.5. A total of 100 mL of pH 5–6 acetate buffer (24.4 g of sodium acetate plus 6 g of acetic acid per 100 mL) was added and the mixture chilled in an ice bath. The solution was filtered to give 2.5 g of crude product, which was then purified by silica gel chromatography in CH_2Cl_2–acetone (10:1) as the eluting solvent to give 320 mg of **8** (R_f 0.5, in solvent system A) as an oil. The oil was crystallized from ether–hexane to give 306 mg (48%) of **8**: mp 60–65 °C; IR (KBr) ν_{max} 3400 (OH), 1660 (C=C–C(O)–), 1620 cm^{-1} (C=C); ^{1}H NMR ($CDCl_3$) δ 4.77 (dd, 1 H, J = 9.3 Hz, C-1′ H), 4.40 (m, 1 H, C-3′ H), 3.65 (dd, 1 H, J = 9.3 Hz, C-4′ H), 3.46 (m, 1 H, C-5′ H), 1.24 (d, 1 H, J = 6 Hz, C-6′ H), 4.02 (m, 1, C-3 H), 7.18 (dd, 1 H, J = 16 and 10 Hz, C-2 OH), 5.98 (d, 1 H, J = 16 Hz, C-22 H), 4.01 (s, 1 H, C-24 H), 0.87 (s, 3 H, C-18 CH_3), 0.93 (s, 3 H, C-19 CH_3), 1.45 and 1.33, (3 each, methyl protons of acetonide group); $[\alpha]^{22}_D$ +15.33 (c 0.300, CH_3OH). Anal. ($C_{32}H_{49}O_6Br$) C, H, N.

Azide **9** was synthesized by addition of 37.5 mg of NaN_3 (5.77 mmol) in 2.2 mL of H_2O to 220 mg (0.36 mmol) of **8** dissolved in 22 mL of DMF. After this was stirred at room temperature in the dark for 3 h, the reaction mixture was poured into ice-cold water and extracted 3 times with ethyl acetate. The ethyl acetate was washed once with water, dried over $MgSO_4$, and evaporated to give 722 mg of crude oil. Purification by preparative TLC (solvent system A, R_f 0.5) followed by recrystallization from CH_2Cl_2–hexane gave 143 mg of **9** as a yellow powder (69%): IR (KBr) ν_{max} 3440 (OH), 1680 (C=C–C=O), 1620 (C=C), 2100 cm^{-1} (N_3); ^{1}H NMR ($CDCl_3$) δ 4.76 (dd, 1 H, J = 9.3 Hz, C-1 H), 4.41 (m, 1 H, C-3′ H), 3.65 (dd, 1 H, J = 9.5 Hz, C-4′ H), 3.47 (m, 1 H, C-5′ H), 1.24 (d, 1 H, J = 6 Hz, C-6′ H), 4.32 (m, 1 H, C-3 H), 7.16 (dd, 1 H, J = 10 and 16 Hz, C-20 H), 5.97 (d, 1 H, J = 16 Hz, C-22 H), 4.32 (s, 2 H, C-24 H), 0.87 (s, 3 H, C-18 H), 0.94 (s, 3 H, C-19 H), 1.41 and 1.33 (s, each 3 H, CH_3 groups of acetonide). Anal. Calcd for $C_{32}H_{49}O_6N_3$: C, 67.22; H, 8.64; N, 7.35. Found: C, 67.59; H, 8.95; N, 6.76.

To synthesize unlabeled **1**, two drops of 5% HCl was added to 190 mg (0.332 mmol) of **9** dissolved in 50 mL of methanol in the dark, under N_2. After 5 h of stirring, the reaction mixture was extracted 3 times with CH_2Cl_2, and the CH_2Cl_2 extracts were dried over $MgSO_4$ and evaporated. Preparative TLC in solvent system A gave 42 mg of **9** (R_f 0.5), a trace of the genin formed by cleavage of the sugar–steroid ether bond (R_f 0.3), and 34 mg (25%) of **1** (R_f 0.2), which was crystallized from CH_2Cl_2–hexane: mp 102–103 °C; IR (KBr) ν_{max} 3430 (OH), 1660 (C=C–C=O), 1620 (C=C), 2100 cm^{-1} (N_3); ^{1}H NMR ($CDCl_3$) δ 4.88 (dd, 1 H, J = 9.3 Hz, C-1′ H), 4.04 (m, 1 H, C-3′ H), 3.71 (dd, 1 H, J = 9.5 Hz, C-4′ H), 3.46 (m, 1 H, C-5′ H), 1.23 (d, 1 H, J = 6 Hz, C-6′ H), 4.03 (m, 1 H, C-3 H), 7.18 (dd, 1 H, J = 10 and 16 Hz, C-20 H), 5.96 (d, 1 H, J = 16 Hz, C-22 H), 4.03 (s, 2 H, C-24 H), 0.87 (s, 3 H, C-18 H), 0.93 (s, 3 H, C-19 H); $[\alpha]^{22}{}_D$ +5.33 (c 0.3, CH_3OH); $\lambda_{max}{}^{ethanol}$ 236 nm (log ϵ = 4.10). Anal. Calcd for $C_{29}H_{45}O_6N_3$: C, 65.51; H, 8.53; N, 7.90. Found: C, 65.71; H, 8.80; N, 7.52.

Synthesis of Tritiated **8**. A total of 42.2 mg (0.086 mmol) of [^{3}H]**6** and 105 mg (0.428 mmol) of phosphonate **7** in 3 mL of absolute ethanol were added to 29 mg (0.43 mmol) of freshly prepared sodium ethoxide in 2 mL of absolute ethanol. The reaction and isolation of crude product were the same as for unlabeled **8**. Pure [^{3}H]**8** was isolated by preparative TLC (solvent system A) to give 16.4 mg (31%) of an oil that cochromatographed with unlabeled **8**.

To synthesize [^{3}H]**9**, 28 mg (0.35 mmol) of NaN_3 in 0.3 mL of H_2O was added to 16.4 mg (0.026 mmol) of [^{3}H]**8** in 3 mL of DMF. The reaction was done as for unlabeled **9**, and after purification by preparative TLC as above, 11.3 mg of [^{3}H]^9 was isolated. The structure of [^{3}H]**9** was confirmed by cochromatography with unlabeled **9** and IR (NaCl).

Hydrolysis of [^{3}H]**9** to give [^{3}H]**1** was done by the same procedure as for unlabeled **1**. One drop of 5% HCl was added to 11.3 mg of [^{3}H]**9** in 10 mL of methanol. After this was stirred for 3 h at room temperature in the dark, preparative TLC gave 6 mg (72% on the basis of recovered **9**) of [^{3}H]**1**, identified by cochromatography with unlabeled **1**: IR (NaCl) ν_{max} 3400 (OH), 2100 (N_3), 1670 (C=C–C=O), 1620 cm^{-1} (C=C). The corresponding ^{3}H-labeled genin was also obtained (2 mg, 32% yield, identified by cochromatography with the unlabeled material) as well as recovery of 2.4 mg (21%) of starting material [^{3}H]**9**.

Na,K-ATPase from hog kidney medulla was prepared by the procedure of Ahmed et al. (1983). The final specific activity was about 1000–1250 μmol of P_i (mg of protein)$^{-1}$ h^{-1}. SDS–PAGE of this preparation was done by the method of Laemmli (1970) in 0.5 × 11 cm tube gels containing a 1-cm, 5% w/v acrylamide stacker gel and a 10-cm, 10% w/v separating gel. Molecular weight standards were purchased from Pharmacia.

The equilibrium dissociation constant of the labeled probe (Figure 3) was determined with an equilibrium centrifugation assay in a Beckman airfuge. Samples containing varying concentrations of [^{3}H]-24-azidodigitoxoside and enzyme were equilibrated for 1 h at 37 °C in 5 mM $MgCl_2$, 3 mM phosphate, 2 mM EDTA, and 30 mM Tris-HCl, pH 7.2, in polyethylene microfuge tubes. An aliquot was removed to determine the total ligand concentration, and 180 μL of sample was then transferred to a cellulose–propionate airfuge tube. After centrifuging for 10 min at 175000g, the supernatants were removed, and aliquots were counted to determine the concentration of free probe. Bound probe was calculated as the difference between total and free probe concentrations. A similar experiment in the presence of 10 μM ouabain permitted the calculation of the dependence of nonspecific binding of ^{3}H-labeled probe on free probe concentration. Specifically bound probe (i.e., displaceable by ouabain) was calculated as the difference between total probe bound and nonspecifically bound probe at any given free probe concentration.

Photoaffinity-labeling experiments were conducted in the following manner. A total of 100 μg of protein plus 5 × 10^{-7} M ^{3}H-labeled probe were incubated for 2 h in the absence or presence of 10 μM ouabain or with [^{3}H]-24-azidodigitoxoside that had been prephotolyzed for 15 min in the buffer system described above (1-mL total volume). The samples were cooled to 4 °C in an ice bath and then diluted to 3-mL total volume by addition of 2 mL of ice-cold 50 mM Tris-HCl buffer, pH 7.5, and photolyzed for 5 min with two UVS-11 mineralights (λ_{max} = 254 nm), one on each side of a 1-cm quartz cuvette, giving an effective path length of 5 mm. Noncovalently bound ^{3}H-labeled probe was displaced by addition of 10 μM digitoxigenin 3β-D-digitoxoside and the labeled enzyme collected by centrifugation at 100000g for 2 h. The pellets were dissolved in 0.2 mL of 2% w/v SDS in 40 mM Tris-HCl–1 mM EDTA, pH 7.5, by an overnight shaking at room temperature. The enzyme was then reprecipitated overnight at −20 °C after addition of 95% ethanol–1% v/v β-mercaptoethanol to a final volume of 4.5 mL. Precipitates were collected by centrifugation (50000g, 30 min), dissolved in sample buffer prepared according to Laemmli (1970), and applied to 0.5 × 11 cm tube gels containing a 1-cm 5% w/v acrylamide stacking gal and a 10-cm, 10% w/v acrylamide separating gel. After SDS–PAGE, according to Laemmli (1970), gels were cut into 0.25-cm slices, digested at 65 °C with 30% H_2O_2 for 6–8 h, and counted for tritium (40% efficiency) in Aquasol purchased from New England Nuclear. All other reagents used were of the highest possible purity commercially available.

Results

Synthesis and Properties of 24-Azidodigitoxoside (**1**). The conversion of **2** to 17-aldehyde **6** followed the lactone-degradation procedure used both by Fullerton et al. (1980) and by Boutagy & Thomas (1971) to make genin analogues. The overall yield from ozonide product **3** to ^{3}H-labeled 17-aldehyde **6** was 77%, starting with 64 mg of **3**. Dimethyl [2-(bromomethyl)carbonylmethyl]phosphonate (**7**) was made by using the Arbuzov reaction with lithium dimethyl methylphosphonate (from BuLi reaction with dimethyl methylphosphonate) and methyl bromoacetate as described by

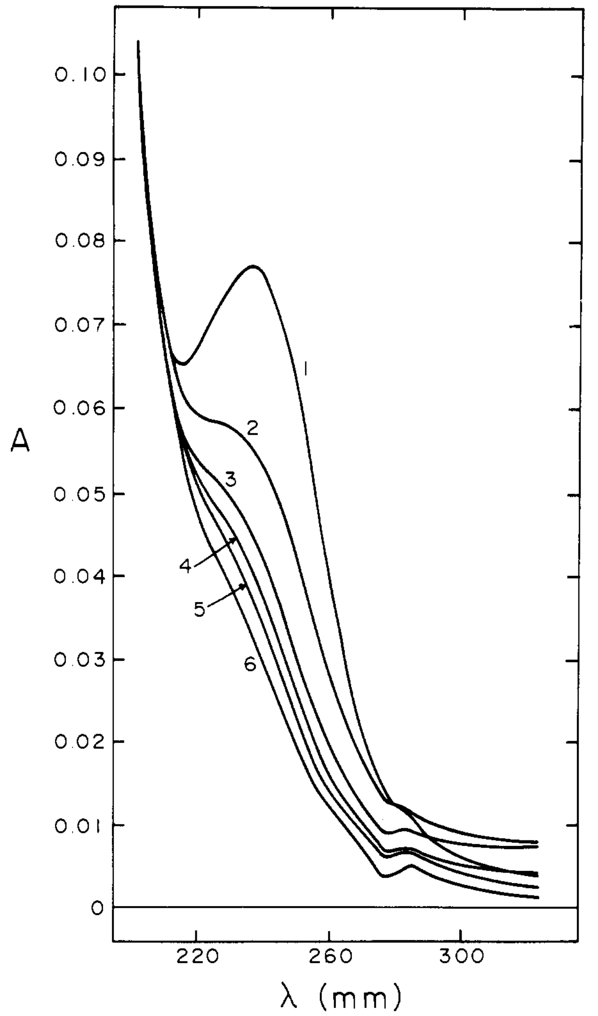

FIGURE 2: Absorbance spectra of [^{3}H]-24-azidodigitoxoside (**1**). The absorbance of a 6 × 10^{-6} M aqueous solution of **1** is shown as a function of photolysis time with two UVS-11 mineralights ($\lambda_{max} = 254$ nm), one on each side of a 1-cm quartz cuvette. Photolysis time equaled (1) 0, (2) 1, (3) 2, (4) 5, (5) 10, and (6) 15 min. $\lambda_{max} = 236$ nm with $\epsilon_{M}^{1cm} = 1.28 \times 10^4$.

Boutagy & Thomas (1974). Wittig reaction of the phosphonate of **7** (from **7** with sodium ethoxide) and ^{3}H-labeled aldehyde **6** in absolute ethanol gave bromomethyl ketone **8** in 31% yield. Finally, displacement of the Br of **8** with NaN_3 in DMF gave [^{3}H]azidoacetonide **9** in 73% yield. Hydrolysis of the acetonide protective group gave **1** in 72% yield and 2% of the corresponding genin. The overall yield from 64 mg of **3** was 11.3 mg of [^{3}H]**1** with specific activity of 1.80 Ci/mmol. The final product was stored in the dark as a dilute solution in ethanol at −20 °C, where it has been stable for at least 6 months.

The UV spectra of the unlabeled 24-azidodigitoxoside (**1**) is shown in Figure 2. The time dependence of the photodecomposition reaction indicates that after 5 min, the molecule had almost completely reacted. TLC analysis of the products showed the presence of four spots, explaining the absence of an isosbestic point in Figure 2. Although photolysis for 5 min in the presence of protein resulted in the formation of some high molecular weight material that did not enter the running gel, this photolysis time was chosen as a compromise between maximal label incorporation and photolytically induced cross-linking reactions.

Binding Characteristics of ^{3}H-Labeled Probe. The results of equilibrium binding studies of the ^{3}H-labeled probe to the Na,K-ATPase, done in the dark, are shown in Figure 3. The specific binding is hyperbolic in nature, indicating that the probe is interacting with a homogeneous population of binding sites. The dissociation constant for the probe, determined from the Scatchard plot (Scatchard, 1949; Figure 3, insert), equaled 0.14 μM. This value agreed quite well with the I_{50} determined for Na,K-ATPase inhibition under similar conditions (0.11 μM, data not shown). If one assumes an equivalent weight of 1.57 × 10^5 (a stoichiometry of one α, β, and γ subunit per ouabain binding site), the total number of ^{3}H-labeled probe sites calculated from the x intercept of the Scatchard plot (0.16 μM) agrees quite well with the expected number of ouabain binding sites (0.21 μM), indicating that one probe binds per ouabain binding site.

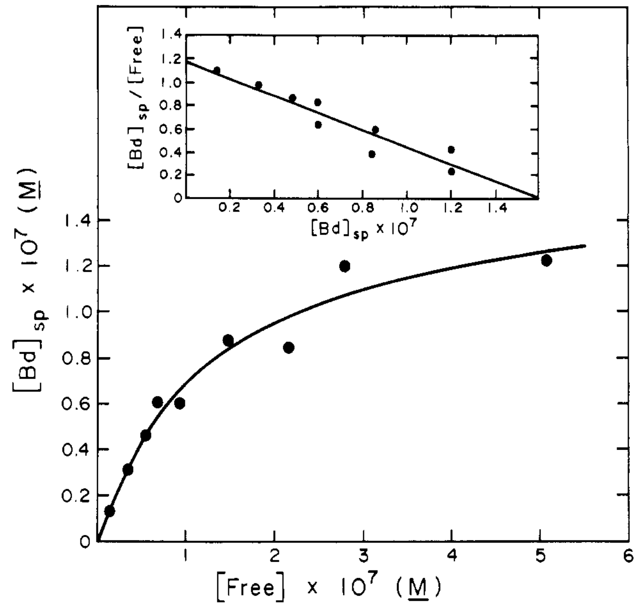

FIGURE 3: Specific binding of [^{3}H]-24-azidodigitoxoside to Na,K-ATPase. The experimental protocol is described in detail under Experimental Procedures. Least-squares analysis of the data in the form of a Scatchard plot (insert) gave a dissociation constant of (1.4 ± 0.3) × 10^{-7} M from the reciprocal of the slope and the concentration of binding sites equal to (1.6 ± 0.3) × 10^{-7} M from the abscissa intercept. The curve drawn through the data is the theoretical curve computed for a single class of binding sites from the values calculated from the Scatchard plot.

Covalent Attachment of ^{3}H-Labeled Probes. The results of SDS–PAGE of Na,K-ATPase labeled with ^{3}H-labeled probe in the absence and presence of 10 μM ouabain and enzyme that was incubated with prephotolyzed ^{3}H-labeled probe are shown in Figure 4. The upper gel shows some labeling of a high molecular weight species found at the top of the gel and at the interphase between the stacking and running gels. A single labeled band was observed at M_r 95 000, which corresponds to the α subunit of the Na,K-ATPase. In the presence of 10 μM ouabain (Figure 4b), labeling of the high molecular weight species was only slightly decreased; however, there was an 80–90% reduction in label incorporation into the α subunit. The final gel (Figure 4c) indicates that no covalent incorporation is observed if the prove is prephotolyzed prior to addition of protein. This implies that there are no long-lived photodecomposition products available to nonspecifically alkylate either the Na,K-ATPase or other protein contaminants in the preparation.

Discussion

Previous work has indicated the importance of the cardenolide C-17 side-chain geometry in Na,K-ATPase inhibition (Fullerton et al., 1979; Ahmed et al., 1983). In order to investigate the binding site of this portion of the molecule, a

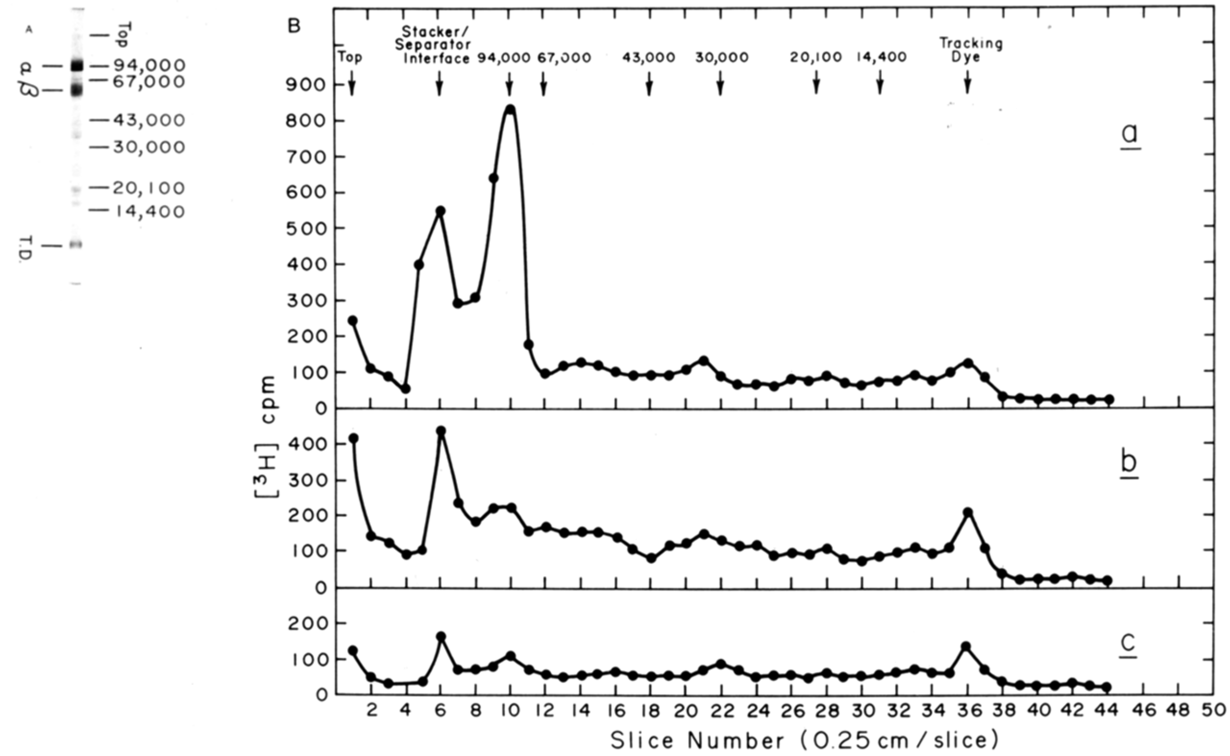

FIGURE 4: (A) SDS–polyacrylamide gel analysis of Na,K-ATPase. The procedure for enzyme preparation and SDS–PAGE is described under Experimental Procedures. The gels show two intensely staining bands at R_f 0.14 (M_r ~95 000) and R_f 0.28 (M_r ~55 000) that correspond to α and β subunits of the enzyme, plus minor impurities at lower molecular weights. (B) Photoaffinity labeling of Na,K-ATPase. Labeling conditions and sample preparation are described under Experimental Procedures. The figure shows ^{3}H cpm incorporated as a function of migration distance for enzyme plus [^{3}H]-24-azidodigitoxoside (**1**) in the absence (upper) or presence (middle) of 10 μM ouabain. The lower panel shows an identical gel of enzyme plus [^{3}H]-24-azidodigitoxoside that had been prephotolyzed for 15 min prior to addition of protein. The upper and middle gels both show labeled material that did not enter the running gel, accumulating at the stacker/separator interphase. The R_f value for the labeled peak in the upper gel (0.13) agreed quite well with that found for the α subunit of the Na,K-ATPase in panel a.

labeled photoaffinity derivative [24-azidodigotoxoside (**1**)] was synthesized with a specific activity of 1.8 Ci/mmol. Prior to synthesis of the tritiated molecule, preliminary experiments with $NaBD_4$ were undertaken in order to show that th C-20 vinyl proton did not exchange during the synthesis.

The equilibrium binding curve of [^{3}H]-24-azidodigitoxoside to the Na,K-ATPase (Figure 3) indicated that the molecule appeared to interact with a homogeneous population of binding sites, $K_d = 0.14\ \mu$M. The agreement of this value with the I_{50} for Na,K-ATPase inhibition indicated that there was a direct correlation between enzymatic activity and the equilibrium binding observed for the labeled molecule. Thus it seems reasonable to assume that the site of action of [^{3}H]-24-azidodigitoxoside was the Na,K-ATPase. It should be noted, however, that the equilibrium binding measurements were somewhat difficult to make. The [^{3}H]-24-azidodigitoxoside exhibited a high degree of nonspecific interaction with both polyallomer and polyethylene airfuge tubes, necessitating the use of the cellulose–propionate tubes. Second, in control experiments where solutions of the labeled probe were centrifuged for longer than 10 min, the molecule appeared to concentrate by a factor of 2 in the airfuge. This behavior may be due to the hydrophobicity of the molecule, resulting in the formation of higher molecular weight aggregates or partial suspension rather than a true solution; however, the exact reason for this behavior is not known. Therefore, as a precautionary measure, the supernatant of the airfuge tube was removed and remixed before taking aliquots to determine the free ligand concentration. In the light of these technical difficulties, it was somewhat surprising that the dissociation constant determined from the equilibrium centrifugation assay was reproducible to within 25% (three experiments) and agreed with the I_{50} from enzyme inhibition experiments.

Control experiments indicated that a suitable compromise between the time required to completely photolyze the 24-azidodigitoxoside (Figure 2) and the generation of high molecular weight peptides that did not enter the running gel was to irradiate with two UVS-11 mineralights, giving an effective path length of 0.5 cm, for 5 min. Even under these conditions (Figure 4b, upper panel), it can be seen that a substantial amount of radioactivity did not enter the running gel. If the protein was not reprecipitated by addition of 95% ethanol–1% v/v β-mercaptoethanol prior to SDS–PAGE, a large amount of radioactivity was found at M_r 12 000. This label could not be displaced by ouabain, and control experiments with [^{3}H]-24-azidodigitoxoside alone or prephotolyzed ^{3}H-labeled ligand in the presence of protein also showed nondisplaceable radioactivity in this region of the gel. Although the reason for this behavior is not known, it may be due to nonspecific incorporation of the probe into either SDS or SDS–lipid micells that migrate to the low molecular weight region of the gel. At this time, protein recovery after precipitation by 95% ethanol–1% v/v β-mercaptoethanol appears to be essentially quantitative (amino acid analysis of the protein distribution between pellet and supernatant showed about 2% of the total amino acids in the supernatant and over 90% of the total amino acids in the pellet). Ethanol was required in this step to remove nonspecifically, noncovalently bound probe found in the low molecular weight region of the gel while the β-mercaptoethanol substantially reduced the formation of high molecular weight aggregates.

The labeling results (Figure 4b) indicate that only the α subunit of the Na,K-ATPase showed any covalent incorporation of radioactivity under type II conditions. Therefore, it appears that the binding site for the steroid-C17 side group of cardiac glycosides is located at or near the α subunit of the Na,K-ATPase. These results agree quite well with the model proposed by Hall & Ruoho (1980) in which the binding site for the steroid portion of the digitalis glycosides resides on or near the α subunit and the sugar binding site(s) is (are) located in a region accessible to both α and β subunits, as well as a possible proteolipid component of the enzyme complex. When an attempt was made to repeat these studies under type I binding conditions (120 mM NaCl, 3 mM Tris-ATP, 3 mM $MgCl_2$, 25 mM Tris-HCl, pH 7.5), the 24-azidodigitoxoside was found to bind to the enzyme 1.5–2.5 times more tightly than under type II conditions; however, covalent incorporation of radioactivity after photolysis was reduced by 50–75% (data not shown). Control experiments indicated that this behavior was not due to a reduction in UV light intensity by ATP, and it is possible to speculate that the difference in labeling efficiency may be due to different conformations of the enzyme under type I and type II conditions. Thus 24-azidodigitoxoside may be a sensitive prove for alterations in the cardiac glycoside C-17 side-group binding site geometry caused by conformational changes of the Na,K-ATPase.

In summary, this paper reports the synthesis and properties of [^{3}H]-24-azidodigitoxoside (**1**). Photoaffinity-labeling experiments utilizing this probe show covalent incorporation in only the α subunit of the Na,K-ATPase, indicating that the binding site for the C-17 side group of cardiac glycosides is located at or near this subunit.

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Registry No. **1**, 87830-63-5; [^{3}H]**1**, 87830-72-6; **2**, 87830-64-6; **3**, 87830-65-7; **4**, 87830-66-8; **5**, 87830-67-9; **6**, 87830-68-0; [^{3}H]**6**, 87830-73-7; **7**, 87830-69-1; **8**, 87830-70-4; [^{3}H]**8**, 87830-74-8; **9**, 87830-71-5; [^{3}H]**9**, 87830-75-9; ATPase, 9000-83-3; butyllithium, 109-72-8; dimethyl methylphosphonate, 756-79-6; lithium dimethyl methylphosphonate, 34939-91-8; methyl bromoacetate, 96-32-2.

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